Epidemiologic and molecular aspects of lung cancer

Miep van der Drift

M.A van der Drift
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Promotor:

Prof. dr. P.N.R. Dekhuijzen

Copromotoren:

Dr. F.B.J.M. Thunnissen (VUMC, Amsterdam)
Dr. B.E.A.Hol (Albert Schweitzer Ziekenhuis, Dordrecht)

Manuscriptcommissie:

Prof. dr. J.H. van Krieken (voorzitter)
Prof. dr. W.T.A. van der Graaf
Prof. dr. P.E. Postmus (VUMC, Amsterdam)

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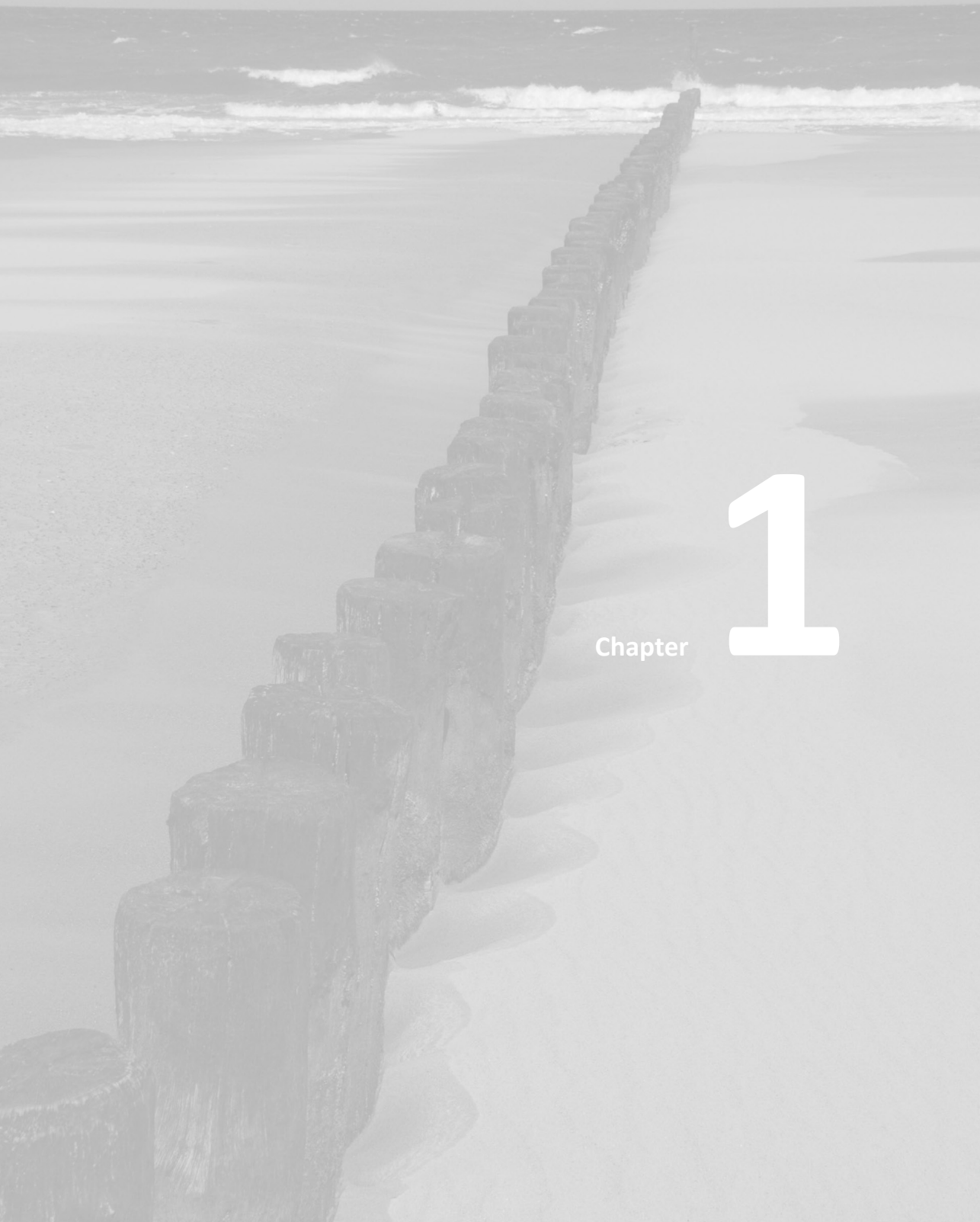
Introduction and outline



Chapter 1

Introduction

Lung cancer gives a high burden of disease to patients and is the leading cause of cancer mortality worldwide. The studies presented in this thesis address changes in treatment and survival in the Netherlands in the past 20 years. It further focuses on the possible use of DNA profiling in body fluids concerning prognosis and facilitating diagnosis of lung cancer.
The characteristics of lung cancer have changed in the past 20 years.[1] About 87% of all lung cancer patients have non-small cell lung cancer (NSCLC); small cell lung cancer (SCLC) rates decreased to 10 to 15%. Since lung cancer is directly related to smoking, trends in incidence follow the smoking habits with a latency period of about 30 years.[2,3] Following the significant decreased tobacco consumption in developed countries, the incidence of lung cancer in males is declining. In the Netherlands the age-standardized incidence rate (ESR) in males decreased from 82 to 54 per 100,000 person years between 1989 and 2009 (www.ikcnet.nl). On the contrary, women increased smoking during the same period resulting in increased incidence rates in women from 11 per 100,000 in 1989 to 29 in 2009. However, among young women the incidence has started to flatten out expected to be followed by a further decreasing incidence in overall female lung cancer.[4,5] Nowadays the incidence of NSCLC in never smokers is increasing, especially in women. Possible causes are genetic, biologic and hormonal factors, and perhaps some factors related to the environment and lifestyle.[6,7]

Prognosis of lung cancer

In the past 20 years, the proportion of adenocarcinoma has increased and more patients presented with stage IV disease.[5,8,9] Stage migration occurred as a result of improved staging techniques such as application of fluorodeoxyglucose-positron emission tomography (FDG-PET) and endoscopic ultrasound biopsies from mediastinal lymph nodes and also because of changes in the TNM (tumor, node, metastasis) classification system in 1997.[10-14] With improved staging, patients with previous early stage disease will be upstaged (stage migration) which results in improved survival for all stage groups (Will Rogers phenomenon). Five-year survival in early stage lung cancer is 40 to 70% by surgical resection, but most patients are unresectable at the time of diagnosis. At the time of diagnosis, NSCLC is often locally or systemically advanced resulting in an overall 5-year survival of only 16%.[15] Median survival of SCLC is even worse and about 1 to 2 years. In the past decades, refinements in surgery and perioperative care, radiotherapy and chemotherapy have been made enabling the combination of different modalities.[16-18] In clinical trials with selected patients, mostly with a good performance status and younger age, these new modalities have shown slight improvements in 1- and 5-year survival. The question remains how these changes in treatment effected survival in daily practice with unselected patients.
Prognostic factors predict survival independent of the treatment applied and

can classify patients as high or low risk. The most important prognostic factor is stage according to the TNM system.[14,19] Larger tumors and involvement of higher lymph node disease have worse survival even in the same stage, which have led to revisions in the TNM system in 2009.[20] Other prognostic factors include clinical aspects (e.g. gender, age, weight loss, cardiovascular disease), elevated lactate dehydrogenase levels, FDG-PET scan and pathologic aspects.[21-26] Increasingly, multiple genetic and epigenetic alterations are under investigation as prognostic factors for survival.[27-31] Cancer results from the accumulation of a variety of events in genes controlling cell growth and differentiation. (Epi)genetic changes consist of alterations in oncogenes such as *KRAS* (Kirsten rat sarcoma 2 viral oncogene homolog), *p53* and *EGFR* mutations, hypermethylation of tumor suppressor genes such as p16 and RASSF1A, loss of heterozygosity (LOH), and microsatellite alterations and deletions.[32-38] Since many years free or circulating DNA in plasma or serum of (lung) cancer patients is under investigation for clinical relevance. Circulating plasma DNA is present in considerably higher concentrations in lung cancer patients compared with healthy controls or patients with benign disease.[29,39] The increased circulating DNA concentration is thought to originate from cancer cells.[40,41] Conflicting data have been reported about circulating DNA as a prognostic factor in NSCLC patients.[42] Some studies showed a correlation between an elevated plasma DNA concentration and poor survival, whereas other studies did not report such a relationship. This might be explained by differences in patient selection, covering both NSCLC and SCLC, and different techniques for sample collection and DNA quantification. In SCLC patients, little is known about the prognostic value of circulating DNA. So far, sensitivity and specificity of (epi) genetic changes has been limiting the clinical value due to the small number of cases and controls, the low frequency of (epi)genetic alterations, choice of appropriate markers and standardized methods.

Diagnosis of lung cancer

(Epi)genetic changes might also serve as useful diagnostic molecular markers. A long-standing goal of cancer researchers has been to develop tests that would facilitate earlier diagnosis and treatment of lung cancer and thereby decrease mortality and improve prognosis. Molecular analysis could be particularly useful for patients with advanced lung cancer in whom cytologic specimens, such as blood, bronchial washing or sputum, are often the only materials available. Flexible bronchoscopy is an essential step in the workup of lung cancer to establish a cytologic or histologic diagnosis. Washings, brushings and forceps biopsies are often combined to increase the diagnostic yield.[43,44] The diagnostic yield of bronchoscopy increases in larger tumors and endoscopic visible tumors. The combined diagnostic yield in patients with *endoscopic visible* (central) tumors, mostly squamous cell carcinoma and small cell carcinoma, varies from 49% to 94%. In these patients, the diagnostic yields for washings and brushings are inferior to the yield of biopsies (52-77% and 71-91%, respectively). The combined diagnostic

yield in patients with *endoscopic nonvisible* (peripheral) tumors, mostly large cell and adenocarcinoma, is much lower and varies from 40% to 56%. In these patients the diagnostic yield of washings is similar to the yield for brushings and for biopsies and varies from 26 to 61%. The optimal timing of bronchial washing (ie whether before or after biopsy and brushing) is not clear.
In case of a negative bronchoscopy, more invasive diagnostic procedures as transbronchial needle aspiration (TBNA), ultrasound guided endobronchial or endoscopic fine needle aspiration (EBUS-FNA or EUS-FNA), transthoracic needle aspiration (TTNA), mediastinoscopy or even thoracotomy are necessary to establish lung cancer diagnosis.[44] If the diagnosis of lung cancer could be established easier, invasive diagnostic procedures could be prevented. Detection of (epi)genetic events in bronchial washings could facilitate in lung cancer diagnosis without additional (invasive) diagnostic procedures. Microsatellite alterations, *KRAS* mutations and *EGFR* mutations and expression can be found in washings of both visible and nonvisible lung tumors.[45-48] In the last decade several studies showed that hypermethylated genes could be detected in bronchial washings.[49-51] Hypermethylation of tumor related genes is a mechanism for silencing tumor suppressor genes.[52,53] RASSF1A (Ras-association domain family 1A gene) methylation has been found in tumors of about 40% of NCSLC patients.[35,54] RASSF1A methylation was positively correlated with the amount of tumor cells in bronchial aspirates. Currently, in bronchial washings without cytologic or histologic diagnosis of malignancy, it is not clear whether methylation can be found.

Besides bronchoscopy, cytologic analysis of sputum can be used in the diagnostic workup of lung cancer. The average diagnostic yield of sputum cytology is 66% (range 22 to 98%) and increases with endoscopic visible tumors, the number of sputum specimens collected per patient, a large and/or high stage tumor (tumors greater than 2.4 cm in diameter) and histology (more in squamous cell carcinomas than in adenocarcinomas).[43,55-57] Because tumor cells comprise only a minor fraction in most sputum samples, the amount of tumor DNA in sputum is likely to be low. It is not known if increased quantities of circulating or free DNA can be found in sputum. Free DNA may originate from malignant cells of lung cancer or upper airway cancer and also from inflammatory cells. Although not frequently used in daily practice due to the limited diagnostic yield, sputum analysis is under investigation in lung cancer screening programs.[58,59]

Outline of this thesis

In this thesis an overview of changes in treatment and survival of NSCLC and SCLC in the last 20 years in the Netherlands will be given. It further focuses how bronchial washings, RASSF1A methylation and circulating DNA facilitate in diagnosing lung cancer. Also the prognostic value of circulating DNA will be discussed.

Chapter 2 describes changes in treatment in daily practice of unselected NSCLC patients in the Netherlands in the past 20 years. In these years, refinements in lung cancer therapy resulted in small improvements in 1-year and 5-year survival in clinical trials. The effects of treatment changes to survival in daily practice are analyzed. Population-based data from the nationwide Netherlands Cancer Registry (NCR) are used. In **chapter 3** changes in treatment in daily practice of SCLC in the Netherlands are described also using the population-based data from the NCR of a 20-year time period, and the effects of therapy on survival are analyzed in these patients.

The following chapters focus on the potential use of DNA profiling in body fluids. **Chapter 4** reports the optimal timing of washing relative to biopsy and brushing in bronchoscopy. The diagnostic yields of washings before and after biopsy and brushings are compared. Also the cost-effectiveness of bronchoscopic procedures in patients with endoscopic nonvisible tumors is studied. The different diagnostic strategies are assessed in terms of yield and costs.
The yield of bronchoscopy in patients with endoscopic nonvisible tumors is usually modest. In these patients with negative bronchoscopy, the diagnostic value of RASSF1A methylation in washings is analyzed in **chapter 5**. RASSF1A methylation in patients is compared with controls. Also, the additional diagnostic value of combined RASSF1 methylation and *KRAS* mutation analyzes in washings is assessed.
The presence of free DNA in sputum and its relationship to the presence of lung cancer is examined in **chapter 6**. The contribution of inflammatory cells to the amount of free DNA in sputum is explored. To discriminate between tumor related free DNA and inflammation, hypermethylation of RASSF1A is assessed.
Free or circulating plasma DNA as a prognostic factor for survival is prospectively analyzed in NSCLC patients (**chapter 7**) and SCLC patients (**chapter 8**). Quantification of baseline circulating DNA is performed at the time of diagnosis by a real-time quantitative polymerase chain reaction (qPCR) targeting the human-globin gene.
The thesis concludes with a summary of the main results of our research in **chapter 9** together with a general discussion and suggestions for future research.

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Progress in standard of care therapy and modest survival benefits in the treatment of NSCLC patients in the Netherlands in the last 20 years

Chapter 2

van der Drift MA, Karim-Kos HE, Siesling S, Groen HJM, Wouters MWJM, Coebergh J, de Vries E, Janssen-Heijnen MLG.

Journal of Thoracic Oncology 2012;7: 291-298

Abstract

Introduction: Lung cancer is the leading cause of cancer mortality worldwide. We analyzed changes in treatment and their potential effect on survival of non-small cell lung cancer (NSCLC) patients in the Netherlands.

Methods: All NSCLC patients diagnosed during 1989-2009 (n=147,760) were selected from the population-based Netherlands Cancer Registry. Differences in treatment over time were tested by the Cochran-Armitage trend test. The effects of sex, age, histology and treatment on relative survival were estimated in multivariable models. Follow-up was completed until January 1st 2010.

Results: Between 1989 and 2009, the proportion of younger patients (younger than 75 years) with stage I undergoing surgery increased from 84% to 89% and among elderly (75 years or older) from 35% to 49%; for stage II, this proportion decreased from 80% to 70% and remained about 25% in respectively younger and older patients. Adjuvant chemotherapy for stage II increased from 0% to 24% in younger patients but remained <5% among the elderly. Chemoradiation increased from 8% to 43% among younger patients with stage III and from 1% to 13% among elderly. In stage IV, chemotherapy in younger patients increased from 10% to 54% and in elderly from 5% to 21%. Five-year relative survival of the total group increased from 14.8% to 17% (especially among females, younger patients, and within each stage), which could be partly explained by changes in treatment and better staging.

Conclusions: Over a 20-year period, application of therapy, which is currently considered as standard, has improved. This resulted in small improvements in survival within all stages.

Introduction

Lung cancer is still the leading cause of cancer mortality worldwide. The incidence of lung cancer in males is declining in developed countries following the significant decreased tobacco consumption; in the Netherlands, the age-standardized incidence rate decreased from 82 to 54 per 100,000 person-years between 1989 and 2009 (www.ikcnet.nl).[1,2] Incidence rates in females increased from 11 per 100,000 in 1989 to 29 in 2009 but are flattening out among young women.[3] The majority (80 to 89%) of all lung cancers are non-small cell lung cancers (NSCLC).[2,4] The distribution of histology and stage has changed in the past 20 years, with an increase in the proportion of patients with adenocarcinoma and stage IV disease.[5-7] Improved staging techniques such as application of fluorodeoxyglucose-positron emission tomography (FDG-PET) and endoscopic ultrasound biopsies from mediastinal lymph nodes, combined with changes in the TNM (tumor, node, metastasis) classification system in 1997, resulted in stage migration.[8-12]
At the time of diagnosis, NSCLC patients often have systemic disease resulting in an overall 5-year survival rate of only 16%.[4,13] During the past 20 years, refinements in surgery and perioperative care, radiotherapy and chemotherapy have been made enabling the combination of different (new) modalities.[14-16] In clinical trials with selected patients, mostly with a good performance status and younger age, these new modalities have shown slight improvements in 1- and 5-year survival. Standard treatment in the Netherlands is described in the "national guideline for staging and treatment of NSCLC" published in 2004 and was recently updated in 2011. The broad categories for treatment are surgery for stages I and II, chemoradiation for stage III and chemotherapy for stage IV (www.ikcnet.nl). The question remains how changes in patient selection for these treatment categories affected survival in daily practice. The period before the introduction of the 2004 guideline (1989-2004) was used as start of our measurements.

The aim of this study was to examine changes in treatment and survival of NSCLC patients in the Netherlands in the last 20 years, on a population-based level, according to stage, age group and sex.

Patients and methods

Data collection

Population-based data from the nationwide Netherlands Cancer Registry was used which has complete national coverage and registers about 90,000 new cancer cases annually.[17] Follow-up was calculated as the time from diagnosis to death or to January 1, 2010. The information on vital status was initially obtained from municipal registries and hospitals and from 1995 onwards from the nationwide population registries network. These registries provide virtually complete coverage of all deceased Dutch citizens.

For this study, all patients diagnosed with microscopically verified NSCLC (C34, ICD-O morphology codes 8010-14, 8020-22, 8030-33, 8046, 8050-78, 8082-84, 8090, 8094, 8123, 8140-41, 8190, 8200, 8211, 8230-31, 8250-63, 8290, 8310, 8323, 8333, 8430, 8470-90, 8550-51, 8560, 8562, 8570-75, 8972, 8980-82) during the period 1989-2009 in the Netherlands were included. Histology was classified as squamous cell carcinoma, adenocarcinoma and large cell carcinoma according to the World Health Organisation classification.[18] A subgroup of other histologies consisted of adenosquamous and sarcomatoid tumors, among others. The study period was divided into four categories: 1989-1993, 1994-1998, 1999-2003, and 2004-2009. Age was divided into three groups (<60, 60 to 74, and ≥75 years). The 6th edition of TNM classification was used for staging of lung tumors.[19] The clinical TNM (cTNM) was used in trend analysis of primary treatment according to stage. For the cases where cTNM was unknown (11%), the pathological stage (pTNM) was added if known (5%). Primary treatment was classified as surgery (with or without chemotherapy and/or radiotherapy), thoracic radiotherapy, chemotherapy and thoracic radiotherapy (chemoradiation), chemotherapy alone and best supportive care (BSC). For survival analysis, pTNM was applied, and if pTNM was unknown, cTNM was used.

Statistical analysis

The percentage of patients receiving certain types of treatment was calculated by age group and time period. Differences in treatment over time were tested by the Cochran-Armitage trend test. Relative survival was used as an estimation of disease specific survival and is calculated as the ratio of the observed rates in cancer patients to the expected rates in the general population.[20] It reflects survival of cancer patients, adjusted for survival in the general population with the same age and gender distribution. Patients younger than 15 years and older than 95 years were excluded from survival analyses, as well as cases diagnosed by autopsy. Traditional cohort-based relative survival analysis was used for the period 1989-2003. As follow-up was available until January 2010, 5 years of follow-up was not available for the period 2004-2009, and therefore period-based relative survival analysis was conducted to obtain 5-year relative survival in this recent period.[21] For stage I and II, 5-year relative survival was calculated, for stage III 3-year and stage IV 1-year relative survival. Survival trends were evaluated by a linear regression model and $p<0.05$ was considered as statistically significant.
Multivariable relative survival analyses were performed to estimate relative excess risk (RER) of mortality for the periods of diagnosis adjusted for follow-up time, age, sex and histology, and stratified by stage. Treatment variables were added to investigate whether the effect of time period on survival could be explained by changes in treatment. The difference in RER is the part that was explained by changes in treatment. SAS software (SAS system 9.2, SAS Institute, Cary, NC) was used to perform the statistical analyses.

Table 1. *Clinical and tumor characteristics of patients with non-small cell lung cancer by period of diagnosis.*

	Number of patients per period of diagnosis (%)				
	1989-1993	1994-1998	1999-2003	2004-2009	p-value*
Total	32,514	33,676	34,102	47,468	
Gender					<0.001
male	27,487 (85)	26,600 (79)	24,805 (73)	31,192 (66)	
female	5,027 (15)	7,076 (21)	9,297 (27)	16,276 (34)	
Age (years)					<0.001
< 60	7,064 (22)	7,733 (23)	8,668 (25)	11,988 (26)	
60-74	17,521 (54)	17,806 (53)	17,002 (50)	23,189 (49)	
≥ 75	7,929 (24)	8,137 (24)	8,432 (25)	12,291 (26)	
Stage (cTNM)					<0.001
in situ	4 (0)	4 (0)	2 (0)	1 (0)	
I	9,150 (28)	9,224 (27)	7,573 (22)	9,675 (20)	
II	1,103 (3)	57 (3)	1,687 (5)	2,056 (4)	
III	11,490 (35)	12,389 (37)	11,238 (33)	13,999 (30)	
IV	7,930 (24)	8,927 (27)	12,106 (36)	20,803 (44)	
unknown	2,837 (9)	2,275 (7)	1,496 (4)	934 (2)	
Histology					<0.001
squamous	17,014 (52)	14,959 (44)	12,556 (37)	13,947 (29)	
adenocarcinoma	8,185 (25)	9,941 (30)	10,815 (32)	17,434 (36)	
large cell	6,686 (21)	8,296 (25)	10,290 (30)	15,471 (34)	
other	629 (2)	480 (1)	441 (1)	616 (1)	

**p-value: period 2004-2009 compared with 1989-1993 tested by the Chi-square test*
cTNM, tumor, node, metastasis

Results

Patient characteristics

Patient characteristics are presented in Table 1. During the period 1989-2009, 147,760 patients were diagnosed with NSCLC. The proportion of females more than doubled from 15% in 1989-1993 to 34% in 2004-2009. The median age has slightly increased among males from 69 years in 1989-1993 to 70 years in 2004-2009 ($p< 0.001$) but remained stable at 64 years among females. Twenty-five percent of the patients was 75 years or older at the time of diagnosis.

The proportion of patients with unknown stage decreased from 9% in 1989-1993 to 2% in 2004-2009. After 1999, the proportion of patients with advanced disease (stage IV) increased from about 27% to 44% in 2004-2009, whereas the proportions of patients with stage I (about 27% to 20%) and III (about 37% to 30%) decreased. Over time, the proportion of nonsquamous cell carcinomas among male patients increased from 42% to 67%, independent of age and among females from 67% to 81% with a stronger increase for those younger than 60 years (results not shown).

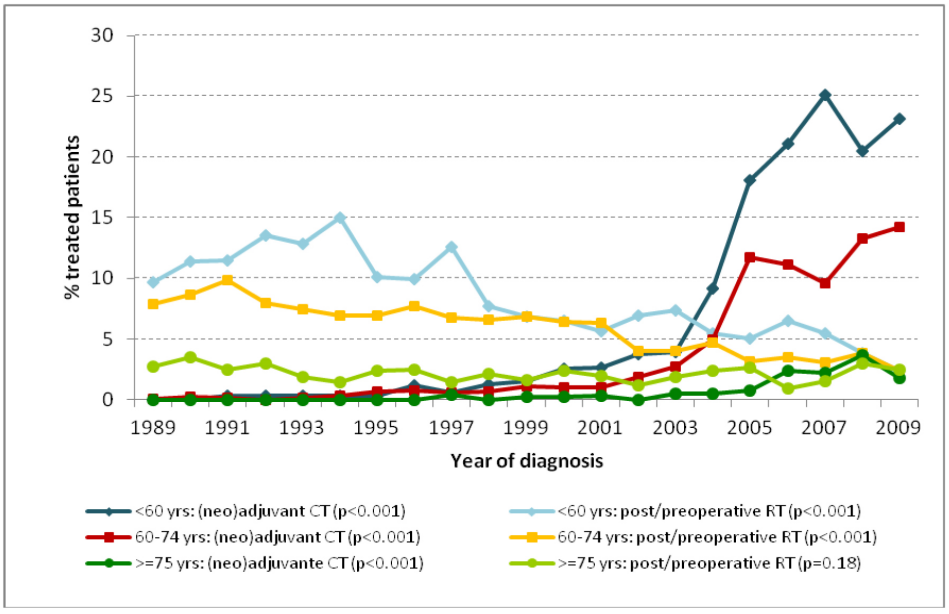
Trends in treatment

Stages I and II

Since 1989, the percentage of patients with *stage I* disease undergoing resection remained about 91% among those younger than 60 years and increased from 77% to 82% among those aged 60-74 years ($p < 0.001$) and from 35% to 49% among patients aged 75 years or older ($p < 0.001$). Since 2003, the proportion of patients younger than 75 years receiving (neo-)adjuvant chemotherapy increased significantly, whereas (neo-)adjuvant radiotherapy decreased (Figure 1a). Among the elderly, (neo-)adjuvant chemotherapy or radiotherapy was hardly used. The percentage of patients receiving thoracic radiotherapy alone has slightly decreased since 1989 from 11% to 9% but remained over 30% for those aged 75 years or older.

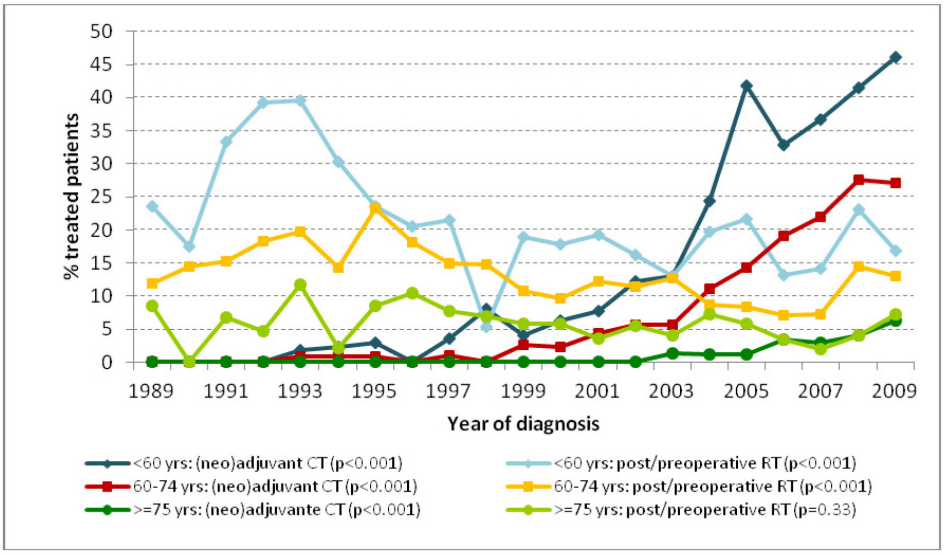
Since 1989, the percentage of patients with *stage II* disease undergoing resection decreased from 89% to 79% among those younger than 60 years ($p < 0.001$), from 71% to 62% among those 60-74 years ($p < 0.001$) and remained about 25% among patients aged 75 years or older. Since 1999, the proportion of patients younger than 75 years receiving (neo-)adjuvant chemotherapy increased significantly, but this treatment was hardly used among the elderly patients (Figure 1b). The proportion receiving (neo-)adjuvant radiotherapy has only decreased among those younger than 75 years (Figure 1b). The percentage of patients receiving thoracic radiotherapy alone has increased since 1989 from 17% to 21% ($p < 0.01$) and remained over 40% for those aged 75 years or older.

Figure 1. *Changes in treatment for NSCLC according to stage and age.*
1a – (neo-)adjuvant therapy stage I



Yrs = years; CT = chemotherapy; RT = radiotherapy

1b – (neo-)adjuvant therapy stage II



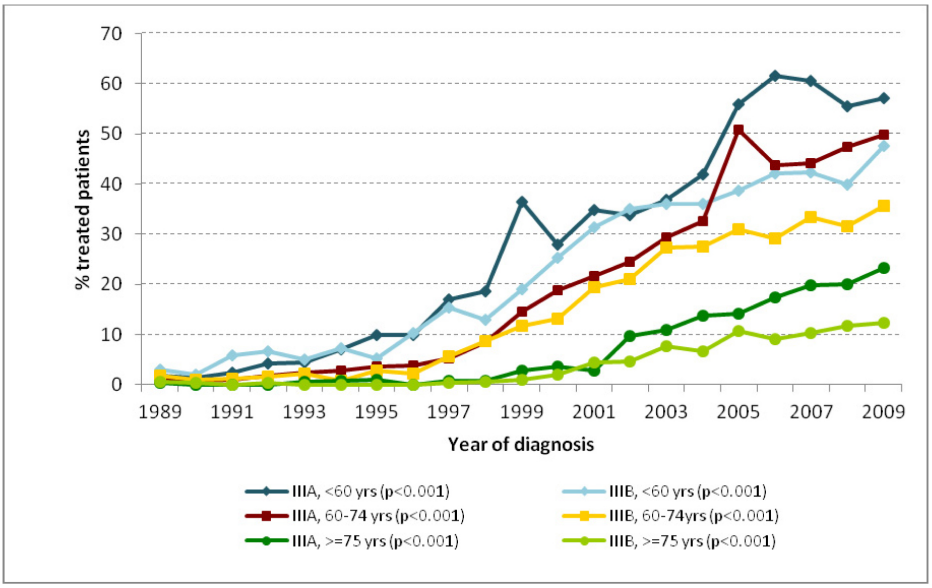
Yrs = years; CT = chemotherapy; RT = radiotherapy

Stages III and IV

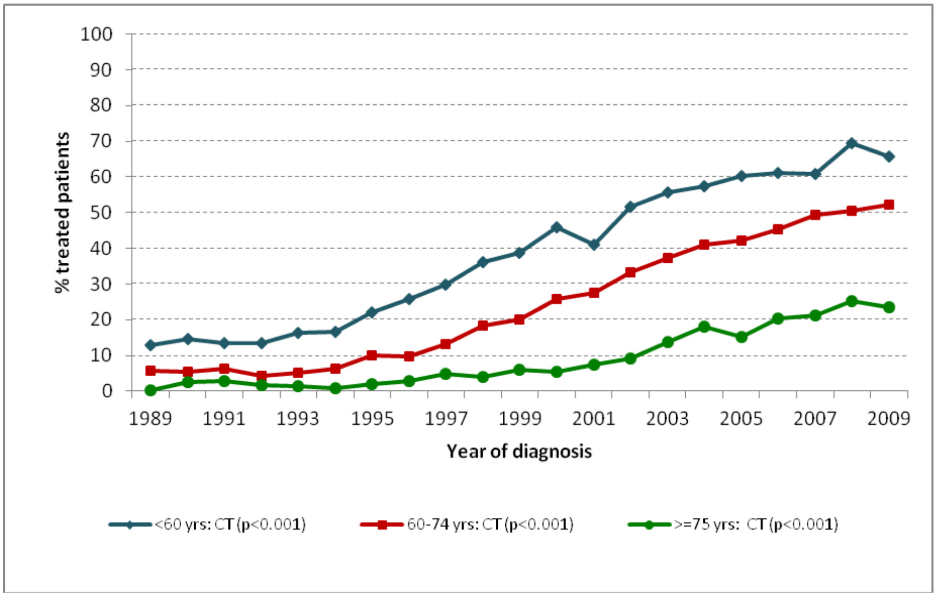
The introduction of chemoradiation for *stage III* was the most prominent change among young patients since 1997, followed by a similar change in the elderly since 2000. In 2004-2009, chemoradiation for stage IIIA was applied in 56% of patients younger than 60 years and in 45% of patients aged 60-74 years, compared with 12% and 5%, respectively, in the period 1994-1998 (Figure 1c). Chemoradiation for stage IIIB was applied in 41% of patients younger than 60 years in 2004-2009 and in 31% of patients aged 60 to 74 years, compared with 10% and 4%, respectively, in the period 1994-1998. Among patients aged 75 years or older, the proportion receiving chemoradiation in stage IIIA was 18% and 10% in stage IIIB in 2004-2009. Among those younger than 75 years, more patients received only chemotherapy than only radiotherapy and vice versa for those aged 75 years or older (results not shown). The proportion receiving chemotherapy alone increased significantly from 2% to 18% in stage IIIA and from 4% to 26% in stage IIIB ($p < 0.001$ in all age groups), whereas the proportion receiving only (palliative) radiotherapy declined significantly over time from 64% to 18% in stage IIIA and from 60% to 15% in stage IIIB ($p < 0.001$ in all age groups).

Few stage III patients were eligible for surgery, whether or not combined with chemoradiation. The proportion of patients younger than 75 years undergoing surgery decreased from 25% to 15% in stage IIIA ($p < 0.001$ in all age groups) and increased from 8% to 10% in stage IIIB ($p < 0.003$ in all age groups). For patients aged 75 years or older this proportion decreased from 10% to 5% in stage IIIA ($p < 0.001$) and did not change in stage IIIB (3%, $p = 0.45$).

1c – chemoradiation therapy stage III



1d – chemotherapy stage IV



Yrs = years; CT = chemotherapy

Since the mid-1990s, the proportion of *stage IV* patients receiving chemotherapy increased significantly among those younger than 60 years from 14% in 1989-1993 to 63% in 2004-2009 and among those aged 60 to74 years from 5% to 47% (Figure 1d). Among patients aged 75 years or older this proportion increased significantly from 5% to 21% since 2000.

Trends in survival

Five-year relative survival for all patients improved significantly since 2005, from 14.8% in 1989-93 to 16.1% in 2004-2009 ($p = 0.003$) and was 17.4% in the year 2009. Five-year relative survival for females improved significantly from 14% in 1989-1993 to 18% in 2004-2009 ($p < 0.001$), whereas 5-year relative survival for males remained stable at 15%. Improvements in survival were observed among all age groups (<75 years from 16% to 18%, $p = 0.01$, and ≥75 years from 9% to 11%, $p = 0.001$), and for patients with squamous cell carcinomas (from 17% to 20%, $p < 0.001$) and nonsquamous cell carcinomas (from 11% to 14%, $p < 0.001$). Statistically significant improvements in relative survival over time were seen in stages I, III and IV (Figure 2). Survival rates according to treatment per stage and age group are presented in Figure 3. Relative survival was better for standard of care therapy in all stages and for all age groups.

After adjustment for sex, age and morphology, the RER of dying within the first 5 years after being diagnosed with stage I in 2004-2009 was significantly lower as compared with 1989-93 (RER = 0.62) (Table 2). After additional adjustment for changes in treatment over time, the prognostic effect for period of diagnosis became slightly smaller, but remained significant (RER=0.69). This was also true for patients with stage II and IIIA/B disease (RER stage II=0.84, after adjustment for treatment RER=0.78, and RER stage IIIA/B= 0.72, after adjustment for treatment RER = 0.88). For patients with stage IV NSCLC, the RER of dying within the first year after diagnosis in 2004-2009 was also significantly lower as compared with 1989-1993 (RER = 0.69), but this effect disappeared after adjusting for changes in treatment over time (RER = 1.06).

Figure 2. *Trends in relative survival of non-small cell lung cancer according to stage and period of diagnosis.*

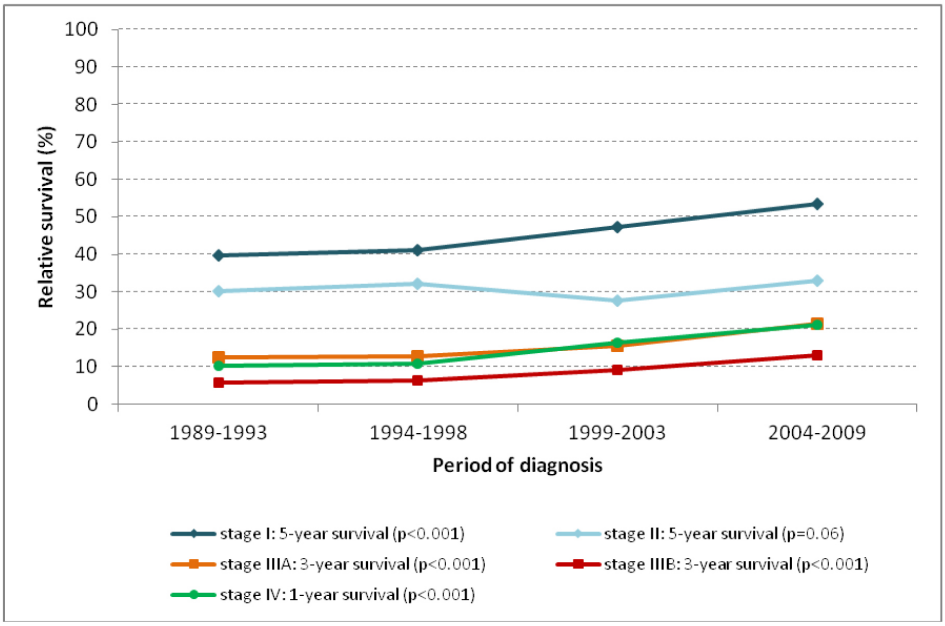


Figure 3. *Relative survival of non-small cell lung cancer patients according to standard of care therapy per stage in 2004-2009.*

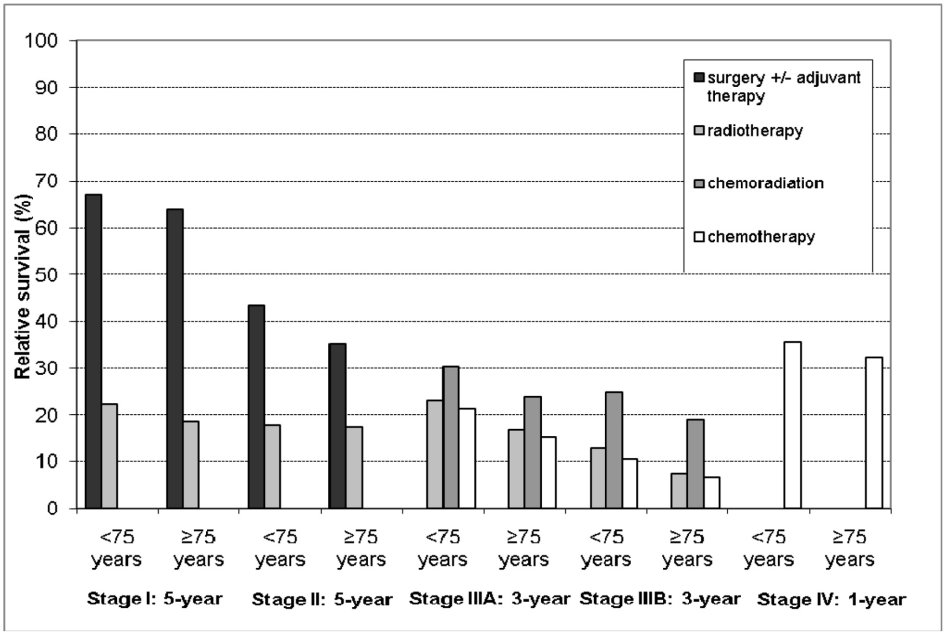


Table 2. *Multivariable relative survival analysis of NSCLC in the Netherlands, 1989-2009.*

		Multivariable model 1 (adjusted for sex, age and histology)		Multivariable model 2 (adjusted for sex, age, histology and treatment)	
	Period of diagnosis	RER	95% CI	RER	95% CI
Stage I (5-year survival)	1989-1993	Ref.		Ref.	
	1994-1998	0.96	0.92 - 1.01	1.00	0.96 - 1.05
	1999-2003	0.82	0.78 - 0.86	0.88	0.84 - 0.93
	2004-2009	0.62	0.59 - 0.65	0.69	0.66 - 0.73
Stage II (5-year survival)	1989-1993	Ref.		Ref.	
	1994-1998	0.94	0.86 - 1.02	0.99	0.91 - 1.07
	1999-2003	1.06	0.98 - 1.14	1.01	0.94 - 1.09
	2004-2009	0.84	0.77 - 0.91	0.78	0.72 - 0.84
Stage III (3-year survival)	1989-1993	Ref.		Ref.	
	1994-1998	1.00	0.97 - 1.03	1.01	0.99 - 1.04
	1999-2003	0.89	0.86 - 0.91	0.98	0.95 - 1.01
	2004-2009	0.72	0.70 - 0.74	0.88	0.85 - 0.90
Stage IV (1-year survival)	1989-1993	Ref.		Ref.	
	1994-1998	0.98	0.94 - 1.01	1.05	1.02, 1.09
	1999-2003	0.79	0.76 - 0.81	1.02	0.99, 1.05
	2004-2009	0.69	0.67 - 0.71	1.06	1.02, 1.09

RER = Relative Excess Risk of dying; 95% CI = 95% Confidence Interval

Discussion

This study showed that the proportion of NSCLC patients receiving the standard treatment according to the Dutch guidelines of 2004 increased significantly over time compared with the period 1989-2004. Nevertheless, a substantial part of the patients did not receive this standard treatment, especially those aged 75 years or older. Five-year relative survival increased slightly since 2004/2005 for the whole group, and especially in females, younger patients and within each stage group, which could only partly be explained by survival benefits due to treatment.
In this study using nationwide population-based data of NSCLC patients, more female patients, more patients with advanced disease and nonsquamous cell carcinomas were diagnosed over time, as was observed elsewhere.[1-4] The proportion of patients with unknown stage decreased, illustrating improvements in staging in recent years.

Trends in treatment

Surgery is the mainstay of curative therapy and offers the best chance for survival.[22] In accordance with other studies and conform (inter)national guidelines (www.ikcnet.nl)[23], we found in *stage I* that almost all patients younger than 60 years underwent surgery. This proportion increased over time for all age groups. (Neo-)

adjuvant chemotherapy in stage I is not recommended except for clinical trials[23], in our study only a minority received additional chemotherapy. Although in *stage II* disease surgery should also be the cornerstone of treatment, only 80% of the patients younger than 60 years underwent surgery and this proportion decreased over time. Resection of stage II tumors is often complicated by the size of the tumors or hilar lymph node metastases, combined with comorbidity: around 50% of these patients have chronic obstructive pulmonary disease.[23-27] Another explanation for the decreased resection rates might be stage migration from stage IIIA to IIB patients, including T3 tumors, after implementation of the 5th edition of the UICC TNM Classification in 1999. Resection rates may improve by centralizing surgical treatment and by implementing multidisciplinary meetings in which treatment strategies can be discussed.[28,29] In the Netherlands, relatively and significantly more patients received (neo-)adjuvant chemotherapy since 2004, in accordance with international observations.[15,30-32]

In *stage III* disease combined chemo- and radiotherapy became standard of care since the early 1990s, replacing the use of radiotherapy alone.[16,33,34] Earlier application of radiotherapy in concurrent schedules together with more accurate radiotherapy techniques have been achieved.[16] Indeed, the proportion receiving chemoradiation among stage III patients increased considerably, although its use was still low (< 50%). Chemoradiation was used more frequently for stage IIIA than stage IIIB patients. This is probably due to the diversity of TNM subsets in stage IIIB including those in which radical radiotherapy cannot be applied, such as pleuritis carcinomatosa (T4 in this TNM staging and nowadays M1a in the new TNM staging of 2009) or large therapy fields including supraclavicular nodal disease (N3) and decreased pulmonary function.[19,35] In *advanced lung cancer* (stage IV) palliative treatment aims to improve or maintain quality of life. Since the 1990s, several studies have shown that platin-based palliative chemotherapy also prolongs survival and is indicated for patients with good performance status and recommended in current guidelines.[36,37] In our study more than 50% of patients younger than 60 years received chemotherapy with remarkable increases since the late nineties.

In line with other studies, about 25% of the NSCLC population was aged 75 years or older.[13] In our study, elderly received less intensive treatment in every stage compared with younger patients, probably reflecting the higher prevalence of multiple co-morbid conditions, frailty and higher operative risks.[26,27,38] Older lung cancer patients have approximately twice as many comorbidities compared with the general population, chronic obstructive pulmonary disease occurring most frequently. Video-assisted thoracic surgery-lobectomy, a new surgical technique, offers possibilities for the elderly, but was hardly applied in our study period because only few centers were offering such treatments.[39] The use of (neo-)adjuvant chemotherapy in stage II was hardly implemented in elderly in the Netherlands as was also shown in a French study.[31] The proportion of older patients receiving only radiotherapy did not change over time, being around 30%

in stage I and almost 50% in stage II, while being almost absent in younger patients. Similar proportions were observed in a Canadian population-based study.[40] Since 2002, there is an increased availability of stereotactic body radiotherapy, a new approach that delivers high radiotherapy doses in short treatment times. Since short-term treatment-related toxicity is low, stereotactic body radiotherapy is appealing for patients with comorbidity, bad performance status and elderly.[41,42] The proportion receiving chemoradiation among older stage III patients increased considerably, although its use was still low (< 20%). Besides age, comorbidity and the diversity of TNM subsets in this stage are main obstacles for applying chemoradiation. In stage III clinical studies, less than 20% of patients aged 70 years or older received chemoradiation.[34] Therefore, it is not known whether the results of these studies can be extrapolated to elderly lung cancer patients.[34] In our study, up to 40% of older stage III patients did not receive any therapy. In line with observations from other studies, elderly stage IV lung cancer patients received less chemotherapy than younger patients, although this proportion has started to increase (up to 24% in our study in 2009).[43-45]

Trends in survival

In our study, 5-year relative survival of all patients with NSCLC together improved slightly since 2004/5 to 17.4%. Better compliance to treatment strategies with clinical service standards such as guidelines could have contributed to better survival at population level.[46,47] With improved staging, patients with previous early stage disease will be upstaged (stage migration) which results in improved survival for all stage groups (Will Rogers phenomenon). The routine use of FDG-PET and integrated PET-CT in the diagnostic workup of patients with early stage lung cancer since 2000 led to a stage-shift towards higher stages.[8,9] Minimally invasive techniques such as esophageal or endobronchial endoscopic ultrasound enhanced the accuracy of mediastinal staging and may also have attributed to a stage-shift.[11] However, in line with other studies, we observed a survival improvement for females and younger patients including all stages which would not be the case if only stage migration plays a role.[27,48,49]. We investigated whether the effect of time period on survival could be explained by changes in treatment by adding treatment variables in the multivariable analyses. Five-year relative survival of patients with *stage I and II* improved over time. This effect became slightly smaller but remained significant after adjusting for changes in treatment in stage I. This indicates that only part of this improvement can be explained by the higher resection rates. Improvements in pre-operative patient selection including multidisciplinary decision making, better staging, better anaesthestic techniques and peri- and postoperative care may have contributed to improved survival as well.[28,50] Several non-randomized studies have shown improvement in overall survival by stereotactic body radiotherapy in stage I and this potential effect has to be awaited in our population-based data.[41,42] Adjusting for changes in treatment in stage II did not affect 5-year relative survival corresponding to

the observed lower resection rates. The increased application of (neo-)adjuvant chemotherapy in this stage is expected to improve survival as was shown in several clinical studies and meta-analysis, but the effects on 5-year survival could not yet be detected.[51]

In the Netherlands, 3-year relative survival for *stage IIIA and IIIB* disease increased significantly over time, largely because of upstaging from stage IIIA to IIB patients after implementation of the 5th edition of the UICC TNM Classification, but also due to the use of chemoradiation, as shown in multivariable analyses. Recent studies indicate that overall survival might be further improved by administrating chemoradiation concurrently instead of sequentially but at the cost of more complications.[34,52] Also patients with significant comorbidity or older age but with a good performance status can achieve similar survival rates as younger patients.[33,53] Other studies showed that in a selected group of downstaged stage III patients including patients with N0 status after chemoradiation, lobectomy may further improve overall survival.[54] In our study, 1-year relative survival for *stage IV* patients younger than 60 years doubled to 21% since 1989. These significant improvements over time disappeared after adjusting for the use of chemotherapy, implying that the increased use of chemotherapy could at least partly explain the improvements in survival. Previous platin-based studies showed improvement of survival and in the last years, applying newer chemotherapy regimens according to either histological subtype or targeted therapy aimed at somatic mutations in receptors or signal proteins (e.g., endothelial growth factor receptor) resulted in improved 1-year survival especially in adenocarcinomas.[37,55,56] Further improvement in survival due to personalized treatment is expected in the near future. In our study, survival of older unselected stage IV patients was poorer compared with younger patients. Among selected patient groups, no survival differences were shown for fit elderly compared with younger patients, and therefore in the current guidelines chemotherapy is also recommended for the fit elderly.[36,45,57]

The increased incidence of nonsquamous cell carcinomas due to changes in the WHO histological typing of NSCLC could also have contributed to improved survival. Among others, the current “non otherwise specified” category has been long time mixed in the large cell subgroup. Also, the incidence of NSCLC in never smokers is increasing, especially in women and nonsquamous cell carcinomas. Possible causes are genetic, biologic and hormonal factors, and perhaps some factors related to the environment and lifestyle.[7,58] Furthermore, the better treatment strategies in adenocarcinomas might have resulted in improved survival.

Our results are based on all NSCLC patients diagnosed in the past 20 years in the Netherlands but nevertheless have the following limitations: detailed information about diagnostic procedures, surgical techniques, thoracic radiotherapy dose, choice of chemotherapy and number of cycles, and comorbidity are not routinely recorded by the cancer registries. Therefore, the effect of newer treatment strategies can only be estimated.

Conclusion

On a population-based level, adoption of standard of care treatment according to the current guidelines increased significantly over time to about 60% of the patients younger than 75 years. Although increased, this proportion was considerably less for those aged 75 years or older (about 20%). Improvements in application of standard therapy were most prominent since 2000 and the Dutch treatment guideline for NSCLC is started to get implemented. Over a 20-year period, small improvements in survival have been made for all stages despite improvement in staging and primary treatment. Since 2004/2005, five-year relative survival increased slightly for the whole group, especially in females, younger patients and within each stage group. Better adherence to therapy whenever possible may further improve survival besides personalized treatment strategies.

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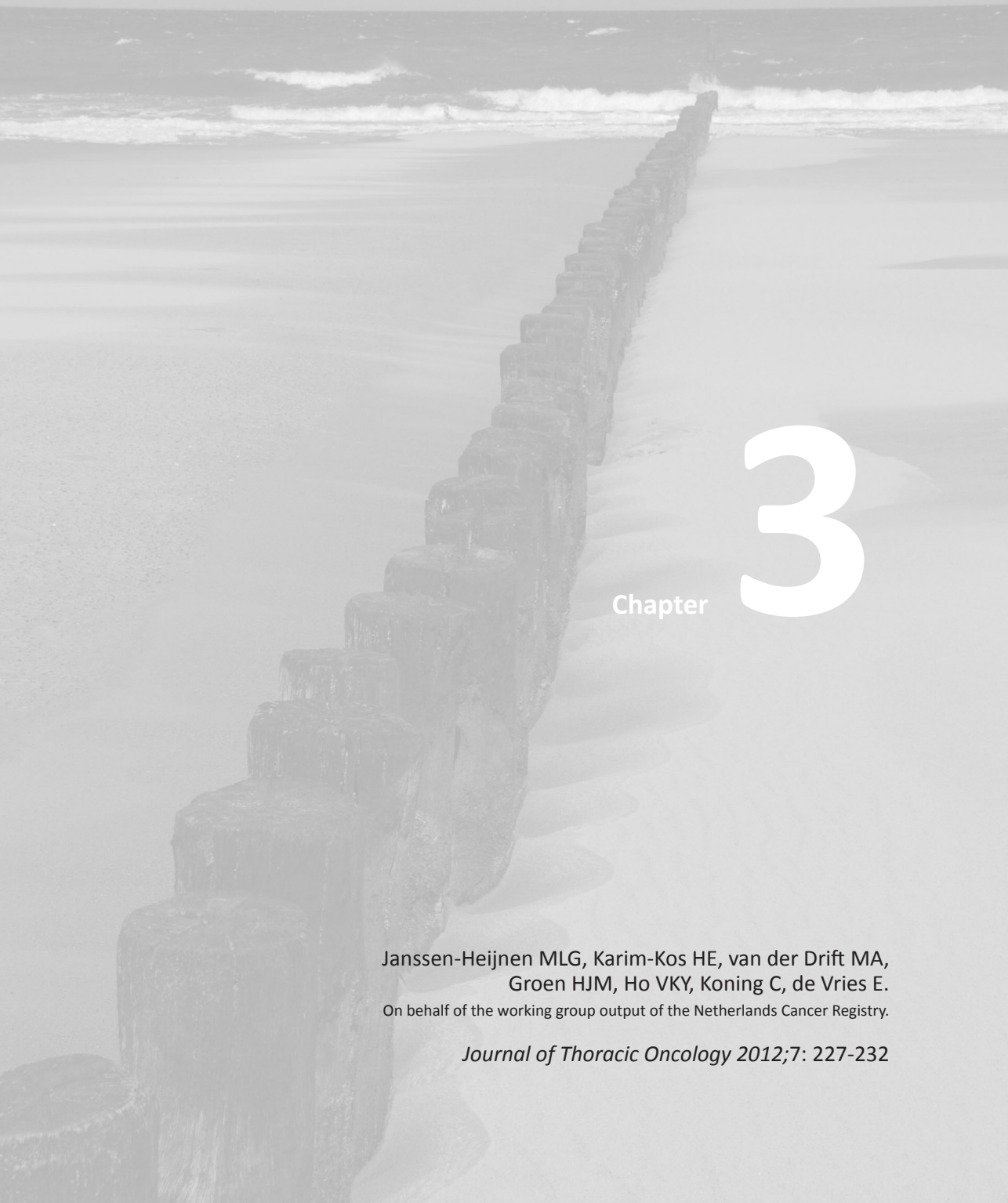
Modest improvements of survival for patients with small cell lung cancer aged 45 to 59 years only, diagnosed in the Netherlands, 1989 to 2008



Chapter 3

Janssen-Heijnen MLG, Karim-Kos HE, van der Drift MA, Groen HJM, Ho VKY, Koning C, de Vries E.
On behalf of the working group output of the Netherlands Cancer Registry.

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Abstract

Introduction: Lung cancer was a major epidemic in the last decades; 10 to 15% of lung cancer consists of small cell lung cancer (SCLC). Several changes in the diagnostic and treatment procedures took place during the last 20 years. This paper focuses on trends in incidence, treatment and survival of SCLC observed since the 1990s.

Patients and methods: All cases with SCLC diagnosed in 1989-2009 in the Netherlands were included (n = 34,100). Follow-up was complete until January 1st 2010.

Results: The proportion of patients with extensive disease (ED) increased from 47% to 63%. The proportion of patients with limited disease (LD) receiving chemoradiation increased from 22% in 1989-2003 to 72% in 2004-2009 among those younger than 45-59 years, from 15% to 58% among those aged 60-74 years, and from 7% to 27% among those aged 75 years or older. Among patients with ED the proportion receiving chemotherapy remained stable over time (84%, 75% and almost 50% for the above mentioned age groups, respectively). Significant improvements in 1-year relative survival occurred for patients aged 45-59 years, but not for the other age groups. Relative survival has significantly increased for both stage groups.

Conclusion: Improved staging resulted in improved survival for both stage groups, whereas survival of the total group has only significantly improved for patients aged 45-59 years. The latter is possibly related with improved treatment strategies. As survival is still very poor, prevention of lung cancer remains important.

Introduction

Lung cancer is now the second most common cancer type in Dutch men and the third most common type in women (www.ikcnet.nl). In the 20th century, the increase in incidence and mortality of lung cancer in the Netherlands was so dramatic that it can be considered as a major epidemic.[1] Although lung cancer mortality among men has been decreasing since the mid 1980s, the recent increase among Dutch women is one of the most prominent in Europe.[2,3]
Ten to 15% of all lung cancer patients have small cell lung cancer (SCLC).[4,5] SCLC is an aggressive tumor which is frequently metastasized at time of diagnosis; median survival time for patients with limited disease (LD) is approximately 23 months and for those with extensive disease (ED) 8-12 months.[6] Because SCLC is considered a disseminated disease, chemotherapy is the cornerstone in the treatment of both limited and extensive disease. Several studies have reported an improvement in survival following the introduction of chemotherapy in the 1970s.[4,7-10] Nowadays, standard treatment for patients with LD who have a good performance score consists of combined chemotherapy and radiotherapy, whereas for those with ED chemotherapy alone is recommended (and depending on locoregional symptoms also thoracic radiotherapy). Due to the high probalility of brain metastases, prophylactic cranial irradiation (PCI) for patients with LD is recommended since the early 2000s.[11] This results in better quality of life. Since the study of Slotman et al., PCI is also recommended for patients with ED.[12] New treatment modalities resulted in small improvements in survival in clinical trials with selected patients, mostly with good performance status and younger age. The question remains how these changes in treatment effected survival in daily practice with unselected patients. This paper focuses on the trends in incidence of SCLC, treatment strategies and survival of unselected patients with SCLC in the Netherlands since 1989.

Patients and methods

Data collection

Population-based data were obtained from the nationwide Netherlands Cancer Registry (NCR), which started in 1989 and is maintained and hosted by the regional Comprehensive Cancer Centers (www.ikcnet.nl). The NCR is based on notification of all newly diagnosed malignancies in the Netherlands by the automated pathologic archive (PALGA). An additional source is the national registry of hospital discharge, which accounts for up to 8% of new cases. Information on patient and tumor characteristics is obtained routinely from the medical records 6 to 9 months after diagnosis. The quality of the data is high, due to thorough training of the administrators and computerized consistency checks at regional and national levels. Completeness is estimated to be at least 95%.[13] Follow-up of vital status of all patients was calculated as the time from diagnosis to death or

until January 1, 2010. The information on vital status was actively obtained from the municipal registries and from the database of deceased persons of the Central Bureau for Genealogy.
For this study, all cases with primary SCLC (C34.0-C34.9 and morphology codes 8041-8045) diagnosed in the period 1989 to 2009 in the Netherlands were included (n=34,100). Patients younger than 15 years and older than 95 years at diagnosis were excluded from the survival analysis, as well as cases diagnosed by autopsy. Treatment and survival were described for three age groups (45-59 years, 60-74 years, and 75 years or older). The study period was divided into four categories: 1989 to 1993, 1994 to 1998, 1999 to 2003, and 2004 to 2009. Clinical stage of disease was classified as limited (tumors confined to one hemithorax without pleural effusion and no distant metastases) and extensive (distant metastases in the contralateral chest or at distant sites and pleural effusion). Treatment of SCLC was classified as chemotherapy + thoracic radiotherapy (chemoradiation, CT + RT), chemotherapy alone, and "no anticancer treatment/best supportive care (BSC)". PCI was described as proportion of those receiving chemotherapy.

Statistical analyses

Annual incidence and mortality rates for the period 1989 to 2009 were calculated per 100,000 person-years, using the annual mid-year population size as obtained from Statistics Netherlands. Rates were age-standardised to the European standard population (European Standardised Rates (ESR)). Changes were evaluated by calculating the estimated annual percentage change (EAPC) and the corresponding 95% confidence interval. To calculate this, a regression line was fitted to the natural logarithm of the rates, using the calendar year as regressor variable.
Stage at diagnosis and primary treatment was described as percentages per age group and time period. Differences between groups were tested with the chi square test. Relative survival was used as an estimation of disease-specific survival. It reflects survival of cancer patients, adjusted for survival in the general population with the same structure for age and gender. Relative survival is calculated as the ratio of the observed rates in cancer patients to the expected rates in the general population.[14] Traditional cohort-based relative survival analysis was used for the period 1989 to 2003, which represents the real survival of patients diagnosed. Because follow-up for patients diagnosed in 2004 to 2009 was only available for a few years, period-based calculation of relative survival analysis was applied to generate the most up-to-date estimates for this period.[15] Relative 1-, 3- and 5-year survival was estimated according to gender, stage, age and period of diagnosis. Survival trends were quantified as the mean annual percentage change within 1989 to 2009 estimated by a linear regression model.

Results

Patient characteristics

Table 1 shows the general characteristics of the patients. In the time period 1989 to 2009 34,100 patients were diagnosed with SCLC: 23,299 (68%) males and 10,801 (32%) females. The male-female ratio decreased from 3.7 in 1989 to 1993 to 1.4 in 2004 to 2009. The median age at diagnosis increased from 68.1 to 69.3 years among males ($p<0.001$) and from 63.6 to 65.6 years among females ($p<0.001$).

Table 1. *Characteristics of patients with small cell lung cancer by period of diagnosis.*

	Period of diagnosis N (%)			
	1989-1993	1994-1998	1999-2003	2004-2009
Gender				
male	6617 (79%)	5737 (72%)	5026 (65%)	5919 (59%)
female	1789 (21%)	2177 (28%)	2682 (35%)	4153 (41%)
Age (years)				
< 44	209 (2%)	182 (2%)	153 (2%)	135 (1%)
45-59	1834 (22%)	1724 (22%)	1833 (24%)	2323 (23%)
60-74	4635 (55%)	4325 (55%)	4029 (52%)	5194 (52%)
≥ 75	1728 (21%)	1683 (21%)	1693 (22%)	2420 (24%)
Stage				
limited	3409 (41%)	3429 (43%)	2863 (37%)	3355 (33%)
extensive	3963 (47%)	3628 (46%)	4159 (54%)	6370 (63%)
unknown	1034 (12%)	857 (11%)	686 (9%)	347 (4%)

The proportion of patients with unknown stage decreased from 12% in 1989 to 1993 to 4% in 2004 to 2009. Among those with known stage, the proportion of patients with ED at diagnosis was about 50% until the late 1990s, but increased to about 65% in 2004 to 2009 (Figure 1). The proportion of patients with LD was significantly higher among women ($p<0.001$). The proportional distribution of stage did not differ significantly between the age groups.

Figure 1. *Trends in stage distribution of SCLC in the Netherlands 1989 to 2009, according to gender.*

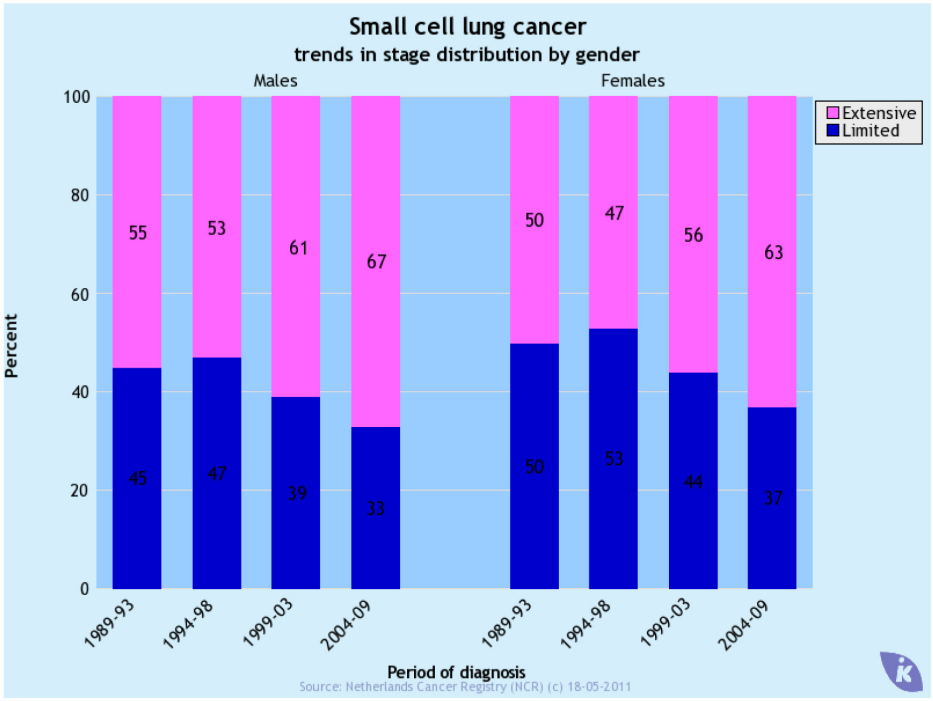
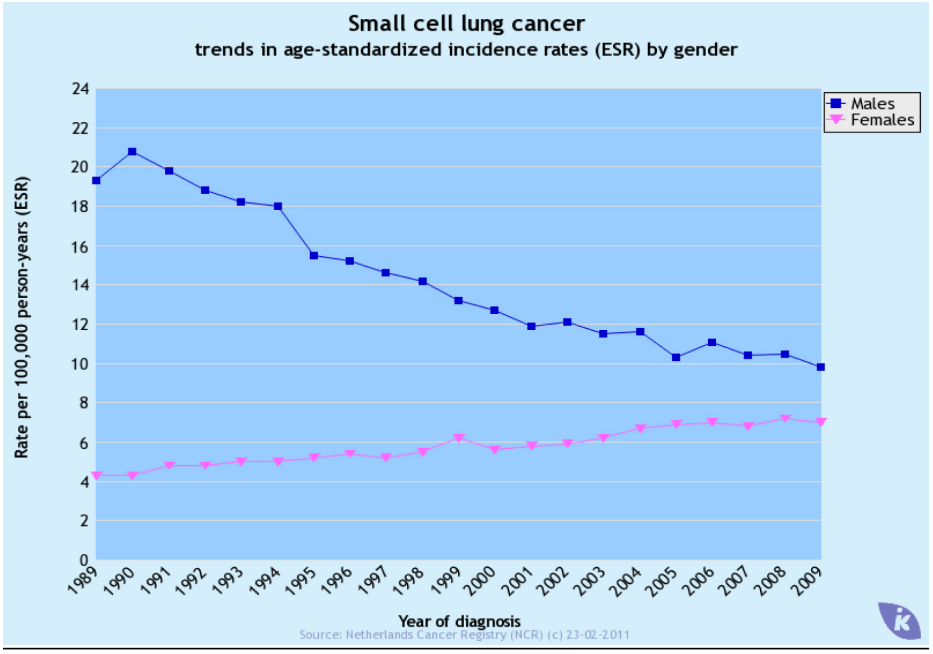


Figure 2. *Trends in age-standardised incidence rates (ESR) of small cell lung cancer (SCLC) in the Netherlands, 1989 to 2009.*



Trends in incidence

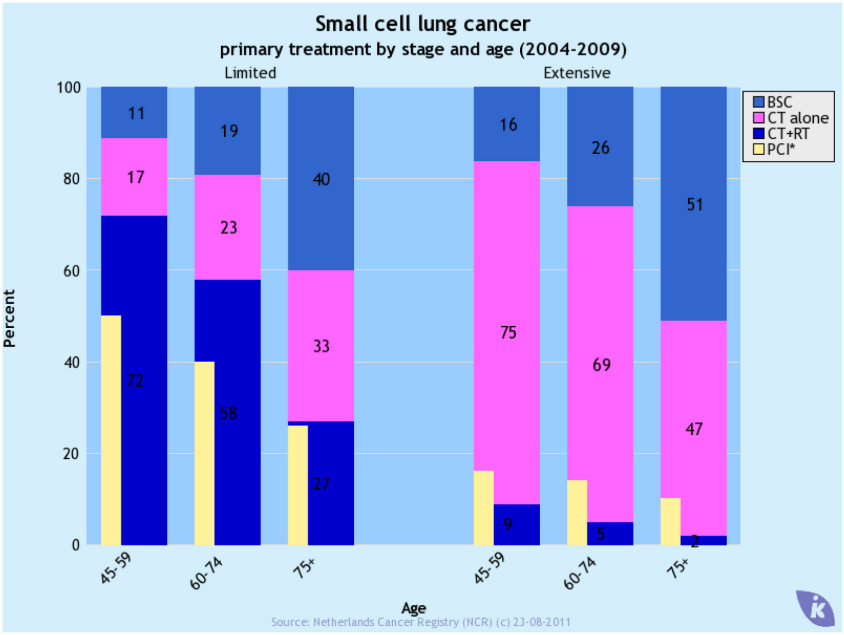
Figure 2 shows the trends in incidence of SCLC. The age-standardised incidence rate (ESR) of SCLC among men decreased from 19 per 100,000 in 1989 to 10 per 100,000 in 2009. The estimated annual percentage change (EAPC) was –3.8%, 95% CI=-4.1, -3.4. Among women the age-standardised incidence has increased from 4.3 to 7.0 per 100,000 (EAPC +2.6%, 95% CI= 2.3, 2.9).

Trends in treatment strategies

Among younger patients with LD diagnosed in 2004 to 2009, the proportion receiving chemoradiation was considerably higher than among those aged 75 or older (Figure 3A). Since the early 1990s the proportion of patients receiving chemoradiation has increased from 22% to 72% among those aged 45 to 59 years, from 15% to 58% among those aged 60 to 74 years, and from 7% to 27% among those 75 years or older (Figure 3B). In the meantime the proportion of patients receiving chemotherapy alone has decreased from 67% to 17% among those younger than 45 to 59 years, from 72% to 23% among those aged 60 to 74 years, and from 50% to 33% among those 75 years or older. Furthermore, the proportion of patients receiving best supportive care remained stable at 12%, 18% and about 40% for those aged 45 to 59, 60 to 74, and 75 years or older, respectively. PCI was introduced in 1999 and increased to 69% in 2009 in patients aged 45 to 59 years who had received chemotherapy, 63% in those aged 60 to 74 years, and 42% of those 75 years or older.

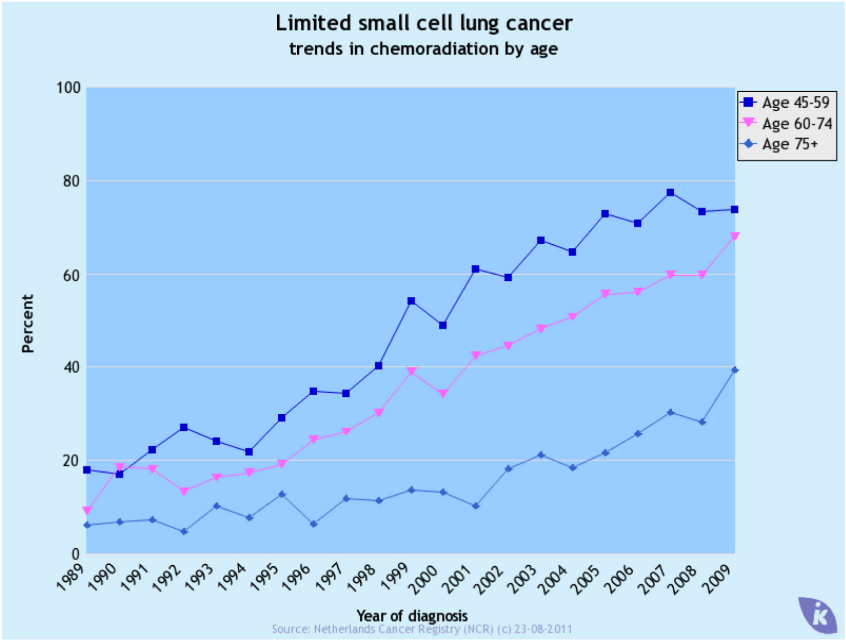
Among patients with ED, the proportion receiving chemotherapy remained stable over time (84%, 75% and almost 50% for aged 45 to 59 years, 60 to 74 years, and 75 years or older, respectively), but was considerably lower among those 75 years or older compared with younger patients (49% versus 84%, figure 3A). In contrast, the proportion receiving only best supportive care was 16% among patients aged 45 to 59 years, but increased to 51% among those 75 years or older. Although PCI was not often applied to patients with extensive disease until 2006, 39% of patients aged 45 to 59 years who had received chemotherapy underwent PCI in 2009. This proportion was 32% for those aged 60 to 74 years and 25% for those 75 years or older.

Figure 3A. *Primary treatment of SCLC in the Netherlands (2004-2009) by stage and age*



*BSC=Best Supportive Care, CT=Chemotherapy, RT=Radiotherapy, PCI=Prophylactic Cranial Irradiation. *Percentage of patients who received chemotherapy*

Figure 3B. *Trends in proportion chemoradiation for limited SCLC by age.*



Trends in survival

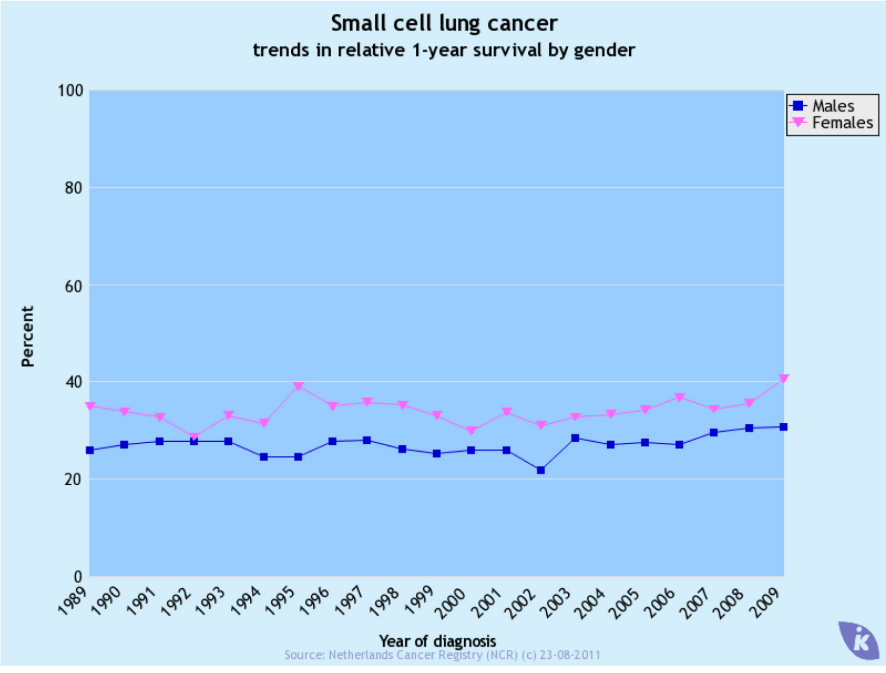
Relative survival for the whole group has only slightly improved, mainly since the mid-2000s. One-year relative survival increased slightly but not significantly from 26% to 32% for males (p=0.09, Figure 4A) and from 35% to 41% for females (p=0.14, Figure 4A), 3-year relative survival increased significantly from 5.5% to 9.2% for males (p=0.001) and from 6.5% to 14% for females (p<0.001), and 5-year survival increased significantly from 3.6% to 6.8% for males (p<0.001) and from 4.2% to 8.9% for females (p=0.001).

When stratifying according to stage, 1-year relative survival for patients with LD was better in 2009 as compared with 1989 (61% versus 39% for males and 67% versus 51% for females, p<0.05). For those with ED, 1-year relative survival has slightly increased from 14% to 19% for males and from 20% to 27% for females (p<0.05).

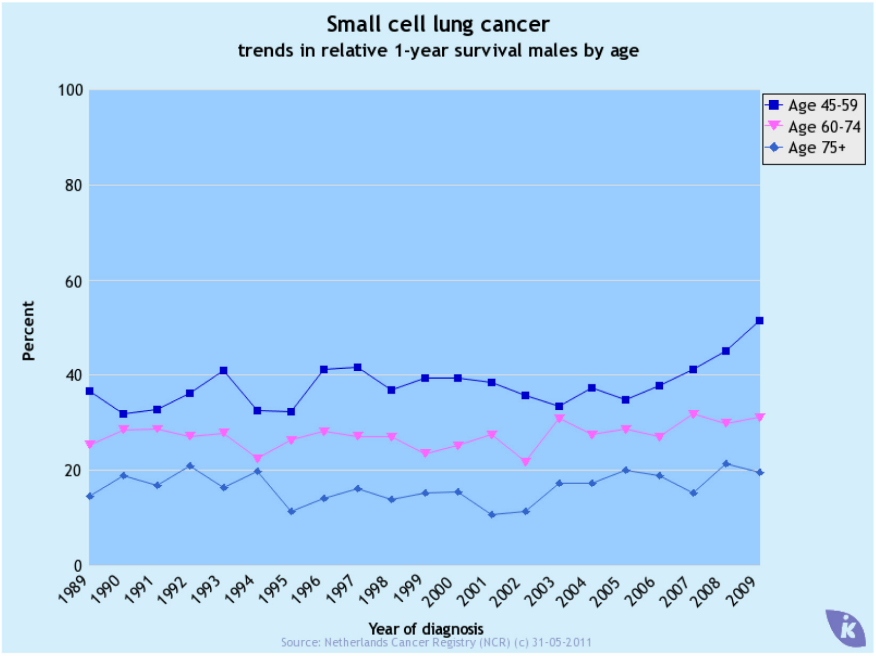
One-year relative survival has only statistically significantly increased over time for patients aged 45 to 59 years (p=0.01, Figures 4B and 4C): from 37% to 51% among males and from 42% to 51% among females. For those aged 60 or older, survival has not significantly improved (p=0.10 and 0.08 for males and females aged 60 to 74 years, respectively, and p=0.49 and p=0.68 for males and females older than 75 years, respectively).

Figure 4. *Trends in 1-year relative survival from SCLC in the Netherlands 2004-2009, by gender and age.*

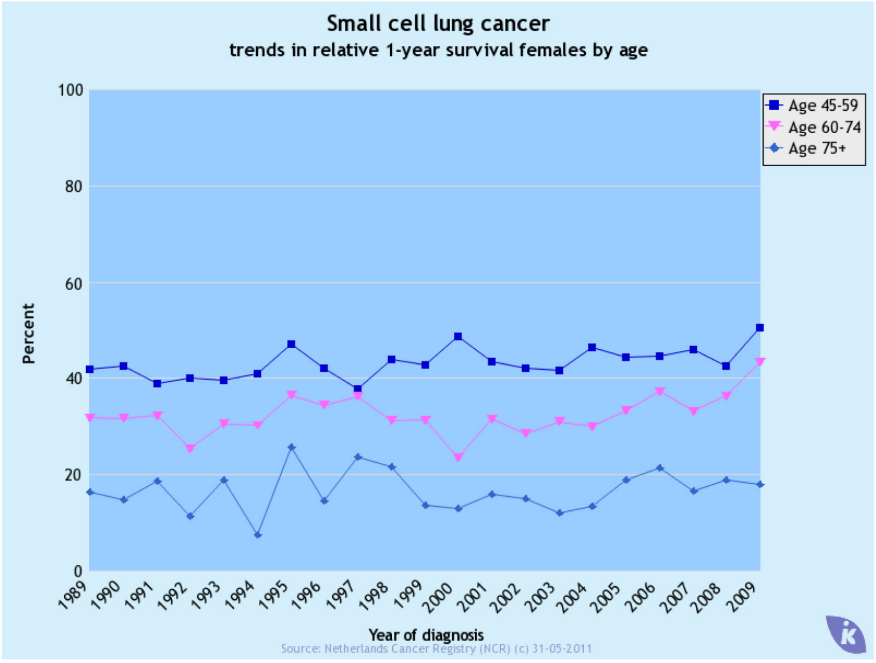
A. By gender



B. Males by age



C. Females by age



Discussion

The incidence of SCLC further decreased among men but increased among women. There was a trend towards more extensive disease. Despite better staging techniques and increased use of new treatment modalities, survival of the total group of unselected patients with SCLC has only improved for patients aged 45 to 59 years.

Trends in incidence

Due to smoking behavior, the incidence of lung cancer among Dutch men increased dramatically until the early 1980s, whereafter it has declined. In the mean time, there has been a shift from squamous cell and small cell carcinoma to adenocarcinoma.[1,16] The incidence of lung cancer among women used to be much lower than the incidence among men, but since the early 1980s, the incidence among women (all histologic subtypes) has been increasing markedly to the point that the incidence rates for men and women have converged toward each other. However, recently the incidence of lung cancer, including SCLC, among young Dutch women has started to decrease.[17]

Our study has shown that the proportion of patients diagnosed with ED has increased since the late 1990s. The same trend has been described in a recent study.[16] This stage shift is probably caused by the introduction of improved diagnostic techniques, such as improved availability and quality of computer tomography (CT) and magnetic resonance imaging (MRI), and positron emission tomography (PET). In the Netherlands, brain CT with contrast or brain MRI is standard of care in order to determine whether or not brain metastases are present. Introduction of PET-scanning differed between regions and took place between the early 1990s and the early 2000s. With the availability of these techniques the detection of previously occult distant metastases is facilitated, which has led to an upstaging from limited to extensive disease. The fact that overall 1-year survival of the total group did not significantly increase, but 1-year survival of both limited and extensive disease has improved over time, is in line with a stage shift.

Trends in treatment strategies

In our study the proportion of patients with LD receiving chemoradiation increased since the early 1990s, in accordance with the previously published studies. Clinical trials have shown that chemoradiation for LD has resulted in better local control and overall survival compared with chemotherapy alone.[18-22] After publication of a Norwegian study showing that a novel multimodal regimen is effective and well tolerated, concurrent chemoradiation became the new standard.[23] Trials of newer chemotherapy variations have failed to produce a regimen that is clearly superior to the two-drug combination of etoposide and cisplatin, which has gradually become the standard of care for both limited and

extensive disease.[18] Nowadays, standard treatment for LD SCLC in the Netherlands is cisplatin-etoposide (CE) combined with concurrent radiation therapy starting on the first day of chemotherapy course two. Sometimes cisplatin was substituted by carboplatin.[24-26]

In our study, the application of PCI increased over time. The brain remains an important site of recurrence. With improved systemic treatment and longer survival the frequency of brain metastases increases. PCI substantially reduces the risk of brain recurrence, but the effect on survival has been debated.[11, 27] In the Netherlands, PCI is applied in case of a partial response or stable disease to chemotherapy or chemoradiation. Because this is an observational study, we cannot evaluate the individual effect of PCI on survival in unselected patients.

In accordance with previous studies[28-34], elderly patients tend to receive less intensive treatment, either due to dose reductions of chemotherapy cycles or dose reductions/less frequent use of radiotherapy.[28, 29, 32, 35-42] The less intensive treatment could be related to expected toxicity. Previous studies report inconsistent findings with regard to increased toxicity for elderly patients with SCLC and for those with serious comorbidity. Most of these studies were clinical trials and may therefore be biased due to trial eligibility criteria (most of them only including relatively healthy elderly patients). A recent publication from our group has shown that 60 to 75% of elderly SCLC patients who were selected for chemotherapy or combined chemoradiation developed toxicity, and two thirds of patients could not complete the full chemotherapy.[34]

Trends in prognosis

Patients with SCLC have a very poor prognosis. In the majority of cases, death from recurrent disease occurs within 2 years of diagnosis. Previous studies have shown some progress in survival since the introduction of chemotherapy: 1-year relative survival rates improved from 18% in the 1970s to more than 30% in the 1990s for patients up to 70 years and from 9% to almost 20% for the elderly.[4,7,43-45] We have shown that survival for the group of SCLC patients as a whole has not further improved since the early 1990s, except for age group 45 to 59 years since the mid-2000s. However, when stratifying according to stage, 1-year relative survival for both patients with LD and ED has improved, as was also found in an American study.[46] The improvements in survival for each stage group with lacking improvement for the whole group might be explained by stage migration. The increased survival for patients aged 45 to 59 years might also be explained by improved treatment strategies.

In the Netherlands, prognosis was more favorable for females as compared with males. In Europe, 5-year survival for lung cancer in general used to be somewhat better for females than males.[47] This might be due to the fact that SCLC was diagnosed earlier among females (results from this study), but also due the fact that the mean age of female lung cancer patients was lower than that of male patients. Furthermore, males have more tobacco-related comorbidity

than females.[48] A favorable prognosis for females was also found for many other tumors.[49, 50]

Relative survival of SCLC clearly decreased with increasing age. This was also found in previous studies.[28, 35] The poorer prognosis among the elderly might be explained by several factors: an increased risk of mortality due to smoking-related comorbid conditions, such as chronic obstructive pulmonary disease (COPD), or a poor performance status, death due to a higher risk of complications of treatment (also related to decreased organ functions[51, 52]), or death of cancer due to less aggressive treatment. The latter could lead to a lower response rate and a higher recurrence rate among elderly patients. Previous studies indicated that prognosis for elderly patients with LD SCLC was not worse compared with younger patients after adjustment for differences in treatment modality, gender and comorbidity.[29, 30, 35, 38, 53] The fact that survival has not significantly improved for elderly patients in our study indicates that elderly did not yet benefit from improved treatment strategies.

Our population-based study made use of a large, high-quality population-based dataset to report on an unselected group of patients with SCLC, and this enables reliable comparison with other studies of unselected patients. Interpreting the prognostic effects of the different treatment modalities would be more difficult, though, since selection for treatment by the physician has played an important role. Caution is therefore warranted in comparing the outcomes with those from selected patient groups in clinical trials.

Conclusions

The diverging trends in incidence of SCLC between Dutch males and females followed the trends in smoking behaviour in the past. Improved staging resulted in improved survival for both stage groups, while survival of the total group has only significantly improved for patients aged 45 to 59 years. The latter is possibly related with improved treatment strategies. As survival is still very poor, prevention of lung cancer remains important.

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A prospective study of the timing and cost-effectiveness of bronchial washing during bronchoscopy for pulmonary malignant tumors

Chapter 4

van der Drift MA, van der Wilt G, Thunnissen FBJM, Janssen JP.

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Abstract

Introduction: The value of obtaining washings during fiberoptic bronchoscopy in the workup of lung cancer is controversial. Moreover, the optimal timing of washing relative to biopsy and brushing is not known. In this study the diagnostic yield of washings before and after biopsy and brushings were compared. The different diagnostic strategies were assessed in terms of yield and costs.

Patients and methods*:* A prospective study was performed from 2001 to 2003 in a secondary care medical centre. Two hundred twenty-one patients underwent flexible bronchoscopy and the diagnostic yield of washings before biopsy and brushing (strategy I) and after biopsy and brushing (strategy II) specimens were assessed. Using the known probabilities and costings for various bronchoscopic procedures, the expected utility of a number of diagnostic strategies was estimated.

Results: Patients (147 males and 74 females) were included in whom a definite cytologic or histologic diagnosis of pulmonary malignancy had been made. The diagnostic yield of strategy I was 72% for visible tumors and 36% for nonvisible tumors. For strategy II it was 74% for visible tumors and in 42% for nonvisible tumors. The comparison of strategies I and II for both visible and nonvisible tumors revealed that 176 cases were concordant (80%); in 19 cases (9%) the cytologic analysis of washings in strategy I was positive for malignancy and negative in strategy II. In 26 cases (12%) washings in strategy II were positive and negative in strategy I ($p = 0.37$). An analysis of the diagnostic yield of both washings in visible tumors and nonvisible tumors showed no significant difference. In 13 patients, a diagnosis of malignancy was established only by washings (6%). Confining the laboratory investigations of washings or brush samples to those cases in which the initial findings of the biopsies are negative (the two-stage procedure) is more cost-effective than examining all biopsy, brushing, and washing specimens. In patients with visible tumors, brushing or washing in addition to biopsy is equally cost-effective; in patients with nonvisible tumors, biopsy combined with washing is the preferred option.

Conclusions: No difference in the diagnostic yield could be demonstrated for washings before or after biopsies and brushings. Although the additional diagnostic yield of washing and brushing during bronchoscopy is relatively low, it is cost-effective to use these procedures in the diagnostic workup of patients who are clinically suspected of having a pulmonary malignancy.

Introduction

To establish a cytologic or histologic diagnosis in suspected lung cancer, flexible bronchoscopy is an essential step in the workup. Washings, brushings and forceps biopsies are often combined to increase the diagnostic yield.[1-5] The usefulness of obtaining washings is still subject to discussion.[6] The diagnostic yield for washings in patients with endoscopic visible (central) tumors varies from 49% to 76% and is similar to the yield for brushings (52% to 77%) but is inferior to the yield of biopsies (71% to 91%).[5,7-10] The diagnostic yield of washings in patients with endoscopic nonvisible (peripheral) tumors varies from 35% to 52% and is similar to the yield for brushings (26% to 52%) and for biopsies (36 % to 61%).[5,7-10]
The optimal timing of bronchial washing (i.e. whether before or after biopsy and brushing) is not clear. In some studies washings were obtained before biopsy and brush samples had been taken[8-10], whereas in others washings were obtained after other specimens were collected.[11-13] Only two abstracts have been published about the timing of bronchial washing at flexible bronchoscopy, but these have, to our knowledge, never been published as full articles.[14,15]
Of increasing interest are the costs of several bronchoscopic techniques to obtain a diagnosis in lung cancer. Since the diagnostic yield of washings and brushings is relatively modest, one might consider omitting these procedures altogether. However, the further diagnostic tests used if the above tests are negative are invasive, imposing a burden on the patient and incurring considerable costs. Therefore, even with a relatively low yield, washings and brushings may still be worthwhile. Govert et al concluded in his study that the collection of biopsies and either washings or brushings is probably the most cost-effective way to obtain a diagnosis in patients with endoscopic visible tumors.[16] The cost-effectiveness of bronchoscopic procedures in patients with endoscopic nonvisible tumors has, to our knowledge, not been studied.
The aim of this study was to compare the diagnostic yield of washings performed before and after brushings and biopsies during flexible bronchoscopy prospectively in patients with lung cancer. Also, different diagnostic strategies were assessed in terms of diagnostic yield and costs.

Patients and methods

From 2001 to 2003, a prospective study in a secondary care medical centre was performed to assess the diagnostic yield of washings before biopsies and brushings (strategy I) and after biopsies and brushings (strategy II). Patients suspected of lung cancer by signs, symptoms or chest radiograph findings underwent flexible bronchoscopy. An endoscopic visible tumor was defined as an exophytic tumor or mass, mucosal infiltration or submucosal lesion. An endoscopic nonvisible tumor was defined as a normal bronchial system or bronchial narrowing due

to extrinsic compression with normal mucosa. Of these, only patients in whom a definite cytologic or histologic diagnosis of pulmonary malignancy was made were included in the study. If the bronchoscopic techniques did not reveal a diagnosis, other techniques such as transbronchial needle aspiration (TBNA), transthoracic needle aspiration (TTNA), mediastinoscopy, thoracotomy and biopsies of extrapulmonary lesions were used. Specimens were considered to be adequate if alveolar macrophages and bronchial epithelial cells were present. The amount of alveolar macrophages may vary, but in most cases they are also present in the case of a centrally located tumor. Strictly speaking, the presence of alveolar macrophages may not be required, but they are a constant finding in the great majority of the washings.
Bronchoscopies were performed by the specialist pulmonologists or by pulmonary residents (under specialist supervision) of our hospital. Flexible bronchoscopes (model 1T240 (working channel 2.6 mm) and model 1T30 (working channel 2.8 mm); Olympus; Tokyo Japan) were used. Unless contraindicated, patients were pre-medicated with diazepam 5 mg orally and intramuscular atropine 0.25 mg, 30 minutes before undergoing the procedure. Local anesthesia was achieved with 5 ml of 4% lidocaine spray applied to the upper airway including the laryngeal area. This was followed with one to two aliquots of 2.5 ml of 4% lidocaine instilled over the mucosa of the trachea, carina and bronchial tree during bronchoscopy.

Bronchoscopic technique

In patients with visible tumors, a first washing (washing I) was performed using 20 ml of saline solution. When the recruitment was low after suctioning due to coughing or if the aliquot was to be separated for microbiologic analysis, another 20 ml was injected and suctioned. After washing I, biopsies were taken of the suspected area and a brushing was performed. Finally, a second washing (washing II) of the suspected area was done, using the same technique as described for the washing I.
In patients with a tumor that was not visible through the bronchoscope, the washings were done in the corresponding segmental bronchus. After the washing I, brushing of the tumor was done under fluoroscopic guidance. If the brush could reach the tumor, biopsies were performed under fluoroscopic guidance. Then, washing II was performed.
The returned aspirates of the washings were fixed in a volume of 20 to 30 ml of fixation fluid (Fixcyt® containing 50% alcohol and 2% carbowax) and sent for cytologic examination. Biopsies were obtained using biopsy forceps (Olympus). Multiple biopsies (two to eight) were obtained for each patient, unless significant bleeding followed after the first specimen was obtained. The biopsy specimens were sent in neutral buffered formalin for histologic examination. Brushings were obtained with a disposable cytology brush (1.7 mm; Endomed; Phoenix, AZ). The material returned on the brush was spread on a slide, which was immediately fixed in 70% alcohol or by a spray fixative (Cerfix Spray Fixative® (containing 2-Propanol, Poly ethylene Glycol 300 and Acetone) Zschimmer and Schwartz, Inc; USA) and

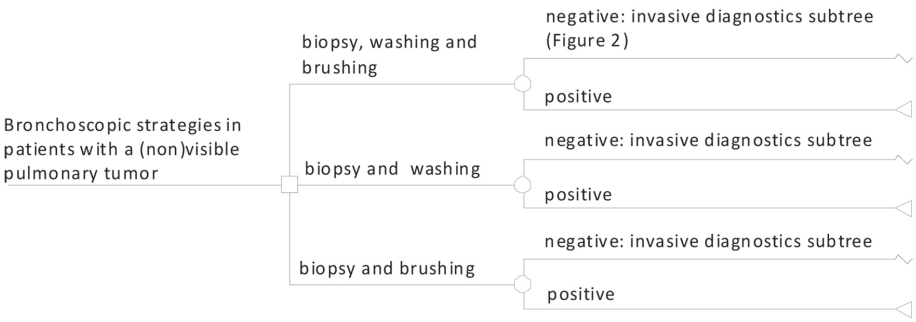
the same brush was then agitated in about 20 to 30 ml of fixation fluid and cut off. The slides, rinsing fluid and brush were combined for cytologic examination of brushing.

Statistical and cost-effectiveness analysis

The level of agreement between both washings was tested with McNemar's test, using a two-sided significance level of 0.05. A decision model was developed to analyze specimen collection strategies of bronchoscopy using appropriate software (DATA, version 4.0; Tree Age software; USA) (Figure 1). The following three approaches were compared: 1. bronchoscopy with biopsy, brushing and washing; 2. bronchoscopy with biopsy and washing; 3. bronchoscopy with biopsy and brushing. The data were analyzed separately for patients with visible and nonvisible tumors. It was assumed that, in the case of negative findings, a diagnostic workup was continued by conducting a second bronchoscopy (in the case of a visible tumor) or by using more invasive diagnostic procedures, including thoracotomy, TTNA, mediastinoscopy, TBNA or biopsies of extrapulmonary lesions (Figure 2). Using the probabilities and costs of the various procedures, the expected value of a number of diagnostic strategies was estimated. Probability estimates were derived from our study; cost data were derived from the financial department of our hospital (Table 1). The expected value of an option is the sum of the values of all the outcomes of that option, with each value being weighed by the probability that the consequence will occur[17]. In our model, confirming the diagnosis of lung cancer was used as outcome, while the monetary costs of the applied procedures were used as value inputs.

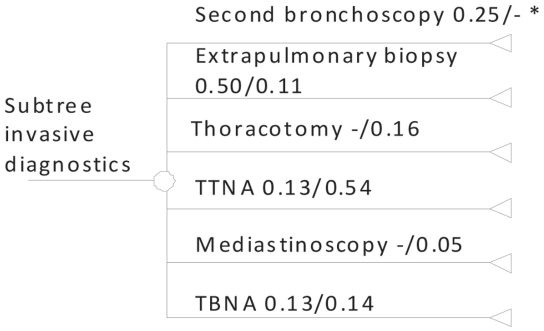
In another decision model in the diagnostic workup, biopsy was routinely performed in combination with brushing and washing, but washing or brushing samples were submitted for laboratory investigations only in the case of a negative biopsy finding (two-stage procedure). The structure of this decision model is depicted in Figure 3.

Figure 1. *Structure of the decision model comparing three different bronchoscopic strategies in the workup of patients with clinically suspected lung cancer.*



Positive = malignancy, negative = no malignancy

Figure 2. *Probability of using either of the invasive diagnostic procedures following a negative biopsy, washing and brushing to establish the diagnosis lung cancer.*



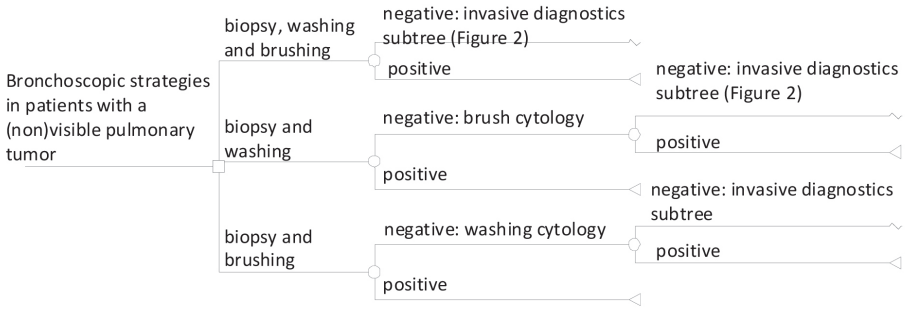
** probability in visible tumor/ nonvisible tumor*
TTNA = transthoracic needle aspiration; TBNA = transbronchial needle aspiration

Table 1. *Unit costs of various diagnostic procedures and of out-patient visits, day care, and hospitalisation.*

Bronchoscopy (including biopsy, washing, and brushing)	625
Cytology washing	103
Cytology brushing	103
Histology biopsy	113
Transbronchial needle aspiration	174
Transthoracic needle aspiration	174
Mediastinoscopy	802
Thoracotomy	1346
Biopsy of extrapulmonary lesion (including histology)	1079
Out-patient visit	235
Day care	423
Hospitalisation (per day)	1875

**Data are presented as prices in Dollars*

Figure 3. *Decision model reflecting the two-stage procedure: cytology of brushing (or washing) only in case of negative findings of biopsy and washing (or brushing).*



Positive = malignancy, negative = no malignancy

Results

Two hundred eighty-one patients suspected of lung cancer underwent flexible bronchoscopy; 221 of these patients were proven to have a new pulmonary malignancy. The visible endobronchial abnormalities (137 patients) were exophytic tumor in 76 patients, mucosal infiltration in 37 patients, and submucosal lesions in 24 patients. Eighty-four patients had a nonvisible tumor. The study population had the following characteristics: mean age 65.6 years, range 38 to 88 years; 147 men and 74 women.

The diagnostic results of visible and nonvisible tumors are presented in Table 2. A diagnosis was obtained from washing, biopsy, or brushing specimens from visible tumors in 129 of 137 patients (94%). The highest diagnostic yield was obtained by forceps biopsy in mass lesions (92%). The combined diagnostic yield of all three techniques for mass lesions was 97%, for infiltration was 92% and for submucosal lesions was 88%. A diagnosis was obtained from washing, biopsy, or brushing specimens from nonvisible tumors in 47 of 84 patients (56%). The diagnostic yield of washings before biopsies and brushings for specimens of visible tumors was higher than the yield for nonvisible tumors (72% and 36%, respectively). The same is true for the yield for performing washings after biopsies and brushings (74% and 42%, respectively). The yields of washing I and II for submucosal lesions was lower (58% and 54%, respectively) than the yield for exophytic tumor (75% and 84%, respectively) or for mucosal infiltration (76% and 62%, respectively).

Table 2. *Diagnostic yield of bronchoscopic visible and nonvisible tumors: different bronchoscopic techniques.*

Bronchoscopic techniques	Cytologic or histologic diagnosis of malignancy		
	Visible tumors (n = 137)	Nonvisible tumors (n = 84)	Total (n = 221)
Washing I	99(72)	30 (36)	129 (58)
Biopsy‡	111/136 (82)	26/64 (41)	137/200 (69)
Brush‡	98 (72)	37/83 (45)	135/220 (61)
Washing II	101 (74)	35 (42)	136 (62)
Total diagnostic yield	129 (94)	47 (56)	176 (80)

**Data are presented as number of patients (%)*
‡Not in all cases biopsies and brushings were performed

The comparison of washing I and II for both visible and nonvisible tumors revealed that 176 cases were concordant (80%); in 19 cases (9%) cytologic analysis of washing I specimens was positive for malignancy, whereas that of washing II specimens was negative, and in 26 cases (12%) washing II specimens were positive whereas washing I specimens were negative (p = 0.37) (Table 3). There were no significant differences in the yields of washing I and II for visible tumors (p = 0.85) or for nonvisible tumors (p = 0.33). A diagnosis of pulmonary malignancy was made exclusively by washing specimens in 13 patients (6%) (Table 4).
The appearance of washing II specimens was blood-stained in almost every patient (89%), and the appearance of washing I specimens was blood-stained in 29% of the patients. The analysis of washing II specimens was inconclusive due to poor cellularity in 5 patients and of washing I specimens in 1 patient. The amount of saline solution used to obtain both washings was 20 ml for 87% of the patients with visible tumors and 68% of the patients with nonvisible tumors. In the other cases, > 20 ml of saline solution was used because of low recruitment or separation for microbiologic analysis. In patients with visible tumors, there were no differences in diagnostic yield for both washings whether 20 ml or > 20 ml of saline solution was used. In patients with nonvisible tumors, the diagnostic yield for washings performed with > 20 ml of saline solution was higher (washing I, 43%; washing II, 52%) than for those performed with only 20 ml (30% and 38%, respectively).

Table 3. *Comparison of diagnostic yield for washing I and washing II for both visible and nonvisible tumors. Note that discordant cases are italic.*

	Washing II					
Washing I	Visible tumors (n = 137)[1)]		Non visible tumors (n = 84)[2)]		Total (n = 221)[3)]	
	Positive	Negative	Positive	Negative	Positive	Negative
Positive	86 (63)	13 (9)	24 (29)	6 (7)	110 (50)	19 (9)
Negative	15 (11)	23 (17)	11 (13)	43 (51)	26 (12)	66 (30)

**Data are presented as number of patients (%). Positive = malignancy, negative = no malignancy. P value = [1)] 0.85, [2)] 0.33, [3)] 0.37, respectively*

Table 4. *Diagnosis of pulmonary malignancy made exclusively by washings.*

	Visible tumors (n = 8)	Nonvisible tumors (n = 5)	Total (n = 13)
Washing I	4	1	5
Washing II	2	1	3
Both washings	2	3	5

**Data are presented as number of patients*

Forty-five patients had a nondiagnostic bronchoscopy of whom 37 patients had a nonvisible tumor. In these patients, a diagnosis of pulmonary malignancy was established by a second bronchoscopy (2 patients), TBNA of mediastinal lymphadenopathy (6 patients), TTNA (21 patients), mediastinoscopy (2 patients), thoracotomy (6 patients) and biopsies of extrapulmonary lesions (e.g. liver, adrenal gland) (8 patients).

The pathologic diagnosis of the pulmonary malignancies were squamous cell carcinoma (76 patients), adenocarcinoma (49 patients), undifferentiated large cell carcinoma (38 patients), small cell carcinoma (41 patients), carcinoid tumor (2 patients), bronchioloalveolar carcinoma (1), metastases from other organs (for example breast carcinoma, renal cell and other carcinomas) (14 patients). There were no false-positive results for the washings. Tumor cell type diagnoses were confirmed by resection samples in 28 patients who underwent thoracotomy.

Costs of various strategies in relation to the diagnostic yield

The diagnostic yield in patients with visible tumors was 0.94, 0.88 and 0.88, respectively, for biopsy, washing and brushing; for biopsy and washing; and for biopsy and brushing. The probability of using an invasive diagnostic procedure after a negative first bronchoscopy is summarized in Figure 2, reflecting the frequency of these procedures in our series. It is assumed that either of these procedures establishes the diagnosis lung cancer. The expected values of the various approaches were $1,247, $1,223 and $1,223, respectively, favoring the performance of bronchoscopy with biopsy and either washing or brushing. In the two-stage procedure, the estimated probability of establishing the diagnosis of lung cancer at the initial stage of the diagnostic workup remained unchanged (0.94, 0.88 and 0.88 respectively). If no malignancy was found, the diagnosis of lung cancer was established by examining either brush or washing samples. The estimated probability of establishing the diagnosis after cytology of the brushing or the washing was in both cases 0.50. In the absence of a diagnosis after the first bronchoscopy, the same more invasive diagnostic options and associated probabilities were modeled. The expected value of these approaches was $1,247, $1,156 and $1,156, respectively, favoring the two-stage procedure, irrespective of whether brushing or washing was used in combination with biopsies.

In patients with nonvisible tumors the diagnostic yields for the three approaches were 0.56, 0.46 and 0.49, respectively. The probability of using an invasive diagnostic procedure after a negative first bronchoscopy is summarized in Figure 2, again reflecting the frequency of these procedures in our series. The expected value of the various strategies was $2,084, $2,243 and $2,178, respectively, hence favoring the approach employing bronchoscopy with biopsy in combination with washing and brushing. In the two-stage procedure, the estimated probabilities of establishing the diagnosis of lung cancer at the initial stage of the diagnostic workup remained unchanged (0.56, 0.46 and 0.49, respectively). The estimated probabilities of establishing the diagnosis after cytology of the washing samples (in the case of a negative test result after brushing) or cytology of the brushing samples (in the case of a negative test result after washing) was 0.12 and 0.18, respectively. In the absence of a diagnosis after the first bronchoscopy, the same more invasive diagnostic options and associated probabilities were modeled. The expected values of these approaches were $2,084, $2,043 and $2,051, respectively, favoring the approach employing bronchoscopy in combination with washing, followed by cytology of the brushing in case of an initially negative test result.

Discussion

The aim of this study was to assess the diagnostic importance of the timing of washings at bronchoscopy in the investigation of patients suspected of having a pulmonary malignancy. The diagnostic yields in patients with visible tumors were 72% after washing I and 74% after washing II, which corresponds to values from other studies.[5,7-10] The extent of visible tumors influences the diagnostic yield. The yields of washing I and II for submucosal lesions were lower (58% and 54%, respectively) than for exophytic tumor (75% and 84% respectively) or for mucosal infiltration (76% and 62%, respectively). Other studies have given similar results[18,19], although the definition of visible tumor varied between studies. The diagnostic yield for washings in patients with nonvisible tumors was lower than was expected (washings I, 36%; washing II, 42%). In other studies it varies from 35% to 52%.[5,7-10]

When comparing washings before and after biopsies and brushings, no significant differences in the diagnostic yield could be demonstrated for both visible and nonvisible tumors in our study. In the literature, no comparable study has been found. Raymond et al described in an abstract no difference in diagnostic yield relative to the timing of washings for central tumors.[14] In peripheral nonvisible tumors, however, the yield for bronchial washing after biopsy and brushing was significantly higher than the yield for washing before biopsy and brushing (45% and 25%, respectively). In another abstract regarding visible tumors, Scriven et al showed a higher yield for washings after biopsy and brushing.[15]

In 45 patients, the diagnostic yield of washing I was different from washing II. The question remains how the difference in yield between both washings can be explained. Arroliga and Matthay suggested that washings should be obtained after forceps and brush biopsies were performed to increase the diagnostic yield by referring to a study performed by Chaudhary et al.[1,12] Chaudhary et al argued that more tumor cells would be freed after manipulation techniques were performed. However, Chaudhary et al. did not compare washings before and after biopsies and brushes in their study. The hypothesis of obtaining more tumor cells by manipulation was not confirmed in our study. Another explanation for the different results of washing I and washing II might be patient-related factors in bronchoscopy e.g. cooperation of the patient or coughing. Although washing II specimens were blood-stained in almost every patient, this did not complicate the cytologic examination by the pathologist. It is not clear if the amount of saline solution that was used to perform a washing influences the diagnostic yield. In our study, in patients with nonvisible tumors there was a trend for a higher diagnostic yield in washings performed with larger amounts of saline, i.e. > 20 ml. Pirozynski et al showed that performing a bronchoalveolar lavage using 200 ml of NaCl was a valuable diagnostic tool in detecting peripheral pulmonary malignant neoplasms, especially in adenocarcinomas, bronchioloalveolar carcinomas and

tumors exceeding 3 cm.[20] Others have suggest that only small volumes, i.e. < 20 ml, are required for a bronchial washing.[6]

In 13 patients (6%), biopsies and brushings showed no malignancy and diagnosis of malignancy was established only by washings. Some studies have reported that adding bronchial washings to biopsies and brushings increases the diagnostic yield[8,9,11-13,21,22], whereas others have reported no additional value of washings.[7,19,23,24] The additional value of a washing as the only test providing a diagnosis varies from 1.5% to 5% in visible tumors and from 7.4% to 9.5% in nonvisible tumors. In our study, the variation was 2.9% (washing II) to 4.4% (washing I) in visible tumors, and 4.8% for both washings in patients with nonvisible tumors.
In one study on the cost-effectiveness of adding a cytologic specimen, a modest increase in the sensitivity of fiberoptic bronchoscopy was found.[16] Although the diagnostic yield of washing and brushing during bronchoscopy is relatively low, it is still cost-effective to use these procedures in the diagnostic workup of patients who are clinically suspected of having a pulmonary malignancy. Clearly, this is due to the relatively high costs associated with the more invasive procedures that are used in cases with negative biopsy, brushing and washing results. The two-stage procedure in which laboratory investigations of washing or brushing are confined to those cases where initial findings are negative could be a more cost-effective option. This is due to the saving of the costs of laboratory investigations, while at the same time fully exploiting the potential of these minimally invasive procedures at the expense of a delay in analysis of the cytologic specimens of about a day. The extra costs of administration for storing brushing - and washing samples were minimal (3.5 dollar) and were therefore neglected. In the two-stage procedure, biopsy combined with brushing or washing was equally cost-effective in patients with visible tumors; in patients with nonvisible tumors, biopsy combined with washing is the preferred option.

Conclusion

The timing of washings during bronchoscopy in the diagnosis of lung cancer (before or after biopsy and brushing) does not influence the diagnostic yield. Although the diagnostic yield of washing and brushing during bronchoscopy is relatively low, it is cost-effective to use these procedures in the diagnostic workup of patients who are clinically suspected of having a pulmonary malignancy. Confining laboratory investigations of washing or brushing to those cases where initial findings of the biopsies are negative (the two-stage procedure) is more cost-effective than examining all biopsy, brushing and washing specimens. In patients with visible tumors, biopsy combined with brushing or washing is equally cost-effective. In patients with nonvisible tumors, biopsy combined with washing is the preferred option.

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Diagnosing peripheral lung cancer: the additional value of RASSF1A methylation and *KRAS* mutation analyses in washings in nondiagnostic bronchoscopy

Chapter 5

van der Drift MA, Prinsen CFM, Knuiman GJ, Janssen JP, Dekhuijzen PNR, Thunnissen FBJM.

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Abstract

Introduction: The diagnostic yield of bronchoscopy in patients with endoscopic nonvisible (peripheral) tumors varies from 40% to 56%. Increasingly, molecular markers in bronchial washings are being investigated to improve the diagnostic yield. The aim of this study was to analyze the diagnostic value of Ras associated domain family 1A gene (RASSF1A) methylation analysis in washings in nondiagnostic bronchoscopy in the analysis of patients with suspected lung cancer who had peripheral tumors. Furthermore, the additional diagnostic value of Kirsten rat sarcoma 2 viral oncogene homolog *(KRAS)* mutations with RASSF1 methylation was analyzed.

Patients and methods: From a prospectively collected series, 129 lung cancer patients and 28 controls were analyzed retrospectively regarding the methylation status of the promoter region of the RASSF1A gene by quantitative methylation-specific PCR (qMSP) and *KRAS* point mutations by using the sensitive Point-EXACCT method.

Results: A total of 40% of the lung cancer patients had peripheral tumors, and 17 patients had a nondiagnostic bronchoscopy. In these patients, RASSF1A methylation was detected in the washings of 4 patients (24%), and *KRAS* mutations were detected in the washings of 2 patients (12%). In total, 29% of the false-negative or doubtful cytology results were accompanied by RASSF1A methylation or *KRAS* mutation results that were highly suggestive of malignancy. The proportion of RASSF1a methylation was significantly higher in central and larger tumors. No relevant RASSF1A methylation was detected in control samples.

Conclusions: Our data suggest that the molecular analysis of 2 biomarkers in nondiagnostic bronchial washings may better guide diagnostic procedures in patients with suspected lung cancer.

Introduction

Flexible bronchoscopy is an essential step in the analysis of lung cancer. Washings, brushings and forceps biopsies are often combined to increase diagnostic yield.[1-3] The combined diagnostic yield of patients with endoscopic visible (central) tumors, mostly squamous cell carcinomas and small cell carcinomas, varies from 49% to 94%. The combined diagnostic yield of patients with endoscopic nonvisible (peripheral) tumors, mostly large cell carcinomas and adenocarcinomas, is lower, varying from 40% to 56%. In cases of nondiagnostic bronchoscopy, supplementary diagnostic procedures, such as transbronchial needle aspiration(TBNA), ultrasound-guided endobronchial or endoscopic fine needle aspiration (EBUS-FNA or EUS-FNA), trans-thoracic needle aspiration (TTNA), mediastinoscopy or even thoracotomy, are necessary to establish a lung cancer diagnosis.[3] If the diagnosis of lung cancer could be established more easily, then (invasive) supplementary diagnostic procedures could be avoided. The detection of (epi) genetic abnormalities in bronchial washings may enhance lung cancer diagnosis without the need for (invasive) supplemental diagnostic procedures.

Increasingly, molecular markers in bronchial washings and bronchoalveolar lavage (BAL), including microsatellite alterations and epidermal growth factor receptor (*EGFR)* mutations, have been under investigation to improve the diagnostic yield of bronchoscopy.[4-8] In the past decade several studies have shown that hypermethylated genes could be detected in bronchial washings.[9-14] Hypermethylation of tumor-related genes is a mechanism for silencing tumor suppressor genes.[15,16,17] RASSF1A (Ras-association domain family 1A gene) has a tumor suppressor function and RASSF1A methylation has been found in the tumors of approximately 40% of non-small cell lung cancer (NCSLC) patients.[18,19,19,20] In bronchial aspirates, RASSF1A methylation was present in 18% to 46% of NSCLC patients compared with 0% to 4% of patients without cancer.[11,12,14,21,22] RASSF1A methylation was positively correlated with the number of tumor cells in bronchial aspirates. Currently, in bronchial washings with no cytologic diagnosis of malignancy, it is not clear whether methylation can be detected.
Activation of the Kirsten rat sarcoma 2 viral oncogene homolog *(KRAS)* by point mutations in codon 12 occurs in approximately 30% of pulmonary adenocarcinomas. In previous reports, the diagnostic value of *KRAS* mutations in bronchoalveolar lavage fluids is limited due to the relatively low prevalence of *KRAS* mutations in NSCLC.[23,24,25]

The aim of this study was to analyze the diagnostic value of RASSF1A methylation in the bronchial washings of patients with peripheral tumors suspected of lung cancer without diagnosis after bronchoscopy. The additional diagnostic value of combined RASSF1A hypermethylation and *KRAS* mutation analyses was assessed.

Patients and methods

Patients

From a prospective lung cancer study endobronchial washing samples were collected from 2001 to 2003 and retrospectively analyzed for the diagnostic value of *KRAS* mutations and RASSF1A methylation.[2] A total of 281 patients suspected of having lung cancer by symptoms or chest X-rays underwent flexible bronchoscopy. Of these, 221 patients were diagnosed with (lung) cancer. Patients were included in this study when sufficient material was available for molecular analysis (129 cancer patients and 28 controls).

Patients in whom no lung cancer was found after analysis served as controls to assess methylation status. The mean follow-up time of the controls was 6.9 years (range: 6-8 years); none of them developed (lung) cancer. Approval of the local ethics committee was obtained. Institutional guidelines do not require written permission because no supplementary procedures were performed and clinical data were anonymized.

Bronchoscopic technique

The routine diagnostic procedure consisted of bronchial brushing and washing for cytologic examination, and biopsies for histologic examination. Peripheral tumors were defined as endobronchial nonvisible tumors, including a normal bronchial system or bronchial narrowing due to extrinsic compression with normal mucosa. In cases of peripheral tumors, brushings and biopsies were performed under fluoroscopic guidance in the corresponding segmental bronchus. Bronchial washings were performed with 20 to 30 ml saline in the segmental bronchus that had been identified endoscopic or radiographic to contain the tumor. The returned aspirates of the washings were fixed in 20-30 ml of fixation fluid (Fixcyt® containing 50% ethanol and 2% Carbowax) and sent for cytologic examination. DNA was isolated from the cytology samples as described previously[26] and RASSF1A hypermethylation and KRAS mutation analyses were performed.

RASSF1A methylation analysis

A maximum of 1 µg of human DNA that was purified from the cytology samples was bisulfite treated using the EZ DNA Methylation –Gold kit (Zymo Research, Baseclear, Leiden, the Netherlands). As a positive control, in vitro methylated human genomic DNA (M.Sss1, New England Biolabs, Westburg, the Netherlands) was used. In bronchial washings, the methylation status of the promoter region of the RASSF1A gene was determined by quantitative methylation-specific PCR (qMSP) as described previously.[27] In short, methylated human DNA was quantified on a LightCycler 1.5 instrument after absolute quantification using the second derivative maximum method (Roche Diagnostics, Almere, the Netherlands). MyoD1 was used as a control gene. All assays were performed at least twice. When two results were the same, the outcome was considered to be conclusive.

In cases of inconclusive outcomes the assay was repeated once more, which was decisive for the outcome.

KRAS mutation analysis

KRAS analysis was performed according to the Point-EXACCT method with a modification that included binding of the capture probe from the microtiterplate to a streptavidin-coated microarray glass slide.[28,29] The oligonucleotide sequences for the 1st and 2nd nucleotide of the triplet of codon 12 and PCR primers were as described previously [28] and supplied by Eurogentec (Seraing, Belgium). A16-atom spacer arm was used to attach the biotin group to the oligonucleotides, whereas a 12-atom spacer arm was used in the attachment between the digoxigenin (DIG) and the penultimate 3' terminal nucleotide. Streptavidin-coated glass slides were purchased from Dot Diagnostics, the Netherlands. Mutant and wild type capture probes for *KRAS* codon 12 were printed in triplicate. Capture probe spotting on the slide, PCR, hybridization, ligation and immunodetection were performed as described previously. Control samples were used each day and consisted of PCR products derived from 1 µg template samples with 25% and 4% *KRAS* mutated A549 or SW480 tumor cells, respectively, and negative control products. Ligation control spots were available on each processed slide for the wild type *KRAS* sequence.

Statistical analysis

Data were analyzed with SPSS/PC+, version 16.0 (SPSS, Inc., Chicago, IL). Descriptive statistics were used for clinical characteristics and comparisons were performed by t-tests or Chi-square tests, as appropriate. A p-value of ≤ 0.05 was regarded as significant.

Results

Baseline characteristics

The clinical characteristics of 129 patients and 28 controls are summarized in Table 1, 70% were men. Fifty-one patients had peripheral tumors. Pathologic analysis showed squamous cell carcinoma in 37% of the patients and non-squamous cell carcinoma in 45%. Mixed tumors consisted of NSCLC and small cell lung cancer (SCLC) cells. In 17 of the patients, no cytologic or histologic diagnosis of lung cancer was made by bronchoscopy. All cytologic samples were adequate. Two samples consisted of atypical cells and were interpreted as nondiagnostic after additional diagnostic procedures were performed. Additional diagnostic procedures to establish a lung cancer pathology diagnosis consisted mostly of TTNA (59%); the other diagnostic procedures are shown in Table 2. The biopsies of the extrapulmonary lesions consisted of a thoracentesis of the pleural fluid (pleuritis carcinomatosa) and a biopsy of an epidural tumor.

The diagnostic yield of the histologic and cytologic bronchoscopic analysis from

central and peripheral tumors is presented in Table 3. As expected, the diagnostic yield of central tumors was significantly higher than that of peripheral tumors for both histologic (85% and 37%, respectively) and cytologic (100% and 47%, respectively) analyses. In 22% of patients with peripheral tumors, no biopsies were performed.

Table 1. *Clinical and tumor characteristics of lung cancer patients and controls.*

Clinical characteristics	Cancer patients (n = 129)			Controls (n = 28)
	Total	Central tumors	Peripheral tumors	
Total number	129	78 (60)	51 (40)	28
Age (min-max) (years)	65 (38-88)	66 (43-88)	64 (38-84)	70 (34-81)
Sex				
Male	90 (70)	56 (72)	34 (67)	20 (71)
Female	39 (30)	22 (28)	17 (33)	8 (29)
Histology				
SCC	48 (37)	31 (40)	17 (33)	
AC ± BAC	37 (29)	17 (22)	20 (39)	
LCC	21 (16)	10 (13)	11 (22)	
SCLC	19 (15)	17 (22)	2 (15)	
Mixed tumors	2 (2)	2 (3)	2 (4)	
Adenosquamous	1 (1)	-	1 (2)	
Carcinoid	1 (1)	1 (1)	-	

SCC = squamous cell carcinoma; AC = adenocarcinoma; BAC = bronchioloalveolar carcinoma; LCC = large cell carcinoma; SCLC = small cell lung cancer

Table 2. *Additional diagnostic procedures to establish lung cancer diagnosis when bronchoscopy was negative (n = 17).*

Diagnostic procedures	Number of patients
Transbronchial needle aspiration (TBNA)	1
Transthoracic needle aspiration (TTNA)	10
Mediastinoscopy	2
Thoracotomy	2
Biopsies of extrapulmonary lesions	2

RASSF1A methylation and KRAS mutation in bronchoscopic samples

The results of the RASSF1A methylation and *KRAS* mutation analyses in patient washings are presented in Table 3. RASSF1a methylation was higher in central tumors (50%) than in peripheral tumors (31%) ($p = 0.03$). The median methylation ratio was 195.4 (range: 0.64–74,949.6) for central tumors and 24.9 (range: 0.3–1,319.2) for peripheral tumors ($p = 0.003$). The number of *KRAS* mutations did not differ between central tumors (9%) and peripheral tumors (6%) ($p = 0.78$). In 6 patients (5%), both RASSF1A methylation and *KRAS* mutations were found, 4 in central tumors and 2 in peripheral tumors.

Table 3. *Diagnostic yield of histologic, cytologic and biomarker analysis from central and peripheral tumors.*

Pathologic analysis	Central tumors (n = 78)	Peripheral tumors (n= 51)	P-value
Histology:			
Positive	66 (85)	19 (37)	< 0.001
Negative	11 (14)	21 (41)	
No biopsy taken	1 (1)	11 (22)	
Histology negative, cytology:			
Positive	12 (100)	15 (47)	< 0.001
Negative	-	17 (53)	
RASSF1A methylation present:			
Total	39 (50)	16 (31)	0.03
Cases with negative biopsy	1	3	
Cases with negative biopsy and cytology	-	4	
K-ras mutation present:			
Total	7 (9)	3 (6)	0.78
Cases with negative biopsy	-	-	
Cases with negative biopsy and cytology	-	2	

KRAS= Kirsten rat sarcoma 2 viral oncogene homolog; RASSF1A = Ras associated domain family 1A gene. Data are presented as No. (%).

In 17 patients with nondiagnostic bronchoscopies, RASSF1A methylation was detected in the washings of 4 patients (24%), or 8% of the overall group of 51 patients with peripheral tumors. The median methylation ratio in this group was 7.6 (range, 1.4-28.6). In 2 patients with nondiagnostic bronchoscopies, *KRAS* mutations (both TGT mutations in codon 12) were detected in the DNA of the washings. In patients with bronchoscopy without a diagnosis in the conventional procedure, the combined added diagnostic value of *KRAS* mutations and RASSF1 methylation was 29%.

Detailed clinical information about the 17 patients with nondiagnostic bronchoscopies is presented in Table 4. RASSF1A methylation was found in 4 of the 17 patients. The mean tumor size in all patients was 3.3 cm (range, 1.5–7.0 cm). Mean tumor size in the patients with methylated samples was 4.6 cm (range, 3–7 cm), compared with a mean tumor size of 2.9 cm (range, 1.5–4.5 cm) in patients in which hypermethylation was not detected (p = 0.04). The *KRAS* mutations were present in larger tumors (4.0 and 4.5 cm) and patients were staged T2N0M0. RASSF1A methylation was present in 1 current smoker, 2 former smokers (> 20 pack years) and 1 never smoker, and *KRAS* mutations were present in current smokers. In 1 out of 17 patients, both RASSF1A methylation and *KRAS* mutations were found.

Table 4. *Clinicopathologic variables for RASSF1A- and KRAS positive cases in those with negative conventional diagnostic bronchoscopy (n=17)*

Tumor and clinical characteristics	**Patients No. (%)**	**RASSF1A methylated**		**No. of *KRAS* mutations (%)**
		No. (%)	***Median ratio***	
Total	17	4 (24)	7.6	2 (12)
Histology				
SCC	4	1/4 (25)	18.2	
AC ± BAC	6	1/6 (17)	1.3	1/6 (17)
LCC	7	2/7 (29)	7.4	1/7 (14)
Stage				
I	7	2/7 (29)	7.6	2/7 (29)
II	1	-		-
III	2	-		-
IV	7	2/7 (29)	14.9	-
Smoking history				
Current	6	1/6 (17)	7.9	2/6 (33)
Former	7	2/7 (29)	4.4	-
Never	2	1/2 (50)	28.5	-
Missing	2	-		-

SCC = squamous cell carcinoma; AC = adenocarcinoma; BAC = bronchioloalveolar carcinoma; LCC = large cell carcinoma

Thirteen controls were current smokers, 9 were former smokers (range: 8-40 pack years), 2 never smoked and smoking information was missing in 4 controls. RASSF1A methylation was found in 1 control with a low methylation ratio (0.3), a smoking woman who was diagnosed with empyema with Streptococcus milleri.
The *KRAS* mutation analysis of the washings was compared with the *KRAS* mutation analysis of the tumor biopsy specimens or resection samples. In the washing of 1 patient, the same mutation was detected as in the tumor. In the other patient, a *KRAS* mutation was found in the washing but not in the biopsy. In a third patient with *KRAS* mutation in the tumor, the mutation was not found in the washing.

Discussion

This study shows that the detection of two biomarkers in endoscopic nonvisible lung cancer without a pathology diagnosis by bronchoscopy may better guide diagnostic procedures in patients with suspected lung cancer.
The rate of RASSF1A methylation found in our prospective collected series of 129 bronchial washings is in agreement with other studies (Table 5).[11,12,14,21,22] The significantly higher proportion of RASSF1a methylation in central tumors compared with peripheral tumors has been confirmed in our study. Not surprisingly, in other studies in which centrally located, endoscopic visible and larger tumors were also examined, a pathologic diagnosis by bronchoscopy is frequently obtained with concomitant increased detection of molecular alterations.[9,14,22] In our study a larger tumor size was found in patients with methylated samples, suggesting that more methylated DNA or tumor cells are shed from larger tumors.

Table 5. *Overview of studies regarding analysis of RASSF1A hypermethylation in bronchial washings of lung cancer patients.*

	Grote [22]	Topaloglu [12]	Kim [11]	Guo [21]	Schmiemann [14]
N	203	41	212	20	169
Histology cases	NSCLC/SCLC	NSCLC	NSCLC	NSCLC	NSCLC
Controls	Benign disease	Benign disease	Unknown	-	Benign disease
DNA assay	QMSP	QMSP	MSP	QMSP	QMSP
Fraction methylated Cases (%)					
Negative	72/157 (46%)	4/14 (29%)	15/85 (18%)	5/20(25%)	35/85 (41%)
bronchoscopy	1/4 (25%)	unknown	unknown	unknown	9/26* (35%)
Controls (no.)	0/46	3/10 (30%)	5/127 (4%)	-	0/102
Methylation ratio	unknown		unknown	unknown	unknown
median		0.17			
cut-off value		0.05			

Fraction methylated refers to the number of tumors showing DNA methylation;
NSCLC = non-small cell lung cancer; SCLC = small cell lung cancer; (Q)MSP = (quantitative) methylation sensitive PCR
** three-marker methylation panel*

Furthermore, in the subset of 17 patients with peripheral tumors in our study without diagnosis by bronchoscopy, RASSF1a methylation was present in 24%. In contrast to our study, most cases in other studies were diagnosed with carcinoma in the washings. To our knowledge, the number of studies examining RASSF1a methylation in peripheral tumors and a nondiagnostic bronchoscopy is small. Grote and colleagues detected RASSF1A methylation in 1 out of 4 lung cancer patients, a patient with SCLC, with nondiagnostic bronchoscopy.[22] Schmiemann found methylation of a three-marker panel (among others of RASSF1A) in 9 of 26 patients with peripheral tumors with a nondiagnostic bronchoscopy.[14] In other studies, the tumor locations or the number of nondiagnostic bronchoscopies was not mentioned.[11,12,21]

A question arises as to whether the presence of RASSF1A methylation in washings is always due to the presence of lung cancer. Previous lung cancer studies comparing RASSF1A methylation in tumor tissues and bronchial washings showed a high concordance, indicating that methylation in these washings originated from the tumors.[11,12,21] Accordingly, RASSF1A methylation was found 21% to 50% of sputum samples of lung cancer patients.[26,30]
Earlier studies showed that RASSF1A methylation can appear months or years before clinical manifestations of lung cancer.[31,32] Methylation may also arise in damaged epithelium as a result of smoking. In approximately 6% of heavy smokers, RASSF1A methylation was found, but the methylation ratio was not mentioned.[33,34] The amount of RASSF1A hypermethylation in patients with lung cancer is much higher than the low proportion of methylation in controls. This difference may allow a threshold level to be set. In our study, one patient with empyema showed methylation with a methylation ratio below the cutoff value, as described by Shivapurkar et al.[27] Other studies showed methylation levels of 0% to 4% in controls (Table 5). Only Topaloglu showed more frequent methylation in control samples (3/10), but the study also showed very low levels of methylation. The methylation yield in BAL could be higher due to the localized harvesting of tumor cells compared with bronchial washings. However, other studies showed that methylation can well be detected in washings with 10 to 30 ml of saline and even be found in cytologic nondiagnostic samples, as shown in our study.[10,11,13,14] Bronchial washing is a routine procedure for cytologic examination, whereas BAL, usually performed with 240 ml saline, is an extended bronchoscopic technique that prolongs the duration of the procedure, carrying a small additional risk to the patient.[35]

In our study, the additional value of *KRAS* mutation analysis in the washings was limited. This result is in agreement with other studies in which *KRAS* mutations were present in 8% to 25% in the bronchial washings of nondiagnostic bronchoscopies.[23-25] In contrast to the focus of our study, in these studies, endoscopic visible tumors were also examined, and tumor cells were found in brush specimens or biopsies with concomitant increased detection of *KRAS* mutations. This finding was not confirmed in our study, where the proportion of *KRAS* mutations in the washings did not differ between central and peripheral tumors. This result is probably due to the low frequency of adenocarcinoma in central tumors and the presence of *KRAS* mutations, mainly in adenocarcinomas. Because it is known that *KRAS* mutations appear in tumor cells and not in healthy subjects, the presence of *KRAS* mutations indicate an increased probability of cancer cells. By combining analyses of *KRAS* mutations and RASSF1A methylation, 29% of the false negative or doubtful cytology was correctly diagnosed as cancer. It cannot be excluded that the addition of other methylation markers may further enhance the accuracy of bronchoscopy. Although these biomarkers may better guide the diagnostic procedures of patients with suspected lung cancer, the final

diagnosis must be confirmed with histologic classification. Selecting patients by histology enables us to apply newer chemotherapy regimens and give personalized treatment. For instance, there is an association between the presence of *KRAS* mutations and a lack of response to EGFR tyrosine kinase inhibitors.[36]

Conclusion

Our data suggest that the detection of RASSF1A methylation in bronchial washings provides important evidence to suggest malignancy in a nondiagnostic bronchoscopy in patients suspected of NSCLC. Quantitative analysis of RASSF1A methylation in combination with KRAS in bronchial washing specimens is a promising diagnostic adjunct in the analysis of lung cancer.

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Can free DNA be detected in sputum of lung cancer patients?

Chapter 6

van der Drift MA, Prinsen CFM, Hol BEA, Bolijn AS, Jeunink MA, Dekhuijzen PNR, Thunnissen FBJM.

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Abstract

Introduction: Free DNA is present in serum of cancer patients in a higher concentration than non-cancer patients. Free DNA in sputum may originate from malignant or inflammatory diseases. The aim of the study was to examine the presence of free DNA in sputum and the relationship to lung cancer. The contribution of inflammatory cells was established as well.

Patients and methods: The amount of free and cellular DNA in sputum was determined using real-time ß-globin PCR in 28 lung cancer patients and 68 controls.

Results: Free DNA was present in sputum samples of the cancer patients and controls. We found no differences in DNA concentration in sputum of patients with and without lung cancer. For all patients combined the amount of free DNA was related to the amount of inflammation. Further, we found increased hypermethylation of RASSF1A in lung cancer patients compared with controls to show that tumor related DNA is present in sputum.

Conclusion: Free DNA can be detected in sputum of lung cancer patients. The amount of free DNA is related to the amount of inflammation, but not to the presence of lung cancer.

Introduction

Lung cancer is the leading cause of cancer mortality worldwide.[1] Despite growing therapeutically options, overall survival is only 15%. If lung cancer could be detected earlier, treatment and prognosis might be improved. Sputum cytology does not play a role in early detection of lung cancer.[2-5] The proportion of tumor cells occurring in sputum of lung cancer patients may vary, but is usually < 1%. The average diagnostic yield of sputum cytology is 66% (range 22-98%).[6-8] The diagnostic yield increases with endoscopic visible (central) tumors, the number of sputum specimens collected per patient, a large and/or high stage tumor (tumors greater than 2.4 cm in diameter) and histology (more in squamous cell carcinomas than in adenocarcinomas).[9-12]
Earlier studies have shown that free DNA fragments are present in a considerably higher concentration in serum of lung cancer patients than in serum of healthy individuals or patients with benign disease.[13-17] Free DNA in serum is not tumor specific and can be derived from tumors of any organ. In sputum free DNA may originate from malignant cells of lung cancer or upper airway cancer and also from inflammatory cells.[18]
The aim of this study was to examine the presence of free DNA in sputum and its relationship to the presence of lung cancer. The contribution of inflammatory cells to the amount of free DNA in sputum was explored. Further, we examined if free DNA originated from cancer cells by determining RASSF1A hypermethylation.

Patients and methods

Patients and sputum samples

A total of 28 lung cancer patients and 68 controls took part in the study. The subjects were recruited from an outpatient pulmonary clinic in 2002 and 2003. Pulmonary function was presented according to the guidelines of the Global Initiative for Chronic Obstructive Lung Disease (GOLD) (88 patients).[19] Smoking history was presented as never, former and current smokers (90 patients). Former smokers had quit smoking for at least one year. The study was approved by the institutional review board and informed consent was obtained from all subjects.
Three day pooled spontaneous sputum samples (n=145) were collected at home in 20 ml of Fixcyt® (48% ethanol/ 13 mM polyethylene glycol 1500 MW).[11,20] Several samples were collected on a different time point and treated as duplicates. 77 Patients provided one sputum sample, 19 patients provided 2 to 9 sputum samples. Upon arrival, the fixed sputa were dithiotreitol (DTT) treated, filtered and centrifuged.[21] After DTT treatment the supernatant was transferred into 4 micro centrifuge tubes and centrifuged at 20.000-x g for 5 minutes. One ml of this supernatant was transferred to a new micro centrifuge tube and stored at -80°C until use. All cell pellets were resuspended in 1.5 ml of FixCyt®. An aliquot of the cell pellet was used for preparation of cellular monolayers and Papanicolaou-

stained. A Feulgen thionin staining was performed for demonstration of smeared DNA.[22] Specimens were considered to be adequate if alveolar macrophages and bronchial epithelial cells were present.[5] The remaining material was stored at -20°C Tumor cells, inflammatory cells, free DNA and RASSF1A hypermethylation were determined in the sputum samples.

Cytology preparation and examination

The slides with cellular monolayers were screened for the presence of atypical cells. Squamous cells were classified in normal cells, mild, moderate and severe atypia, carcinoma in situ or invasive carcinoma.[23,24] Invasive carcinoma was classified as squamous cell carcinoma, adenocarcinoma, large cell carcinoma or small cell carcinoma according to the World Health Organization.[23,24] In addition, the amount of inflammatory cells (143 samples) was divided in 4 categories: samples without inflammatory cells (0), 1-25% (1), 25-50% (2) and >50% neutrophilic granulocytes (3).

DNA analysis

Sputum cell pellets were resuspended in phosphate-buffered saline (PBS) and DNA isolation was performed with spin columns (QIAamp DNA Blood Maxi kit, Qiagen) according to the "Blood and Body Fluid Spin Protocol" of the manufacturer. Sputum supernatant (1 ml) was used without the addition of PBS but with increased amounts of QIAGEN kit components proportionally. The same DNA isolation protocol was used for supernatant as for cells. DNA was eluted with 100 µl buffer and 10 µl was used for the quantification of human DNA using a real-time beta-globin polymerase chain reaction (PCR) method targeting a 71 bp fragment within exon 3 of the human ß-globin gene.[25] All quantitations were performed in triplicate and mean DNA was expressed as the amount of DNA in ng per 1 ml of sputum/Fixcyt solution.

We investigated RASSF1A hypermethylation as marker of tumor related DNA.[26] In our hands RASSF1A is more frequently present in sputum of lung cancer patients than in controls (data not shown). RASSF1A hypermethylation analysis was performed as described previously.[27,28] A part of the samples could not be analyzed because of limited remaining DNA quantities (19 lung cancer samples and 37 control samples). Both first and second step PCR reaction comprised of 30 cycles. The amount of modified DNA used in the first PCR step was up to 400 ng for the DNA that originated from sputum cell pellets and 0.04 to 319 ng for DNA from sputum supernatants (the maximal amount of the DNA material available). All assays were at least performed in duplicate. When two results were the same, the outcome was considered to be conclusive. In case of an inconclusive outcome a third assay was done, which was decisive for the outcome.

Statistical analysis

For free and cellular DNA the mean and standard error (SE) were determined. For statistical comparison a nonparametric Mann-Whitney U or Kruskal-Wallis H was used. Frequencies of hypermethylation of RASSF1A in the sputum samples were compared with a chi-square test. A p-value of < 0.05 was regarded as significant. Data were analyzed with SPSS/PC+, version 12.0 (SPSS, Inc., Chicago, IL).

Results

Patient characteristics

The mean age (range) of cancer patients and controls did not differ significantly (68 years (range 46-88) and 66 years (range 40-87), respectively; $p = 0.36$). In cancer patients there were significantly more women compared with controls ($p < 0.001$) (Table 1). Mean forced expiratory volume in one second (FEV1) (range) did not differ significantly between cancer patients and controls (1.83 L (0.82–3.67) and 1.73 L (0.60–3.68) respectively, $p = 0.55$). 26 of the 28 lung cancer patients had chronic obstructive pulmonary disease (COPD) (GOLD stages: I: 11 (42.3%); II: 9 (34.6%); III: 6 (23%)). 57 of the 68 controls had COPD (GOLD stages: I: 18 (29%); II: 19 (30.6%); III: 17 (27.4%); IV: 3 (4.8%)). Five controls (8.2%) did not have COPD. In 2 lung cancer patients and 6 controls GOLD stages were unknown. In cancer patients there were significantly more former smokers than in controls ($p < 0.001$) and in controls were significantly more current smokers ($p < 0.001$). In 1 lung cancer patient and 5 controls smoking history was unknown.

Cytologic examination

The cytologic examination of the sputum samples of the lung cancer patients and controls is presented in Table 2. Most sputum samples of controls showed no abnormalities (93.8%). Malignant cells were present in sputum of 16 lung cancer patients (57%). In Table 3 histologic subtypes and tumor stage according to the revised lung cancer staging system are presented, 46% had squamous cell carcinoma.[29] Tumor stage of 4 patients was unknown. At bronchoscopy 11 patients had an endoscopic visible (central) tumor, 14 patients had an endoscopic nonvisible (peripheral) tumor, 1 patient did not have a bronchoscopy and information of 2 patients was not available.

Table 1. *DNA concentration in sputum of lung cancer patients (n=28) and controls (n=68).*

Clinical characteristics	**Free DNA* (ng/ml)**				**Cellular DNA (ng/ml)**	
	Cancer patients		Controls		Cancer patients	Controls
	n	Mean ± SE	n	Mean ± SE	Mean ± SE	Mean ± SE
Sputum samples p-value	49	312 ± 61 0.30	96	1033 ± 227	5751 ± 1027 0.10	11108 ± 1587
Sex *Male* *Female* p-value	 18 10	 288 ± 87 130 ± 55 0.27	 51 17	 696 ± 184 660 ± 276 0.69	 4600 ± 1932 2760 ± 861 0.59	 7956 ± 1523 9384 ± 3240 0.74
Smoking *Current* *Former* *Never* p-value	 13 13 1	 265 ± 101 216 ± 83 234 0.93	 34 22 7	 638 ± 237 824 ± 257 370 ± 187 0.69	 2786 ± 604 5219 ± 2685 3740 0.66	 7004 ± 1512 10352 ± 2817 11804 ± 7223 0.45

**: p < 0.001 versus cellular DNA*

Table 2. *Sputum DNA according to cytologic analysis.*

Cytologic analysis	**Free DNA (ng/ml)**					**Cellular DNA (ng/ml)**		
	Cancer patients		Controls			Cancer patients	Controls	
	n	Mean ± SE	n	Mean ± SE	p-value	Mean ± SE	Mean ± SE	p-value
normal cells	19	373 ± 109	79	1181 ± 272	0.62	4614 ± 1016	12329 ± 1878	0.09
mild atypia	1	124	5	157 ± 53	NT	2503	6053 ± 1993	NT
moderate atypia	-	-	6	821 ± 187	NT	-	10147 ± 2605	NT
severe atypia	11	168 ± 48	1	91	NT	8069 ± 2681	779	NT
CIS or invasive carcinoma	17	365 ± 120	-		NT	6026 ± 2114	-	NT
not representative	1	6	5	7 ± 2	NT	440	79 ± 46	NT

Data are presented for sputum samples of lung cancer patients (n=49) and controls (n=96).
CIS: carcinoma in situ; NT = not tested

Table 3. *Sputum DNA of lung cancer patients (n=28) according to tumor characteristics.*

Tumor characteristics	n	Free DNA mean ± SE (ng/ml)	Cellular DNA mean ± SE (ng/ml)
Histologic subtypes			
squamous cell carcinoma	13	304 ± 98	5695 ± 2628
adenocarcinoma	9	213 ± 116	2659 ± 882
large cell carcinoma	2	6 ± 0.4	311 ± 129
small cell lung carcinoma	4	152 ± 76	2506 ± 999
p-value		0.24	0.30
Stage			
0	1	528	6935
I	4	228 ± 103	4022 ± 1665
II	5	489 ± 217	9428 ± 6676
III	4	76 ± 35	2853 ± 1885
IV	6	88 ± 39	2376 ± 1006
SCLC-LD	2	136 ± 126	3423 ± 1838
SCLC-ED	2	168 ± 134	1580 ± 965
p-value		0.33	0.82

SCLC-LD: small cell lung carcinoma-limited disease; SCLC-ED: small cell lung carcinoma-extensive disease. P-values: comparisons among subdivisions

DNA analysis

The mean concentration of free DNA was significantly lower than the mean concentration of cellular DNA in lung cancer patients ($p < 0.001$) and controls ($p < 0.001$; Table 1). No differences in free and cellular DNA concentration were found in sputum between patients with and without lung cancer ($p = 0.30$ and $p = 0.10$, respectively). Sex, age and smoking status did not correlate with the free or cellular DNA concentration in both lung cancer patients and controls. In cancer patients, GOLD stage did not influence mean free or cellular DNA ($p = 0.42$ and $p = 0.60$, respectively). In controls, mean free DNA was significantly higher in patients with higher GOLD stage ($p = 0.01$), but mean cellular DNA did not differ significantly between GOLD stages ($p = 0.66$).

The presence of malignant cells in the sputum samples of lung cancer patients was not related to the concentration of free DNA ($p = 0.66$) or DNA in the cellular fraction ($p = 0.66$) (Table 2). The samples with atypical / malignant cells were evenly distributed over the four inflammatory categories. In cancer patients mean free and cellular DNA did not differ significantly among histologic subtypes ($p = 0.24$ for free and $p = 0.30$ for cellular DNA), tumor stage ($p = 0.33$ and $p = 0.82$, respectively) (Table 3) or centrally or peripherally located tumors at bronchoscopy ($p = 0.19$ and $p = 0.44$, respectively).

The concentration of free and cellular DNA in the four inflammatory categories is shown in Table 4. Free DNA was present in all sputum samples of cancer patients and controls. For all patients combined the amount of free and cellular DNA was related to the amount of inflammation ($p = 0.01$ and $p < 0.001$, respectively). The *free DNA concentration* in sputum of lung cancer patients was significantly increased in the subgroup with more than 50% neutrophilic granulocytes compared with the samples with 1-25% neutrophilic granulocytes ($p = 0.006$). The free DNA concentration in controls was significantly increased in the subgroup with more than 50% of neutrophilic granulocytes compared with the samples with 25-50% neutrophilic granulocytes ($p = 0.03$). The *cellular DNA concentration* in lung cancer patients was similar in the subgroups of inflammation ($p = 0.26$). The cellular DNA concentration in controls was significantly increased in the subgroup with more than 50% of neutrophilic granulocytes compared with samples with less than 50% ($p \leq 0.001$). Smoking did not influence the amount of inflammation in both cancer patients and controls ($p = 0.15$ and $p = 0.11$, respectively).

Table 4. *Sputum DNA of lung cancer patients (n=48) and controls (n=95) according to the inflammatory categories.*

Neutrophilic granulocytes	**Free DNA (ng/ml)**					**Cellular DNA (ng/ml)**		
	Cancer patients		Controls			Cancer patients	Controls	
	n	Mean ± SE	n	Mean ± SE	p-value	Mean ± SE	Mean ± SE	p-value
0%	6	172 ± 67	7	571 ± 532	0.53	2078 ± 547	1238 ± 498	0.23
1–25%	15	93 ± 27	22	688 ± 321	0.10	6510 ± 1729	3684 ± 889	0.06
25–50%	9	428 ± 204	26	451 ± 177	0.96	3460 ± 2122	6897 ± 1233	0.04
>50%	18	498 ± 110*	40	1701 ± 479¶	0.87	7774 ± 2049	19780 ± 3224#	0.03

**: p = 0.006 versus 1–25% neutrophilic granulocytes; ¶: p = 0.03 versus 25–50% neutrophilic granulocytes; #: p = 0.002 versus 0% neutrophilic granulocytes and p < 0.001 versus 1–25% and 25-50% neutrophilic granulocytes*

RASSF1A analysis

The result of the RASSF1A analysis is shown in Table 5. Hypermethylation of RASSF1A of both free and cellular DNA was significantly increased in lung cancer patients compared with controls ($p < 0.001$). In *free DNA* methylation of RASSF1A was present in 20% of the lung cancer patients and not in controls. In *cellular DNA* methylation of RASSF1A was present in 42.9% of the lung cancer patients and in 1.4% of the controls.

Table 5. *RASSF1A methylation analysis of sputum DNA of lung cancer patients (n=28) and controls (n=68).*

RASSF1A	Free DNA (n)		Cellular DNA (n)	
	Cancer patients	Controls	Cancer patients	Controls
Methylation present	5*	0	12*	1
Unmethylated	18	52	16	67
Not conclusive	2	0	0	
Missing data	3	16	0	0

*Data are presented in numbers. *: p < 0.001 versus controls*

Discussion

In this study, we demonstrated the presence of free DNA in all sputum samples. No differences were found in free DNA concentration in sputum of lung cancer patients compared with controls. However, we found that the amount of free DNA was related to the amount of inflammatory cells.

The average diagnostic yield of sputum cytology is approximately 66%.[6-8] In our study with 3-day pooled samples the diagnostic yield was comparable (57%). The present study was performed to investigate if free DNA can be detected in sputum and its relationship to lung cancer. To the best of our knowledge there are no previous data on free DNA in sputum and the relation to lung cancer. Theoretically, free DNA may be present in the epithelial lining fluid. Free DNA may be derived from all airway epithelial cells underneath the lining fluid, from the alveolar macrophages and disease related cells such as inflammatory cells and tumor cells. Because tumor cells comprise only a minor fraction in most sputum samples, the amount of tumor DNA in sputum is likely to be low. In our study the concentration of free DNA was 10 fold lower compared with the concentration of cellular DNA. No significant differences were found in both free and cellular DNA concentration in sputum of patients with lung cancer compared with controls. In this study we performed hypermethylation of RASSF1A to show that tumor related DNA is present in sputum. Hypermethylation for RASSF1A was present in 13 lung cancer patients (46.4%) in contrast to hypermethylation in 1 control (1.4%). Honorio et al. also found that hypermethylation of RASSF1A was detectable in sputum in about 21% NSCLC and in 50% small cell lung cancer (SCLC) sputum samples.[30] Our finding of significant increased RASSF1A hypermethylation in sputum in a small number of lung cancer patients is interesting to explore in a further study in a larger group of patients.

It is likely that the majority of degenerating tumor cells in sputum originate from the lung tumors that are endobronchial visible at bronchoscopy. However, about 38% of the lung tumors are not visible at bronchoscopy.[31] In addition the possibly shredded tumor cells have to be transported together with the mucus on the

surface of the proximal airways and coughed up. In the present study mean free and cellular DNA in lung cancer patients did not differ significantly among centrally or peripherally located tumors at bronchoscopy.
In an inflammatory disease such as COPD, with or without acute component, the epithelial lining fluid increases in volume due to increased mucus production, increased permeability of the respiratory mucosa, the presence of inflammatory cells and secreted anti-inflammatory components.[7,32] Lytic inflammatory cells may contribute to the free DNA fraction. We found that free DNA was present in all sputum samples of both the lung cancer patients and controls regardless the amount of inflammation, thus also in those samples without inflammation. Most lung cancer patients are ex- or current smokers and about 50% have COPD.[33] In our study 93% of lung cancer patients had COPD. In addition it appeared that the amount of free DNA increases proportionally with the amount of inflammatory cells. This is in line with the findings of Gautschi and coworkers, who observed a strong correlation between neutrophils and free DNA in serum of NSCLC patients.[18] The increased amount of free DNA due to neutrophilic granulocytes was so large that a possible minor increase of free DNA due to a malignant process was not likely to be detected. In controls, mean free DNA was significantly higher in patients with higher GOLD stage. Probably this is related to the increased amount of neutrophilic granulocytes in sputum.[34] Although in cancer patients there were more former smokers and in controls there were more current smokers, smoking status did not correlate with free or cellular DNA concentration in both lung cancer patients and controls.

Conclusion

The results of the present study show that free DNA can be detected in sputum of lung cancer patients and controls. The amount of free and cellular DNA was related to the amount of inflammatory cells present as a result of COPD, but not to the presence of cancer.

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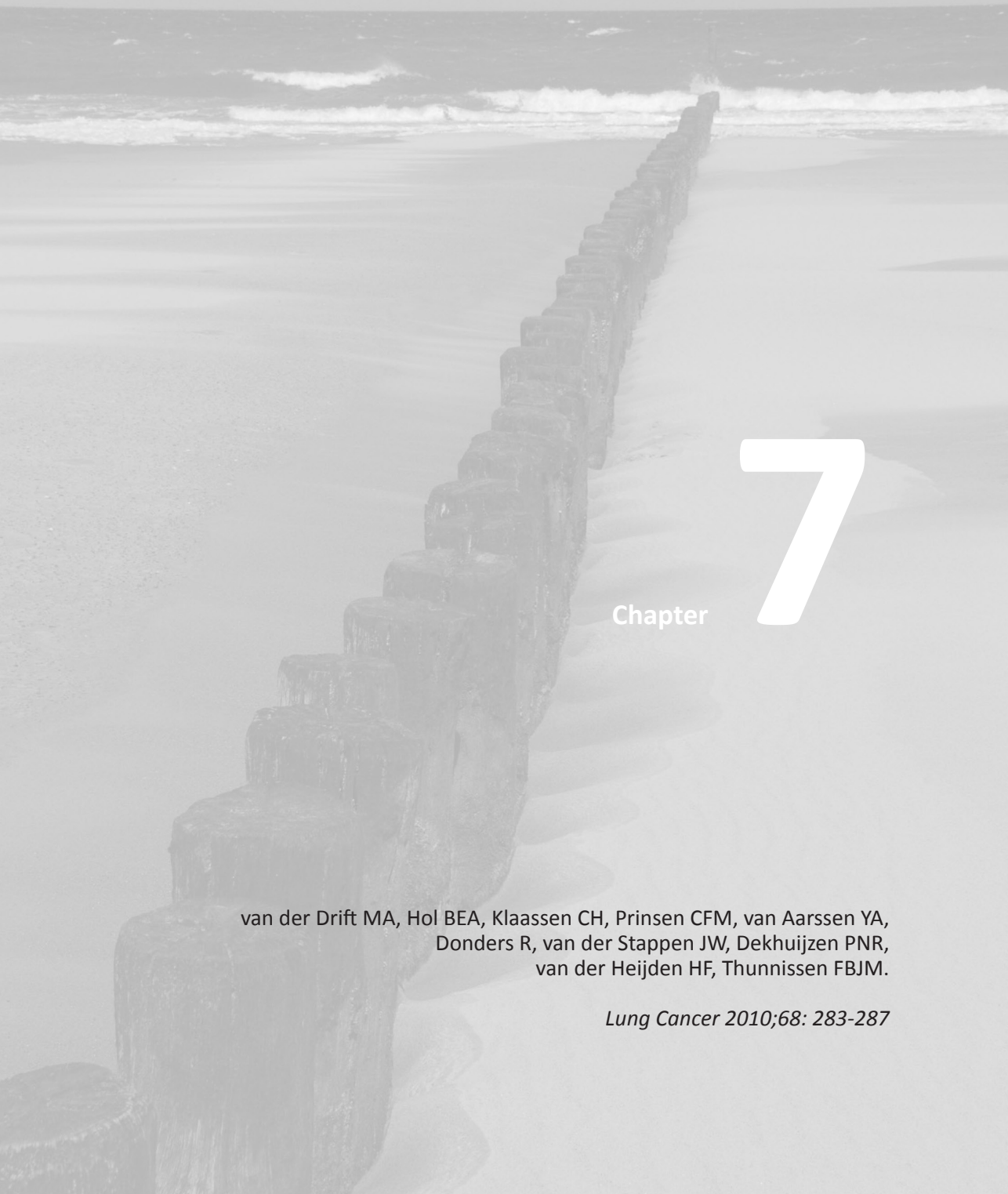
Circulating DNA is a non-invasive prognostic factor for survival in non-small cell lung cancer



Chapter 7

van der Drift MA, Hol BEA, Klaassen CH, Prinsen CFM, van Aarssen YA,
Donders R, van der Stappen JW, Dekhuijzen PNR,
van der Heijden HF, Thunnissen FBJM.

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Abstract

Introduction: Circulating plasma DNA is present in a considerably higher concentration in lung cancer patients than in controls. Conflicting data are reported about circulating DNA as a prognostic factor. The aim of this study was to prospectively analyse the relationship of circulating plasma DNA with overall survival (OS) of previously untreated non-small cell lung cancer (NSCLC) patients.

Patients and methods: 46 untreated NSCLC patients and 21 controls with a follow-up time of 6.5 years were analysed. Quantification of baseline circulating plasma DNA was performed by a real-time quantitative polymerase chain reaction (qPCR) targeting the human ß-globin gene. Survival analysis was performed using the Kaplan-Meier method and compared with a Cox-regression analysis.

Results: The median DNA concentration of the patients who died (87%) was significantly higher compared with the patients that survived at the end of follow-up (55 ng/ml versus 23 ng/ml, p = 0.02). In patients with higher DNA concentration overall survival was significantly worse. In this study no relation of DNA concentration with tumor characteristics, age, gender or pulmonary inflammatory conditions was found.

Conclusion: In this study a high circulating plasma DNA concentration at time of diagnosis in NSCLC patients was a prognostic factor for poorer survival. Circulating DNA may be used as a non-invasive biomarker to refine the prognostic profile in NSCLC patients.

Introduction

Lung cancer is the leading cause of death by cancer worldwide. At the time of diagnosis, lung cancer is often locally or systemically advanced and overall survival is 15%.[1] Prognostic factors predict survival independent of the treatment applied and can classify patients as high or low risk. The most important prognostic factor is stage according to the TNM system.[2,3] Other prognostic factors include clinical aspects (e.g. gender, age, weight loss, cardiovascular disease), elevated lactate dehydrogenase levels[4-6], FDG-PET scan[7] and pathological aspects.[8-10] Increasingly, multiple genetic and epigenetic alterations are under investigation as prognostic factors for survival.[11-14]

Since many years free or circulating DNA in plasma or serum of (lung) cancer patients is under investigation for clinical relevance. Circulating plasma DNA is present in considerably higher concentrations in lung cancer patients compared with healthy controls or patients with benign disease.[15-19] The increased circulating DNA concentration is thought to originate from cancer cells.[20-22] Conflicting data have been reported about circulating DNA as a prognostic factor in lung cancer patients. Some studies showed a correlation between an elevated plasma DNA concentration and poor survival[23,24], whereas other studies did not report a such a relationship.[25-27] This might be explained by differences in patient selection, covering both non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), and both treated and untreated patients. Besides, techniques for sample collection and DNA quantification differed between these studies. Thus, at present, the prognostic value of circulating DNA for survival has not been established yet. Also the relationship of circulating DNA with age, gender, histology, stage and pulmonary inflammatory conditions is not clear.

The aim of our study was to analyse if elevated circulating DNA is a prognostic factor for survival in NSCLC patients. The present study reports 46 untreated NSCLC patients and 21 controls with a follow-up time of 6.5 years. A real-time quantitative polymerase chain reaction (qPCR), the current standard method to quantitate DNA, was applied to determine the baseline plasma DNA concentration at the time of diagnosis.

Patients and methods

Study design

In the Canisius-Wilhelmina hospital, Nijmegen, 77 consecutive patients suspected of having lung cancer by signs, symptoms or chest X-ray were prospectively recruited between June 2001 and September 2003. Patients in whom no lung cancer was diagnosed served as controls; none of them developed lung cancer during follow-up. 10 Patients with SCLC were excluded. Overall survival (OS) was computed from the date of diagnosis to the date of death or the date of last follow-up. Survival data were obtained until January 2009. Smoking history

was presented as current, former and never smokers. Former smokers had quit smoking for at least one year. Information about smoking of 1 control patient was missing. Pulmonary function was presented as classification of chronic obstructive pulmonary disease (COPD) according to the guidelines of the Global Initiative for Chronic Obstructive Lung Disease (GOLD).[28] The study was approved by the institutional review board and informed consent was obtained from all participants.

Sample collection, DNA isolation and quantification

A 10 ml sample of peripheral blood was collected in EDTA tubes from patients before surgery, chemo- or radiotherapy and from controls. Within 1 h after collection, EDTA-blood samples were subjected to two centrifugation steps. After initial centrifugation of the original tube for 10 min at 2,000 x *g* at 20°C, the supernatants were transferred to micro centrifuge tubes and centrifuged again for 5 min at 16,000 x *g* at 20°C. Subsequently, the supernatant was stored at –20°C before analysing the DNA content.

DNA was isolated from 1 ml of plasma using the MagNA Pure LC Total Nucleic Acid Isolation Kit - Large Volume (Roche diagnostics) and eluted in 100 µl of elution buffer. The DNA concentration was measured by using a previously described highly reproducible real-time quantitative polymerase chain reaction (qPCR) targeting the human ß-globin gene.[29] Primer and probe sequences were used as described. The qPCR was performed on a LightCycler 1.2 instrument (Roche diagnostics) in a final volume of 20 µl containing 0.25 µM of amplification primers, 0.1 µM detection probe and 3 mM $MgCl_2$ (total concentration) in 1x LightCycler FastStart DNA Master HybProbe (Roche Diagnostics). A calibration curve was made from pure human lymphocyte DNA that was quantified by UV absorbance measurements. The qPCR program consisted of an initial denaturation of 10 min at 94°C followed by 40 cycles of 0 s at 95° and 15 s at 60°C with maximal ramping rates. Data were analyzed using the second derivative maximum method. DNA was expressed in ng per ml of plasma. Mean storage of DNA samples before analysis was 12.2 (range 5.4 – 20) months for NSCLC patients and 11.2 (range 5.2 – 14.5) months for controls. Storage time did not influence DNA concentration (data not shown).

Statistical analysis

Data were analyzed with SPSS/PC+, version 16.0 (SPSS, Inc., Chicago, IL). Descriptive statistics were used for clinical characteristics and comparisons were performed by t-tests or Chi-square tests, as appropriate. Differences in DNA concentrations between NCSLC patients and controls were analyzed using a nonparametric Mann-Whitney U or Kruskal-Wallis test, as appropriate. Survival analysis, according to three tertiles of DNA distribution, was performed using the Kaplan-Meier method and compared with a Cox-regression analysis according to Sozzi et al.[30] For the diagnostic discrimination of DNA concentrations between

cancer patients and controls the area under the curve of the receiver operating characteristic curve (AUC-ROC) was assessed non-parametrically. A p-value of ≤ 0.05 was regarded as significant.

Results

Baseline characteristics

46 NSCLC patients and 21 controls were analysed. Patient characteristics are summarized in Table 1. In the patient group there were significant less males than in the control group (65% and 90%, respectively; p = 0.04). Smoking history was equally divided between patients and controls (p = 0.85). 30 Patients (65%) and 11 controls (52%) had COPD. The mean forced expiratory volume in one second (FEV1) percentage predicted did not differ between patients and controls (p = 0.06) and GOLD stages were divided equally between both groups (p = 0.54).
In patients, the median DNA concentration was significantly higher than in controls (52 ng/ml (range 5-3597) and 29 ng/ml (range 0-175), respectively; p = 0.03).

***Table 1**. Clinical characteristics of non-small cell lung cancer (NSCLC) patients (n = 46) and controls (n = 21).*

Clinical characteristics	NSCLC patients % (no.)	Controls % (no.)	p-value
Male / female	65% (30) / 35% (16)	90% (19) / 10% (2)	0.04
Age (min-max) (years)	66.1 (47.6-88.4)	59.3 (41.7-82.6)	0.04
Smoking history			0.85
Current	65% (30)	57% (12)	
Former	33% (15)	38% (8)	
Never	2% (1)	-	
Unknown	-	5% (1)	
Mean FEV1% predicted (min-max)	70.7% (36.2-112.4)	80.6% (32.6-117.9)	0.06
COPD			0.54
absent	35% (16)	48% (10)	
GOLD 1	13% (6)	19% (4)	
GOLD 2	41% (19)	29% (6)	
GOLD 3	11% (5)	4% (1)	

FEV1 = mean forced expiratory volume in one second; COPD = chronic obstructive pulmonary disease; GOLD = Global Initiative for Chronic Obstructive Lung Disease

Follow-up and relationship to DNA concentration

Maximum follow-up time was 6.5 years. At the date of last follow-up 40 patients (87%) and 2 controls (9.5%) had died. 12 patients staged I-III relapsed after therapy or developed metastases (41.4%) after median 15 months (range 1.5-51.5).
Median OS was significantly decreased for patients compared with controls (13.7 months (range 1.1-78.4) and 67.6 months (range 2.0–78.2), respectively; p < 0.001). The median DNA concentration of the patients who died (n = 40) was significantly higher compared with the patients that survived (n = 6) at the end of

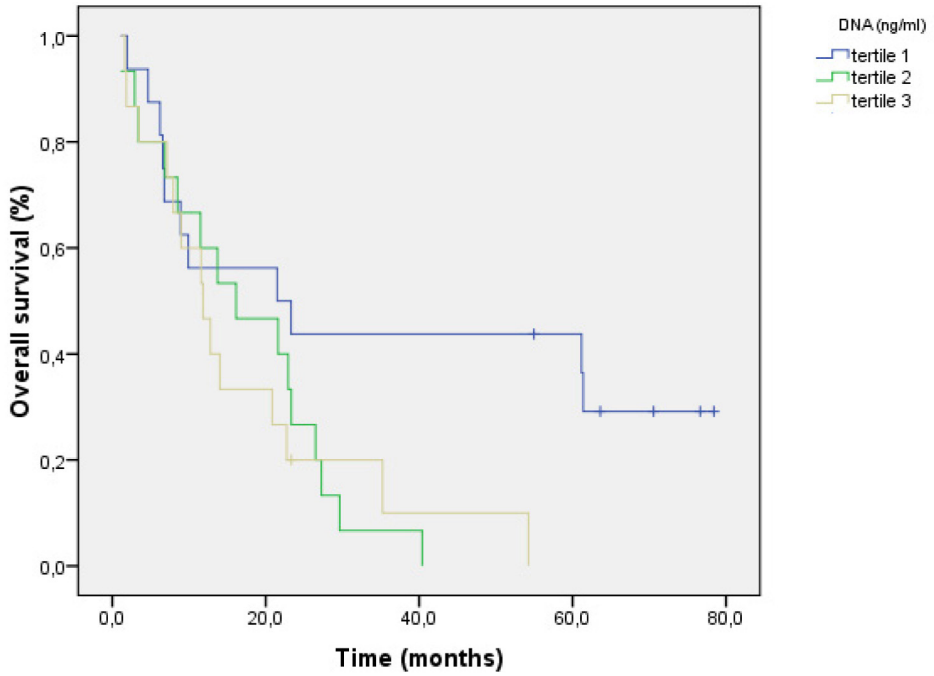
follow-up (55 ng/ml versus 23 ng/ml, p=0.02). Survival analysis, using the Kaplan-Meier method and compared with a Cox-regression analysis, was performed according to three tertiles of DNA distribution (Table 2). In patients with DNA concentration in the middle and upper tertiles (≥ 32 ng/ml) overall survival was significantly shortened (Figure 1).
The AUC-ROC for discrimination of DNA concentrations between patients and controls was 0.66 (95% confidence interval 0.53 to 0.80, p = 0.03). A DNA cut-off level of > 32 ng/ml differentiated between lung cancer patients and controls with a specificity of 52% and sensitivity of 67%.

Table 2. *Survival analysis in non-small cell lung cancer (NSCLC) patients according to three tertiles of DNA distribution.*

Tertile (no.)	DNA range (ng/ml)	Median survival time (months) (SE)	p-value (Cox regression)	HR
Overall (46)	5 - 3597	13.7 (5.2)	0.06	
1 (16)	0 - 31	21.5 (13.4)		
2 (15)	32 – 66	16.2 (6.5)	0.04*	2.5
3 (15)	67 - 3597	11.8 (2.5)	0.03**	2.6

*HR = hazard ratio; *p = 0.04 versus tertile 1; **p = 0.03 versus tertile 1*

Figure 1. *Overall survival and correlation to circulating DNA in plasma of lung cancer patients.*



Clinical characteristics and relationship to DNA concentration

Table 3 summarizes the DNA concentration according to the clinical and tumor characteristics. Median DNA concentration differed neither between men and women in patients (p = 0.71) nor in controls (p = 0.91). Age was not related to DNA concentration in patients (p = 0.25) nor in controls (p = 0.43). Smoking was not related to DNA concentration in patients (p = 0.85), but in controls DNA concentration was significantly increased in former smokers compared with current smokers (p = 0.02). GOLD stages were not related to DNA concentration in patients (p = 0.83) nor in controls (p = 0.88).

(Locally) advanced disease was observed in 58.7% of the patients. In patients no relation was observed between DNA concentration and histology (p = 0.35) or tumor stage (p = 0.39). 3 Controls developed cancer during follow-up (metastasized colon carcinoma, mamma carcinoma and renal cell carcinoma) with DNA levels ranging 6-26 ng/ml.

Table 3. *Median DNA concentration (ng/ml) according to clinical and tumor characteristics of the non-small cell lung cancer (NSCLC) patients (n=46) versus controls (n=21).*

Clinical characteristics	**NSCLC patients**		**Controls**	
	DNA (IQ range)	**p-value**	**DNA (IQ range)**	**p-value**
Overall	52 (64)		29 (27)	0.03
Gender				
male	53 (59)	0.71	29 (29)	0.91
female	48 (107)		36 (-)	
Age				
< 60 year	32 (48)	0.25	28 (45)	0.43
≥ 60 year	55 (62)		34 (19)	
Smoking				
Current	52 (72)	0.85	24 (29)	0.02
Former	49 (29)		40 (36)	
Never	777 (-)			
COPD		0.83		0.88
absent	53 (67)		28 (45)	
GOLD 1	60 (57)		27 (139)	
GOLD 2	49 (64)		32 (11)	
GOLD 3	27 (83)		44	
Histological subtypes		0.35		
SCC (n=21)	49 (54)			
AC (n=20)	42 (89)			
LCC (n=5)	84 (81)			
Stage in NSCLC		0.39		
I (n=11)	52 (38)			
II (n=6)	31 (75)			
III (n=12)	34 (77)			
IV (n=15)	61 (100)			
unknown(n=2)	93 (-)			

IQ = interquartile; SCC = squamous cell carcinoma; AC = adenocarcinoma; LCC = large cell carcinoma; disease; IQ = interquartile

Discussion

The aim of this study was to analyse circulating plasma DNA as a prognostic factor for survival of NSCLC patients at time of diagnosis. The results show that a high circulating DNA concentration correlates with poor survival. We did not find a relationship of DNA concentration with clinical parameters as age, gender, histology, stage, smoking or pulmonary inflammatory conditions.
Since many years circulating DNA in plasma of lung cancer patients is under investigation as a promising non-invasive diagnostic or prognostic biomarker. The source of DNA circulating in plasma is uncertain. It is presumed that circulating DNA in healthy subjects or patients with benign disease is derived from lymphocytes, leucocytes or other damaged inflammatory cells. In cancer patients a considerable part of circulating plasma DNA is derived from cancer cells as is shown by the presence of specific oncogene or tumor-suppressor gene mutations, hypermethylation of several genes or other (epi)genetic changes.[22,31] Several mechanisms of DNA release have been proposed such as apoptosis or lysis of cancer cells and leakage in the blood stream.[18,21,23,32]

***Table 4**. Overview of studies regarding analysis of circulating DNA in plasma/ serum of lung cancer patients as a prognostic factor.*

	Fournie [23]	**Sozzi [25]**	**Beau-Faller [26]**	**Gautschi [24]**	**Ludovini [27]**	**Sozzi [30]**
N	68	81	34	185	76	38
Histology	NSCLC + SCLC	NSCLC	NSCLC + SCLC	NSCLC	NSCLC	NSCLC
(Un)treated at baseline	untreated	untreated	untreated	both treated and untreated	untreated	untreated
DNA source	plasma	plasma	plasma	plasma (+ serum)	plasma	plasma
DNA assay	nick translation DNA labeling	dipstick	fluorescence assay	qPCR	qPCR	qPCR
Gene (DNA)				G-3-PD	hTERT	hTERT
DNA (ng/ml) ***mean*** ***median*** ***cut-off value***	 30 25	 318 25	 157 -	 3.7 10	 60 3.25	 4.8 6.3
Correlation ↑ DNA with ↓ survival	p=0.005	p=0.55	No correlation (p unknown)	p<0.001	p=0.27	p=0.0066
Correlation ↑ DNA	stage IV, SCLC	none	none	↑ stage, ↑ LDH	recurrence	none

NSCLC = non-small cell lung cancer; qPCR = real-time polymerase chain reaction; G-3-PD = glyceraldehyde-3-phosphate dehydrogenase; hTERT = human telomerase reverse transcription

For the quantification of DNA, we selected a real-time PCR assay designed for the ß-globin sequence that performed consistently in other experiments.[29,33] Real-time PCR for DNA quantification can be regarded as the standard method.[34] PCR based technology was introduced in the late 1980s and refinements in the last

decade resulted in the quantification of extremely small amounts of nucleic acids. Different genes can be used for amplification, depending on the experience of the institute.
In our study, the median DNA concentration in lung cancer patients was significantly higher compared with controls, which is in agreement with former studies.[15-19] The AUC-ROC for discrimination of DNA concentrations between patients and controls was 0.66, which is comparable with AUC levels in other studies.[35-37] The DNA concentration of the patients who had died during follow-up was significantly higher compared with those patients that survived at the end of this period. Survival analysis, according to three tertiles of DNA distribution, showed a significantly worse survival in patients with DNA concentration in the middle and upper tertiles. Several studies did not show a correlation of DNA concentration to survival[25-27], whereas others showed that a higher DNA concentration did correlate to poorer survival.[23,24] In a recently published lung cancer screening study, survival was also worse in patients with plasma DNA concentration in the upper tertile.[30] According to the 3 tertiles in our study, we found a virtual cut-off level of 32 ng/ml, but the sensitivity and specificity were disappointing (67% and 52%, respectively). Sensitivity and specificity estimates for different DNA cut-off values were also computed and as to be expected higher cut-off levels went together with increased specificity but lower sensitivity and vice versa. In previous studies DNA cut-off levels ranged from 3.25 to 100 ng/ml and were dependant of the assays and genes used to isolate circulating DNA (Table 4).[15,16,35-40] Nevertheless, the cut-off level in our study is not at variance with most of these studies.[35-38] As the DNA concentration consists of continuous variables, dividing DNA according to tertiles seems to have a stronger clinical value than determining cut-off levels with an ample variety of sensitivity and specificity.

Possible explanations for the discrepancy in survival data are patient and sample selection. An overview of studies regarding analysis of circulating DNA in plasma or serum of lung cancer patients as a prognostic factor is presented in Table 4. In some studies both NSCLC and SCLC patients were combined.[23,26] Since NSCLC and SCLC differ in biology, treatment and prognosis, only NSCLC patients were selected in our study. Some studies were performed with both newly diagnosed and relapsed NSCLC patients.[16,24] It is not yet clear how DNA concentration is influenced by therapy. A lower DNA concentration was found after surgery compared with baseline DNA concentration.[19,25,27] Those patients with an increase in DNA concentration after surgery showed recurrence of malignancy. On the other hand, Gautschi et al. did not find a decrease in DNA concentration after treatment with chemotherapy.[24] To avoid an altered DNA concentration after treatment in our study, baseline DNA concentration of yet untreated lung cancer patients was determined.
Another potential bias for differences in outcomes in similar survival studies is the method of DNA isolation. As discussed before, qPCR for DNA quantification can be

regarded as the standard method and can detect a large range of small amounts of nucleic acids. Other techniques have a detecting range with lower upper DNA levels, for instance a range of 0 - 1000 ng/ml for the radioimmunoassay and pico green quantification[16,41] or 0 - 2000 ng/ml for nick translation DNA labeling.[23] In our study DNA values up to 3597 ng/ml were detected. The possible significance of higher DNA values might have been missed in studies with older assays.

There is some doubt about the quality of DNA after prolonged storage. In a brief report Sozzi showed that DNA levels declined at about 30% per year.[42] In our study mean storage of DNA samples before analysis was one year. When analysing DNA at different time points of storage between 6 months and one year, DNA concentration did not differ significantly. This latter observation was also reported by Frattini.[43]

DNA concentration in our study was not influenced by tumor characteristics, age, gender and conditions associated with inflammation. Our results show that neither histological subtypes nor stage in NSCLC patients were related to DNA concentration which is in agreement with previous studies.[19,25,26,44] The studies that reported higher DNA concentration in advanced NSCLC were performed in a selected group of patients of whom a substantial part had received prior treatment.[16,24] As discussed before, DNA concentration might be influenced by treatment. The mean age of cancer patients was significantly higher than the mean age of controls in our study. The mean age of 66.7 years in lung cancer patients is in agreement with the appearance of lung cancer at higher age in the population. Age was not related to DNA concentration in lung cancer patients, which is in agreement with one[19], but not another study by the same author.[25] Our results showed that gender and smoking in cancer patients were not related to DNA concentration which is in agreement with other studies. Unexpectedly, in control patients DNA concentration of former smokers was increased compared with current smokers. Possibly this is due to the small range of DNA concentration in controls compared with the larger range in cancer patients.

As circulating plasma DNA is also higher in patients with benign disease, the relationship of DNA with pulmonary conditions associated with inflammation, such as COPD and smoking, was analysed. In our study 65% of the patients had COPD, compared with 50% in another study.[45] In previous studies, the amount of circulating DNA increased proportionally with the amount of inflammatory cells in sputum and serum, but not in plasma.[24,33] In these studies free DNA concentration was not related to GOLD stage, which is in line with the results of our study.

In our study 3 controls developed cancer during follow-up and had low DNA levels. In a recently published lung cancer screening study, median baseline concentration of plasma DNA did not differ between individuals who developed lung cancer during follow-up versus cancer-free controls.[30]

Conclusion

In this study a high circulating plasma DNA concentration at time of diagnosis was a prognostic factor for poorer survival of NSCLC patients. No relationship was found between DNA concentration and histology, stage, age, gender or inflammatory pulmonary conditions as COPD or smoking. Circulating DNA may be used as a non-invasive biomarker requiring only a blood sample to refine the prognostic profile in NSCLC patients.

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Brief report of circulating DNA as a potential prognostic factor for survival in small cell lung cancer

Chapter 8

van der Drift MA, Hol BEA, Klaassen CH, Prinsen CFM, van Aarssen YA,
Donders R, van der Stappen JW, Dekhuijzen PNR,
van der Heijden HF, Thunnissen FBJM.

Submitted

Abstract

Introduction: The prognosis of small cell lung cancer (SCLC) is poor. Most prognostic factors are based on clinical characteristics and studies to (epi)genetic changes as prognostic factors are limited. Circulating plasma DNA, increased in cancer patients compared with controls, is an unfavorable prognostic biomarker in patients with non-small cell lung cancer (NSCLC). Little is known about the prognostic value of circulating DNA in SCLC. The aim of this study was to explore circulating DNA as a prognostic factor for survival in SCLC.

Patients and methods: In 10 SCLC patients and 21 controls quantification of baseline circulating plasma DNA was performed by a real-time quantitative PCR targeting the human ß-globin gene. Survival analysis was performed using the Kaplan-Meier method and compared with a Cox-regression analysis. Follow-up time was 6.5 years.

Results: In SCLC patients, the median DNA concentration was significantly higher compared with controls (163 ng/ml and 29 ng/ml, respectively; p = 0.004). We found a correlation between higher plasma DNA concentration and poorer survival in SCLC patients (p = 0.04, HR 1.6). The median DNA concentration of the patients, who died within 10 months after diagnosis, was significantly higher compared with the patients, who died after 10 months (p =0.05).

Conclusion: Our results suggest that circulating DNA should be evaluated as a potential prognostic biomarker in SCLC.

Introduction

Lung cancer is the most fatal solid cancer in developed countries. Small cell lung cancer (SCLC) is an aggressive tumor and frequently metastasized at time of diagnosis. The prognosis of SCLC is poor despite multimodality therapy, even for limited disease. Most prognostic factors are based on clinical characteristics. Favourable clinical prognostic factors for survival of SCLC patients include younger age, female gender, former smoking, better performance status and limited disease.[1] Studies to (epi)genetic changes, e.g. increased expression of proteins, as prognostic factors for survival in SCLC patients are limited.[2,3] A small study in SCLC patients showed a better median survival in the presence of both microsatellite alterations and TP53 mutations in plasma.[4]

Increasingly, circulating tumor-derived DNA and RNA markers in blood are under investigation.[2] Circulating plasma DNA is increased in patients with cancer or – to a lesser degree – benign disease.[5] Most studies about circulating plasma DNA as a diagnostic or prognostic biomarker in lung cancer were performed in patients with *non-small cell lung cancer* (NSCLC). The unfavorable prognostic value of a high circulating plasma DNA concentration at time of NSCLC diagnosis was discussed in several studies.[5-7]

In SCLC patients, only a few studies to circulating DNA were performed. They reported increased quantities of circulating DNA in *SCLC* patients at time of diagnosis compared with controls. However, the prognostic value of circulating DNA in these patients is not clear.[8,9] In these studies initially developed non-quantitative methods were used in a mixed population of NSCLC and SCLC patients. We performed an exploring analysis in a selected group of SCLC patients to investigate the potential role of circulating DNA as a prognostic factor for survival. A real-time quantitative polymerase chain reaction (qPCR), the current standard method to quantitate DNA, was applied to determine the baseline plasma DNA concentration at the time of diagnosis.

Patients and methods

Patients

Patients referred for (suspected) lung cancer were prospectively recruited for circulating DNA analysis and selected for this study upon diagnosis of SCLC. The patients in whom no lung cancer was diagnosed served as controls.[7] Overall survival was computed from the date of diagnosis to date of event occurrence or date of last follow-up until January 2009. Clinical characteristics including tumor characteristics and conditions associated with inflammation as smoking status and chronic obstructive lung disease (COPD) were registered. Pulmonary function was presented according to the guidelines of the Global Initiative for (Gold).[10] The study was approved by the institutional review board and informed consent was obtained from all participants.

DNA extraction and quantification

A 10 ml baseline peripheral blood sample was collected in EDTA tubes and centrifugated in two steps within 1 h after collection. After initial centrifugation of the original tube for 10 min at 2,000 x *g* at 20°C, the supernatants were transferred to micro centrifuge tubes and centrifuged again for 5 min at 16,000 x *g* at 20°C. The supernatant was stored at –20°C before DNA analysis. DNA was isolated from 1 ml of plasma using the MagNA Pure LC Total Nucleic Acid Isolation Kit - Large Volume (Roche diagnostics) and eluted in 100 µl. The DNA concentration was determined by using a highly reproducible real-time quantitative polymerase chain reaction (qPCR) targeting the human ß-globin gene.[7] The qPCR was performed on a LightCycler 1.2 instrument (Roche diagnostics) in a final volume of 20 µl containing 0.25 µM of amplification primers, 0.1 µM detection probe and 3 mM $MgCl_2$ (total concentration) in 1x LightCycler FastStart DNA Master HybProbe (Roche Diagnostics). A calibration curve was made from pure human lymphocyte DNA that was quantitated by UV absorbance measurements. The PCR program consisted of an initial denaturation of 10 min at 94°C followed by 40 cycles of 0 s at 95° and 15 s at 60°C with maximal ramping rates. Data were analyzed using the second derivative maximum method. DNA was expressed in ng/ml of plasma.

Statistics

Data were analysed with SPSS/PC+, version 16.0 (SPSS, Inc., Chicago, IL). Descriptive statistics were used for clinical characteristics and comparisons were performed by t-tests. Differences in DNA concentrations between patients and controls were analysed using a nonparametric Mann-Whitney U test. Differences in survival between patients and controls were compared with a log-rank test (Kaplan-Meier). A Cox regression model was used to assess the prognostic effect of DNA concentration on survival in SCLC patients. A p-value of ≤ 0.05 was regarded as significant.

Results

Clinical characteristics

Characteristics of the 10 SCLC patients, 80% staged extensive disease, and 21 controls are summarized in Table 1, including 87% males. The mean age of patients was just significantly higher than the mean age of controls (p = 0.05). 80% of the patients and 57% of the controls were current smokers. Former smokers had quit smoking for at least one year. Information about smoking of 1 control patient was missing. 3 Patients (30%) and 11 controls (52%) had COPD. The mean forced expiratory volume in 1 s (FEV1) in patients was just significantly lower compared with controls (p = 0.05).

DNA concentration and clinical characteristics

In patients, the median DNA concentration was significantly higher compared

with controls (p = 0.004) (Table 2). The median DNA concentration was 2284 ng/ml in patients with limited disease (n=2) and 163 ng/ml (IQ range 3517) in patients with extensive disease (n=8). Comparison of median DNA concentration in SCLC patients and controls according to clinical and tumor characteristics was not calculated due to small patient numbers.

Table 1. *Clinical characteristics of small cell lung cancer (SCLC) patients (n = 10) and controls (n = 21).*

Clinical characteristics	**SCLC patients**	**Controls**
Male / female	8 / 2	19 / 2
Age (range) (years)	68.1 (54.1-77)	59.3 (41.7-82.6)
Smoking history *Current* *Former* *Unknown*	 8 2 -	 12 8 1
Stage *Limited / extensive disease*	 2 / 8	
Mean FEV1(L)	1.82 (0.92-3.15)	2.52 (0.87-4.24)
COPD *absent* *GOLD 1* *GOLD 2* *GOLD 3*	 7 - 1 2	 10 4 6 1

Data are presented as number of patients, except for age and FEV1 (= mean forced expiratory volume in 1 s). COPD = chronic obstructive pulmonary disease; GOLD = Global Initiative for Chronic Obstructive Lung Disease

Table 2. *Median DNA concentration (ng/ml) according to clinical and tumor characteristics of the small cell lung cancer (SCLC) patients (n=10) and controls (n=21).*

Clinical characteristics	**SCLC patients**		**Controls**	
	no.	**DNA (IQ range)**	**no.**	**DNA (IQ range)**
Overall *min-max*	10	163 (4524)* 13-5802	21	29 (27) 0-175
Sex *male* *female*	 8 2	 263 (4631) 99	 19 2	 29 (29) 36
Smoking *Current* *Former*	 8 2	 99 (4631) 263	 12 8	 24 (29) 40 (36)
Stage *Limited disease* *Extensive disease*	 2 8	 2284 163 (3517)		

*IQ = interquartile; *p-value = 0.004 versus controls*

Relationship of DNA concentration to survival

At the date of last follow-up all SCLC patients and 2 controls (9.5%) had died. None of the control patients developed lung cancer or any other solid tumor during a follow-up period of median 67.6 months (range 3-78). Survival analysis is presented in Table 3. Mean overall survival was significantly decreased for patients compared with controls, as expected. A higher DNA concentration was related to worse survival (p = 0.04, HR 1.6). The median DNA concentration of the patients that died within 10 months after diagnosis (n=6) was higher compared with the patients that died after 10 months (n=4) (p=0.05). This result was also found when we corrected for stage.

Table 3. *Survival analysis in 10 small cell lung cancer (SCLC) patients and 21 controls according to DNA distribution.*

Subjects	Median DNA (ng/ml)	Mean survival time (months) (SE)	p-value (Cox regression)	HR
Controls Patients	29 163	71.3 (4.6) 9.6 (1.8)	0.04	1.6
Patients † *< 10 months* † *> 10 months*	 *58* *2425*	 *6.0 (1.2)* *14.9 (2.1)*	0.05	

Discussion

The results of this exploring study show a correlation between higher plasma DNA concentration and poorer survival in SCLC patients, which is in agreement with other studies[4,8], but not all.[9] Fournie et al. and Beau-Faller et al. used non quantitative detection, whereas nowadays qPCR for DNA quantification can be regarded as the standard method.[11] Different genes can be used for DNA amplification, depending on the experience of the institute, and do not influence the amount of circulating DNA. In our institute a qPCR assay targeting the ß-globin gene performed consistently in other experiments and was used in the present study.[7]

Interestingly, in our study the median DNA concentration of the patients who died within 10 months after diagnosis was notably higher compared with the patients who died after 10 months. As far as we know, this has not been shown before in SCLC patients. The median survival for patients treated for extensive SCLC is about 8-13 months.[12] Determining the DNA concentration in a simple blood sample might be helpful in informing SCLC patients and discussing prognosis. This is supported by Hou et al. showing that a higher number of circulating tumor cells (CTCs) is related to survival.[13] Until now, studies examining circulating DNA or circulating DNA derived epigenetic changes as prognostic factors for survival in

SCLC are limited. Assessing epigenetic changes in blood besides circulating DNA might be complementary in determining SCLC diagnosis and prognosis.[4,14] The ultimate goal is to develop targeted therapy based on (epi)genetic markers and making progress in patient's prognosis.
In this study a significantly higher median DNA concentration with broader IQ range values was found in SCLC patients compared with the NSCLC patients in our previous study which is in agreement with others investigating circulating DNA as a diagnostic biomarker.[7-9] Since NSCLC and SCLC differ in biology, treatment and prognosis, we hypothesized that the DNA concentration might differ between these two types of lung cancer. The circulating DNA is thought to originate from lysis, cell necrosis and apoptosis of cancer cells.[5] Tumor volume doubling times obtained with X-thorax are considerable lower for SCLC compared with NSCLC (median time 65 days and 90-185 days, respectively).[15] SCLC is morphologically characterized, besides a limited amount of cytoplasm, by a high number of mitoses (>50 / 2 mm^2) and apoptotic figures.[16] The rapid growth of SCLC involves a high turnover of tumor cells to apoptosis and lysis, and may explain why this leads to higher DNA levels compared with NSCLC patients.

The limiting factor of our study is the small sample size. Of the lung cancer patients, only 10-15% have SCLC and the incidence is declining.[1] Other studies concerning SCLC also described comparable patients numbers.[8,9] Although in former NSCLC studies no relationship of age, sex or pulmonary inflammatory conditions with DNA concentration was found, our study is too small to investigate these relations and therefore, this has to be confirmed in future SCLC studies with larger number of cases, allowing subgroup analysis.

Conclusion

We found increased quantities of circulating DNA in SCLC patients compared with controls and NSCLC patients with a state of the art qPCR technique. A high circulating plasma DNA concentration in SCLC patients was related to poor survival. Our results suggest that circulating DNA should be evaluated further as a potential prognostic biomarker in SCLC in larger studies.

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Summary and general discussion

Chapter 9

Summary

Lung cancer is considered as a major epidemic in the 20th en 21th century. It is the second most common cancer type in Dutch men and the third most common type in women and the leading cause of death by cancer worldwide (www.ikcnet.nl).1 The incidence of lung cancer in males is declining in developed countries following the significant decreased tobacco consumption, but the proportion of females more than doubled from 15% in 1989 to 1993 to 34% in 2004 to 2009. In this thesis, an overview of changes treatment and prognosis of non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) in the Netherlands in the last 20 years is presented. Further, it has been investigated how biomarkers in different body fluids guide diagnosis and prognosis in lung cancer.

Epidemiology of lung cancer

The majority (85-90%) of all lung cancers are NSCLC. In **chapter 2**, changes in treatment and prognosis of all NSCLC patients selected from the population-based Netherlands Cancer Registry (NCR) were described in the period 1989 to 2009 (n=147,760). Twenty-five percent of the patients was 75 years or older at the time of diagnosis. The proportion of NSCLC patients treated with standard of care therapy, as described in the Dutch guideline for staging and treatment of NSCLC published in 2004 (www.ikcnet.nl), increased in the past 20 years, except for stage II. The proportion of younger patients (< 75 years) with stage I undergoing surgery increased from 84% to 89% and among elderly (≥ 75 years) from 35% to 49%; for stage II this proportion decreased from 80% to 71% and remained about 25% in respectively the younger and older patients. Application of adjuvant chemotherapy for stage II increased to 24% in younger patients, but remained <5% among the elderly. Younger patients with stage III received chemoradiation more often up to 43% and up to 13% in elderly, compared with 8% and 1% in 1989 to 1993, respectively. In stage IV, chemotherapy in younger patients increased from 10% to 54%, and in elderly from 5% to 21%. Five-year relative survival of the total group increased from 14.8% to 17.4% and especially among females (18%), younger patients (18%) and within each stage, which could be partly explained by changes in treatment and partly by better staging. In conclusion, over a 20-year period, application of therapy in NSCLC patients, which is currently considered as standard, has improved. This has led to small improvements in survival within all stages.

Ten to fifteen percent of lung cancer consists of SCLC. Changes in treatment and survival in daily practice of *SCLC* in the Netherlands since 1989 were described in **chapter 3** also using the population-based data from the NCR (n =34,100). The proportion of younger patients (< 75 years) with limited disease (LD) receiving chemoradiation, the current standard therapy, increased from 19% to about 65% in 2004 to 2009 and from 7% to 27% among elderly (≥ 75 years). Among patients

with extensive disease (ED) the proportion receiving chemotherapy remained stable over time (around 82% for younger patients and almost 50% for the elderly). Significant improvements in 1-year relative survival occurred for patients aged 45 to 59 years, but not for the other age groups. Relative survival has significantly increased for both stage groups. In conclusion, application of standard of care therapy increased over time, but was less for elderly and for patients with limited disease. Despite better staging techniques and the use of new treatment modalities, survival of unselected patients with SCLC only improved for patients aged 45 to 59 years.

Biomarker analyses and DNA profiling in lung cancer

A suspected clinical and/or radiologic diagnosis of lung cancer needs to be confirmed by cytologic or histologic analysis. Mostly, a flexible bronchoscopy is performed including washings, brushings and forceps biopsies to establish lung cancer diagnosis. The diagnostic yield of bronchoscopy increases in larger tumors and endoscopic visible tumors. In **chapter 4**, the optimal timing and cost-effectiveness of washing relative to biopsy and brushing in bronchoscopy was analyzed prospectively in central (n=137) and peripheral (n=84) tumors of 221 patients. The diagnostic yield of washings *before* biopsies and brushings did not differ from the diagnostic yield of washings *after* biopsies and brushings in central, visible tumors (about 73%) and peripheral, non-visible tumors (about 39%). In 6% of the patients a diagnosis of malignancy was only established by washings. In case of a negative bronchoscopy, more invasive procedures were performed to establish a cancer diagnosis. Using known probabilities and costs for various bronchoscopic procedures, the expected utility of a number of diagnostic strategies was estimated including the invasive procedures. Confining laboratory investigations of washings or brush samples to those cases where initial findings of the biopsies are negative (the two-stage procedure) is more cost-effective than examining biopsies, brushings and washings altogether. In patients with visible tumors, brushing or washing in addition to biopsy is equally cost-effective; in patients with non-visible tumors, biopsy combined with washing is the preferred option. Apparently no difference in the diagnostic yield could be demonstrated for washings before or after biopsies and brushings. Although the additional diagnostic yield of washing and brushing during bronchoscopy is relatively low, it is cost-effective to use these procedures in the diagnostic work-up of patients who are clinically suspected of having a pulmonary malignancy.

Increasingly, molecular markers in bronchial washings are investigated to improve the diagnostic yield of peripheral, endoscopic non-visible, tumors. In **chapter 5**, the diagnostic value of RASSF1A methylation and *KRAS* mutations in washings in nondiagnostic bronchoscopy was analyzed in 51 patients with suspected lung cancer who had peripheral tumors and compared with 28 controls without a cancer diagnosis. The proportion of RASSF1a methylation was significantly higher

in central and larger tumors. In patients with nondiagnostic bronchoscopy (n=17), RASSF1A methylation was detected in the washings of 4 patients (24%), and *KRAS* mutations were detected in the washings of 2 patients (12%). In total, 29% of the false-negative or doubtful cytology results were accompanied by RASSF1A methylation or *KRAS* mutation results that were highly suggestive of malignancy. No relevant RASSF1A methylation was detected in control samples. These data suggest that the molecular analysis of 2 biomarkers in nondiagnostic bronchial washings may better guide diagnostic procedures in patients with suspected lung cancer.

Besides bronchoscopy, cytologic analysis of sputum can be used in the diagnostic work-up of lung cancer. Because the average diagnostic yield of sputum cytology is modest, also molecular markers are increasingly investigated in the (early) diagnosis of lung cancer. Therefore, the aim of **chapter 6** was to examine the presence of free DNA in sputum and its relationship to the presence of lung cancer. Free, or circulating DNA, is present in considerably higher concentrations in plasma of lung cancer patients compared with healthy controls or patients with benign disease. Free DNA was detected in sputum samples of 28 lung cancer patients and 68 controls without differences in DNA concentration. For all patients combined the amount of free DNA was related to the amount of inflammation. To discriminate between tumor related free DNA and inflammation, hypermethylation of RASSF1A was assessed. Increased hypermethylation of RASSF1A was found in lung cancer patients compared with controls. In conclusion, the amount of free DNA in sputum appeared to be related to the amount of inflammation, but not to the presence of lung cancer.

Although many studies report about the *diagnostic* value of circulating DNA in lung cancer, conflicting data are reported its *prognostic* value. Therefore, the aim of **chapter 7** was to prospectively analyze the relationship of circulating plasma DNA with overall survival (OS) of previously untreated non-small cell lung cancer (NSCLC) patients (n=46). The median baseline DNA concentration, quantitated by a real-time quantitative polymerase chain reaction (qPCR) targeting the human-globin gene, of the NSCLC patients who died (87%) was significantly higher compared with the patients that survived at the end of a follow-up time of 6.5 years (55 ng/ml versus 23 ng/ml, p = 0.02). In patients with higher DNA concentration overall survival was significantly worse. In this study no relation of DNA concentration with tumor characteristics, age, gender or pulmonary inflammatory conditions was found. In conclusion, a high circulating plasma DNA concentration at time of diagnosis in NSCLC patients was a prognostic factor for poorer survival. As little is known about the prognostic value of circulating DNA in SCLC patients, circulating plasma DNA as a prognostic factor for survival was analyzed prospectively in SCLC in **chapter 8**. In this small exploring study a correlation between higher plasma DNA concentration and poorer survival in 10

SCLC patients was found (p = 0.04, HR 1.6). The median DNA concentration of the patients that died within 10 months after diagnosis (n=6) was higher compared with the patients that died after 10 months (n=4) (p=0.05). Circulating DNA in SCLC patients should be further evaluated as a potential prognostic biomarker.

General discussion

The traditionally poor prognosis of lung cancer is accompanied by a considerable proportion of advanced stage at the time of diagnosis, which increased significantly in the last 20 years. Currently, (more than) half of the population of NSCLC and SCLC patients have advanced disease (this thesis). In the last decade, stage migration occurred as a result of improved diagnostic techniques, such as improved availability and quality of computerized tomography (CT) scans and fluorodeoxyglucose-positron emission tomography (FDG-PET) scanning. With the availability of these techniques the detection of previously occult distant metastases is facilitated, which has led to an upstaging from early to advanced disease.[2,3] Minimally invasive techniques such as esophageal or endobronchial endoscopic ultrasound enhanced the accuracy of mediastinal staging and also changes in the TNM (tumor, node, metastasis) classification system in 1997 have attributed to a stage-shift.[4-6] Increasingly, refinements of the diagnostic yield of bronchoscopy, sputum and plasma including assessment of molecular markers are under investigation to improve lung cancer diagnosis. Molecular analysis in body fluids could be particularly useful for patients with advanced lung cancer in whom cytologic specimens are often the only materials available. In this thesis was investigated how bronchial washings, RASSF1A methylation and circulating DNA in different body fluids facilitate in diagnosing lung cancer.

Biomarker analyses and DNA profiling in lung cancer

Bronchial washings

Bronchoscopy is an invaluable diagnostic tool in the diagnosis and staging of lung cancer.[7,8] Bronchoscopic biopsies of *central* tumors provide a high yield of 71 to 91%. Since the additional diagnostic yield of brushings and washings in these tumors is modest, one might consider omitting these procedures altogether restricting the increasing costs of lung cancer diagnosis in the current health care system. However, a negative bronchoscopy implicates additional invasive diagnostic procedures with increased health care risk and costs. In this thesis was shown that confining the cytologic investigations of washings or brushings only in patients with a nondiagnostic biopsy was most cost-effective. The same result was found in patients with peripheral tumors. Bronchoscopic biopsies of *peripheral* tumors provide a lower yield of about 36 to 61%. Brushings and washings increase the diagnostic yield to a modest extent. Biopsies combined with washings was the most cost-effective in peripheral tumors. In both central and peripheral tumors the timing of washings did not influence the diagnostic yield. Considering logistic

aspects, one can perform all techniques to obtain material for the pathologist, but work cost-effectively by investigating washings or brushings only if the biopsy is not diagnostic. However, considering patient aspects and current quality indicators demanding short waiting times for diagnostic procedures and treatment, one can investigate all material as fast as possible.[9] It also gives opportunity to analyze additional molecular analysis.

RASSF1A methylation and KRAS mutation

Methylation analysis in (lung) cancer is upcoming. Besides RASSF1A methylation, other methylated genes are described such as p16, FHIT, O-6-methylguanine-DNA methyltransferase (MGMT) and death-associated protein kinase (DAPK).[10-13] Methylation of more than one of these genes occurs frequently and seems an important phenomenon in silencing tumor suppressor genes. Assays for the detection of hypermethylation have several advantages: abnormal DNA methylation represents a stable tumor-associated marker, DNA methylation occurs mainly at specific CpG islands and with a methylation-specific (MSP) PCR the methylation status of CpG sites can rapidly be assessed with a high sensitivity and specificity.[14,15] Hypermethylation and *KRAS* mutations can be found in tumor tissue and various body fluids such as sputum and bronchial washings (this thesis).[16,17] Both have shown to be early events in the development of lung cancer.[12] The diagnostic value of these parameters were analyzed in washings of central and peripheral tumors. In line with other studies, the additional diagnostic value in central tumors is limited due to the already high yield of common cytologic and histologic analysis of bronchoscopic sampling.[18] In peripheral tumors with nondiagnostic bronchcoscopy was found that the detection of RASSF1A methylation in bronchial washings provides important evidence to suggest malignancy in patients suspected of NSCLC. Considering the above, we suggest that the bronchoscopic work-up for peripheral tumors may consist of performing biopsies and washings. The pathologist may perform a step by step analyses: if biopsies are negative, cytologic analysis of washings is performed, and if negative, RASSF1A analysis is performed. If positive, additional invasive procedures should be performed until lung cancer diagnosis is made definitely. If not, it depends on symptoms and radiologic signs if either further diagnostic procedures are necessary, or to perform evaluation chest CT-scan after 3 months. The presence of RASSF1A methylation is highly suggestive of lung cancer. In several studies, the amount of RASSF1A hypermethylation in patients with lung cancer is much higher than the low proportion of methylation in controls (0-4%).[16,18,19] This difference may allow a threshold level to be set. Besides facilitating in diagnosing lung cancer, RASSF1A methylation appears to be related to unfavorable prognosis.[13,20,21] Hypermethylation seems to be reversible by pharmacologic agents. Further studies to anti-lung cancer drugs aimed at RASSF1A methylation are ongoing.

Besides RASSF1A methylation analysis, the diagnostic value of *KRAS* mutations in washings of patients with peripheral tumors was analyzed. Because it is known

that *KRAS* mutations appear in tumor cells and not in healthy subjects, the presence of *KRAS* mutations indicate an increased probability of cancer cells.[22] However, *KRAS* mutations are predominantly found in adenocarcinomas (about 30%), which occur in about a third of the NSCLC patients. Thus, the prevalence of *KRAS* mutations in NSCLC is not high. In line with other studies, the additional value of *KRAS* mutation analysis in the washings of nondiagnostic bronchoscopy was limited.[23,24] Although the diagnostic value of *KRAS* mutations is limited, it is considered as a poor prognostic factor. *KRAS* mutations are associated with resistance to chemotherapy and targeted therapy aimed at the epidermal growth factor receptor (EGFR).[25,26]

Circulating DNA

Molecular changes, such as point mutations, microsatellite alterations and hypermethylated sequences, can also be found in extracellular DNA besides tumor cells. Extracellular or circulating DNA is increased in plasma of cancer patients as compared with healthy controls or patients with benign disease, but it is not cancer specific.[27-29] So, finding increased quantities of circulating in plasma is highly suspective of cancer, but needs to be confirmed by cytologic or histologic analysis. Therefore, the diagnostic value of circulating plasma DNA for lung cancer patients is limited in daily practice, although interesting in combination with other clinical parameters and/or qualitative molecular markers.[14,30,31] Until now, the presence of extracellular DNA in sputum and its relationship to lung cancer was unknown. We found that free DNA was present in sputum samples of the cancer patients and controls and related to the amount of inflammation, but not to the presence of cancer (this thesis). Further, increased hypermethylation of RASSF1A in lung cancer patients compared with controls was found to show that tumor related DNA is present in sputum. Sputum cytology does not play a role in early detection of lung cancer yet, but is of increasing interest in combination with low dose CT in large screening studies.[32-35] If lung cancer could be detected earlier, treatment and prognosis might be improved.

In spite of the reservations about the diagnostic capacities of circulating DNA, it offers potential utility as a prognostic biomarker. Circulating DNA may be used as a non-invasive biomarker to refine the prognostic profile in NSCLC and possibly in SCLC patients. The results in this thesis show that a high circulating plasma DNA concentration correlates with poor survival in NSCLC patients and perhaps in SCLC patients. No relationship of DNA concentration with clinical parameters as age, gender, histology, stage and smoking was found. Also no correlation with pulmonary inflammatory conditions was found in contrast to our sputum analysis. Recent studies also show that circulating plasma DNA is a prognostic factor for survival in NSCLC, but the clinical value remains controversial.[30,36,37] There are several limitations about circulating DNA. First, in guiding prognosis we are dealing with a broad range of circulating DNA, it is not an on-and-off phenomenon. The need for a valid cut-off value is clear, but not established

yet. Since circulating DNA is analyzed with different techniques, such as qPCR, pico green quantification and radioimmunoassay, and with different targeting genes, it is difficult to define a cut-off value which is valid for all NSCLC patients worldwide.[38-40] Also, the significance of a DNA concentration just above the cut-off value is doubtful. So, it may be valuable for a group of patients, but not for the individual patient yet. With increased refinements in predictive markers and personalized treatment, the role of circulating DNA as a predictive marker needs to be explored. In the last years, several studies showed that circulating DNA was predictive of the response to chemotherapy, but not to targeted therapy.[30,37,41,42] Besides extracellular DNA, circulating tumor cells (CTC's) can be found in plasma.[43] Studies in breast, prostate and colorectal cancer have demonstrated the prognostic significance of CTC's. Recently, an increased number of CTC's was found in stage IV lung cancer patients and correlated with worse survival.[44,45] Data interpretation is difficult due to variation in detection methods and reproduction across laboratories, comparable with the extracellular DNA studies. Finding the true boundaries of clinical opportunities for both circulating DNA and CTC's is a repeating challenge.

Population-based survival studies in lung cancer

Besides the prognostic value of circulating plasma DNA for survival of lung cancer patients, changes in survival according to treatment in the last 20 years was part of this thesis. Five-year relative survival of all patients with NSCLC together in the Netherlands improved slightly since 2004/5. In SCLC 1-year relative survival improved for patients aged 45 to 59 years, but not for the other age groups. Stage migration contributed to the observed small improvements in survival within all stages.[46] Also better compliance to treatment strategies with clinical service standards such as guidelines could have contributed to better survival at population level.[47] Although application of standard of care therapy increased over time, it was significantly less for elderly. Both therapy and survival results in clinical trials, including younger patients with good performance status, is still at a distance from the application of therapy and survival data observed in daily practice in unselected patients. Here, evidence based medicine meets patient based medicine. Individual treatment choices are limiting survival in elderly, driven for example by comorbidity and fulfilled life expectancy. The young, however, are subject to strict protocols driving them to prolonged survival.

Although multiple molecular biomarkers are under investigation as prognostic and predictive factors for survival, new personalized treatment strategies are developed and physicians act increasingly according to the current treatment guidelines. Physicians and researchers are increasingly aware of the positive effect on survival of psychosocial support and palliative care.[48] Metastatic lung cancer is a debilitating disease that results in a high burden of symptoms and poor quality of life. In palliative care, with its focus on management of symptoms,

psychosocial support, and assistance with decision making, the quality of care can be improved with lower rates of depressive symptoms and lower use of medical services. Optimal integration of psychosocial support and palliative care with standard oncologic care in patients with lung cancer should be the focus in oncologic clinical practice and future research.

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Nederlandse samenvatting

Chapter 10

Nederlandse samenvatting

De diagnose longkanker geeft een hoge ziektelast bij patiënten en is de belangrijkste doodsoorzaak door kanker wereldwijd (www.ikcnet.nl).[1] Het is het 2e meest voorkomende kankertype bij Nederlandse mannen en het 3e bij vrouwen. In de Westerse landen neemt de incidentie van longkanker bij mannen af door een verminderd tabaksgebruik, maar het aantal vrouwen met longkanker is verdubbeld van 15% in 1989-93 tot 34% in 2004-09. In dit proefschrift wordt een overzicht gegeven van de veranderingen in behandeling en overleving van niet-kleincellig longkanker (NSCLC) en kleincellig longkanker (SCLC) in Nederland in de laatste 20 jaar (hoofdstukken 2 en 3). Daarnaast is de bijdrage van biomarkers in verschillende lichaamsvloeistoffen in de diagnostiek en prognose van longkanker onderzocht (hoofdstukken 4, 5, 6, 7 en 8).

Epidemiologie van longkanker

Het merendeel van de longkankerpatiënten heeft niet-kleincellig longkanker (85-90%). In **hoofdstuk 2** worden veranderingen beschreven in de behandeling en prognose van alle niet-kleincellig longkankerpatiënten in Nederland, verzameld in de landelijke database van de Nederlandse Kanker Registratie (NKR), in de periode 1989-2009 (n=147.760). Op het moment van diagnose was 25% van de patiënten ≥ 75 jaar. Het aantal niet-kleincellig longkankerpatiënten behandeld met de huidige standaardbehandeling, zoals beschreven in de Nederlandse richtlijn voor stadiëring en behandeling van niet-kleincellig longkanker (2004, www.ikcnet.nl), is toegenomen in de laatste 20 jaar, behalve voor stadium II. Het aantal jongere patiënten (< 75 jaar) behandeld met chirurgie in stadium I nam toe van 84% tot 89% en bij ouderen (≥ 75 jaar) van 35% tot 49%; in stadium II daalde dit aantal van 80% tot 71% bij jongere patiënten en bleef rond 25% bij ouderen. De toepassing van adjuvant chemotherapie in stadium II bij jongere patiënten nam toe tot 24%, maar bleef < 5% bij ouderen. In stadium III steeg de proportie jongere patiënten behandeld met chemoradiatie van 8% tot 43% en bij ouderen van 1% tot 13%. De proportie jongere patiënten met stadium IV behandeld met chemotherapie nam toe van 10% tot 54%, en bij ouderen van 5% tot 21%. De 5-jaars relatieve overleving van de totale groep steeg van 14.8% tot 17.4% en vooral bij vrouwen (18%), jongere patiënten (18%) en binnen elk stadium, wat verklaard kon worden door zowel veranderingen in behandeling als door een betere stadiëring. Concluderend is de toepassing van de huidige standaard behandeling voor niet-kleincellig longkanker gedurende de laatste 20 jaar verbeterd. Dit heeft geleid tot kleine verbeteringen in overleving binnen alle stadia.

Tien tot 15% van de longkankerpatiënten heeft kleincellig longkanker. Veranderingen in de periode 1989-2009 in de behandeling en prognose van alle kleincellig longkanker patiënten in Nederland, eveneens verzameld in de NKR, zijn beschreven in **hoofdstuk 3** (n =34.100). De proportie jongere patiënten

(< 75 jaar) met een vroeg stadium, limited disease, behandeld met de huidige standaard behandeling met chemoradiatie nam toe van 19% tot ~65% in 2004-2009 en bij ouderen (≥ 75 jaar) van 7% tot 27%. Het aandeel patiënten met een gevorderd stadium, extensive disease, behandeld met chemotherapie veranderde niet gedurende 20 jaar (~82% van de jongere patiënten en ~50% van de ouderen). De 1-jaars relatieve overleving van patiënten tussen 45-59 jaar verbeterde significant, maar niet voor de andere leeftijdsgroepen. De relatieve overleving verbeterde significant voor beide stadia. Concluderend is het gebruik van de huidige standaard behandeling van kleincellig longkanker in de laatste 20 jaar verbeterd, maar minder duidelijk voor ouderen en patiënten met limited disease. Ondanks verbeterd stadiëringsonderzoek en het gebruik van verbeterde en nieuwe behandelcombinaties, is de overleving van ongeselecteerde kleincellig longkanker patiënten alleen verbeterd voor de leeftijdsgroep 45-59 jaar.

Biomarker en DNA analyse in lichaamsvloeistoffen bij longkanker

Een klinische of radiologische verdenking op longkanker moet bevestigd worden met cytologisch of histologisch onderzoek. Materiaal wordt vaak verkregen via een flexibele bronchoscopie waarbij spoelingen, brush (borsteltechniek) en biopten worden verricht. De diagnostische opbrengst van een bronchoscopie neemt toe bij grotere tumoren en bij endobronchiaal zichtbare tumoren. In **hoofdstuk 4** is de optimale timing en kosten-effectiviteit van spoelingen in relatie tot biopten en brush tijdens bronchoscopie prospectief onderzocht bij centrale (n=137) en perifere (n=84) tumoren van 221 patiënten. De diagnostische opbrengst van spoelingen *voor* biopten en brush verschilde niet van de diagnostische opbrengst van spoelingen *na* biopten en brush bij centrale, zichtbare tumoren (~ 73%) en bij perifere, niet-zichtbare tumoren (~ 39%). Bij 6% van de patiënten werd alleen op basis van spoelingen de diagnose kanker gesteld. Indien de bronchoscopie geen diagnose van kanker opleverde, werd meer invasief onderzoek verricht. De verwachte meerwaarde van het aantal diagnostische strategieën inclusief de invasieve procedures werd geschat met behulp van de bekende kosten van de diverse bronchoscopische procedures. Het laboratoriumonderzoek van spoelingen en brush beperken tot de patiënten waarbij de biopten negatief zijn (de twee-staps procedure) is kosteneffectiever dan alle biopten, brush en spoelingen gezamenlijk onderzoeken. Bij patiënten met zichtbare tumoren is het toevoegen van ofwel brush ofwel spoelingen aan biopten even kosteneffectief; bij patiënten met niet-zichtbare tumoren heeft biopten gecombineerd met spoelingen de voorkeur. Deze resultaten tonen dat er geen verschil is in diagnostische opbrengst tussen spoelingen voor of na het nemen van biopten en brush. Hoewel de toegevoegde waarde van spoelingen en brush tijdens een bronchoscopie relatief beperkt is, is het kosteneffectief om deze procedures toe te passen tijdens de diagnostische work-up van patiënten met verdenking op een pulmonale maligniteit.

In toenemende mate worden moleculaire markers in endobronchiale spoelingen onderzocht om de diagnostische opbrengst van perifere, niet-zichtbare

tumoren te verbeteren. In **hoofdstuk 5** is de diagnostische waarde van RASSF1A methylering en KRAS mutaties onderzocht in spoelingen van niet-diagnostische bronchoscopiëen bij 51 patiënten met verdenking op longkanker met perifere tumoren. Dit is vergeleken met 28 controle patiënten zonder maligniteit. De proportie RASSF1a methylering was significant hoger in centrale en grotere tumoren. Bij patiënten met een niet-diagnostische bronchoscopie (n=17) werd RASSF1A methylering gevonden in de spoelingen van of 4 patiënten (24%), en *KRAS* mutaties werden gevonden in de spoelingen van 2 patiënten (12%). In totaal ging 29% van de vals negatieve of twijfelachtige cytologie resultaten gepaard met RASSF1A methylering of *KRAS* mutaties met hoge verdenking op een maligniteit. Er werd geen relevante RASSF1A methylering gevonden bij controle patiënten. Deze resultaten suggereren dat de moleculaire analyse van 2 biomarkers bij niet-diagnostische bronchiale spoelingen leidend kunnen zijn in verdere diagnostische procedures bij patiënten met verdenking longkanker.

Naast bronchoscopie kan cytologische sputumanalyse worden gebruikt in het diagnostisch traject van longkanker. Omdat de gemiddelde opbrengst van sputumcytologie bescheiden is, wordt in toenemende mate de diagnostische waarde van moleculaire markers in sputum geanalyseerd. **Hoofdstuk 6** beschrijft een studie naar de aanwezigheid van vrij, of extracellulair, DNA in sputum en de relatie tot longkanker. Extracellulair, of circulerend, DNA komt in aanzienlijk hogere concentraties voor in plasma van (long)kankerpatiënten vergeleken met gezonde controles of patiënten met een goedaardige aandoening. Extracellulair DNA werd gevonden in sputum monsters van 28 longkankerpatiënten en 68 controle patiënten zonder verschil in DNA concentratie. In de hele groep was extracellulair DNA gerelateerd aan de mate van inflammatie. Toegenomen methylering van RASSF1A werd gevonden bij longkankerpatiënten in vergelijking met controle patiënten ter onderscheiding van tumor gerelateerd DNA met extracellulair DNA door inflammatie. Op basis van deze resultaten werd geconcludeerd dat de hoeveelheid extracellulair DNA in sputum gerelateerd is aan de mate van inflammatie, maar niet aan de aanwezigheid van longkanker.

Hoewel in veel studies de diagnostische waarde van circulerend DNA in longkanker is onderzocht, zijn er tegenstrijdige resultaten over de prognostische waarde van circulerend DNA. Het doel van **hoofdstuk 7** was om prospectief te onderzoeken wat de relatie van circulerend DNA met de overleving is bij 46 niet-kleincellig longkankerpatiënten. De baseline DNA concentratie, afgenomen voor behandeling, werd bepaald door een real-time quantitatieve polymerase chain reactie (qPCR) op het humane beta-globine gen. De DNA concentratie van de overleden niet-kleincellig longkankerpatiënten (87%) was significant hoger vergeleken met de patiënten nog in leven aan het einde van de follow-up tijd van 6.5 jaar (55 ng/ml versus 23 ng/ml, $p = 0.02$). De overleving van patiënten met een hoge DNA concentratie was significant slechter. In deze studie werd geen relatie gevonden tussen de DNA concentratie en tumor karakteristieken, leeftijd, geslacht of pulmonaal inflammatoire condities. Geconcludeerd werd dat een

hoge circulerend DNA concentratie in plasma op het moment van de diagnose niet-kleincellig longkanker een prognostische factor is voor slechte overleving.
Aangezien er weinig bekend is over circulerend DNA als prognostische waarde voor overleving van kleincellig longkankerpatiënten, werd dit prospectief onderzocht in **hoofdstuk 8**. In deze kleine explorerende studie werd een correlatie gevonden tussen een hogere plasma DNA concentratie en slechte overleving bij 10 kleincellig longkankerpatiënten (p = 0.04, HR 1.6). De mediane DNA concentratie van de patiënten die binnen 10 maanden na diagnose overleden (n=6) was hoger vergeleken met de patiënten die overleden na 10 maanden (n=4) (p=0.05). Circulerend DNA dient verder onderzocht te worden in toekomstig onderzoek als potentiële prognostische biomarker bij kleincellig longkanker patiënten.

Algemene discussie

De slechte prognose van longkanker wordt in belangrijke mate veroorzaakt door een groot percentage gevorderd stadium op het moment van diagnose. Dit percentage is significant toegenomen in de laatste 20 jaar en vandaag de dag heeft meer dan de helft van de niet-kleincellig en kleincellig longkankerpatiënten een gevorderd stadium (dit proefschrift). In de laatste 10 jaar vond stadiummigratie plaats als een gevolg van verbeterde diagnostische technieken, zoals toegenomen beschikbaarheid en kwaliteit van computerized tomography (CT) scans en positron emissie tomografie (PET) scans. Dankzij deze technieken is de ontdekking van eerder niet zichtbare metastasen op afstand toegenomen, wat ertoe heeft geleid dat een aanzienlijk aantal patiënten met aanvankelijk een vroeg stadium, een gevorderde stadium bleek te hebben.[2,3] Minimaal invasive techniqen zoals slokdarm- of endobronchiale endoscopische echografie verbeterde de nauwkeurigheid van mediastinale stadiëring. Daarnaast hebben veranderingen in de TNM classificatie 1997 bijgedragen aan veranderingen in stadiëring.[4-6]
In toenemende mate vindt verfijning van de diagnostiek plaats om de diagnose longkanker makkelijker te kunnen stellen, inclusief analyse van moleculaire markers. Moleculaire analyse in lichaamsvloeistoffen kan vooral van toegevoegde waarde zijn bij patiënten met gevorderd longkanker bij wie vaak cytologisch, maar geen histologisch materiaal beschikbaar is. In dit proefschrift is onderzocht hoe bronchiale spoelingen, RASSF1A methylering en circulerend DNA in verschillende lichaamsvloeistoffen kunnen bijdragen aan de diagnose en prognose van longkanker.

Biomarker analyse van longkanker in lichaamsvloeistoffen

Bronchiale spoelingen

Bronchoscopie is een belangrijk diagnostisch instrument in de diagnose en stadiëring van longkanker.[7,8] Bronchoscopische biopten van *centrale* tumoren hebben een hoge opbrengst (71% tot 91%). Aangezien de toegevoegde diagnostische waarde van brush en spoelingen in deze tumoren bescheiden is, kan men overwegen om

deze procedures achterwege te laten en hiermee de toenemende kosten in de huidige gezondheidszorg in de diagnostiek van longkanker te beperken. Echter, een negatieve bronchoscopie leidt tot aanvullende invasieve diagnostische procedures met als gevolg toegenomen gezondheidsrisico's en kosten. De resultaten van de studie in hoofdstuk 4 laten zien dat het uitvoeren van een cytologische analyse van brush en spoelingen alleen bij patiënten met een niet-diagnostisch weefselonderzoek het meest kosteneffectief is. Een vergelijkbaar resultaat werd gevonden bij patiënten met perifere tumoren. Bronchoscopische biopten van *perifere* tumoren hebben een lagere opbrengst (36 to 61%). Brush en spoelingen verhogen de diagnostische opbrengst in beperkte mate. Het meest kosteneffectief in perifere tumoren was het nemen van biopten gecombineerd met spoelingen. De timing van spoelingen had geen invloed op de diagnostische opbrengst van zowel centrale als perifere tumoren. De logistieke aspecten in acht nemende, kan men alle technieken uitvoeren om materiaal te verzamelen voor de patholoog, maar kosteneffectief te werken door brush en spoelingen alleen na te kijken indien de biopten niet conclusief zijn. Als we anderzijds rekening houden met de huidige kwaliteitsindicatoren betreffende korte doorlooptijden voor diagnostiek en behandeling, moet al het materiaal zo snel mogelijk onderzocht worden.[9] Dit biedt ook mogelijkheden om moleculaire markers aanvullend te onderzoeken.

RASSF1A methylering en KRAS mutatie

In toenemende mate wordt methylering in (long)kanker onderzocht. Behalve RASSF1A methylering, zijn andere gemethyleerde genen beschreven zoals p16, FHIT, O-6-methylguanine-DNA methyltransferase (MGMT) en death-associated protein kinase (DAPK).[10-13] Methylering van één of meer van deze genen komt vaak voor en blijkt een belangrijk fenomeen te zijn in het stilleggen van tumor suppressor genen. Het gebruik van hypermethyleringstechnieken heeft diverse voordelen: abnormale DNA methylering vertegenwoordigt een stabiele tumor-geassocieerde marker, DNA methylering vindt vooral plaats op specifieke CpG eilandjes en de methyleringsstatus van deze CpG eilandjes kan snel worden bepaald met een methylerings-specifieke (MSP) PCR met een hoge sensitiviteit en specificiteit.[14,15]

Hypermethylering en *KRAS* mutaties kunnen zowel in tumorweefsels als in verschillende lichaamsvloeistoffen zoals sputum en bronchiale spoelingen worden gevonden (dit proefschrift).[16,17] Beide veranderingen blijken vroeg in de ontwikkeling van longkanker voor te komen.[12] In hoofdstuk 5 is de diagnostische waarde van deze parameters onderzocht in spoelingen van centrale en perifere tumoren. Zoals ook in andere studies is gevonden, bleek de toegevoegde diagnostische waarde in centrale tumoren beperkt door de reeds hoge opbrengst van regulier cytologisch en histologisch bronchoscopisch onderzoek.[18] In niet-diagnostische bronchcoscopiëen van perifere tumoren bleek dat door de aanwezigheid van RASSF1A methylering in bronchiale spoelingen de verdenking

op niet-kleincellig longkanker toenam.
Gebaseerd op deze resultaten kan de diagnostiek van perifere longtumoren als volgt worden uitgewerkt. Tijdens een bronchoscopie worden biopten en spoelingen afgenomen. De patholoog onderzoekt alleen de cytologische monsters van de spoelingen indien de biopten negatief zijn. Indien de cytologie ook negatief is, wordt een RASSF1A methyleringsanalyse uitgevoerd. Indien positief, dan volgt zoveel aanvullend invasief onderzoek totdat de diagnose longkanker definitief gesteld is. Indien negatief, dan hangt het van de symptomen en beeldvorming (CT- en PET-scan) af of aanvullend diagnostisch onderzoek wordt verricht, of dat de afwijkingen na 3 maanden worden geëvalueerd middels een CT-scan. De aanwezigheid van RASSF1A methylering is sterk suggestief voor diagnose longkanker. In diverse studies was de mate van RASSF1A methylering in patiënten met longkanker veel hoger dan de lage hoeveelheid methylering in controle patiënten (0-4%).[16,18,19] Dit maakt het noodzakelijk om een drempelwaarde vast te stellen in verder onderzoek. Naast de toegevoegde waarde in de diagnostiek, is RASSF1A methylering ook gerelateerd aan een ongunstige prognose van longkanker.[13,20,21] Hypermethylering kan worden teruggedraaid door farmacologische stoffen en onderzoek naar anti-longkanker medicatie gericht op RASSF1A methylering is gaande.
Naast RASSF1A methyleringsanalyse, is in hoofdstuk 5 de diagnostische waarde van *KRAS* mutaties in spoelingen van patiënten met perifere tumoren onderzocht. Aangezien bekend is dat *KRAS* mutaties wel in tumorcellen maar niet in gezonde controle patiënten voorkomen, duidt de aanwezigheid van *KRAS* mutaties op de aanwezigheid van kanker.[22] De prevalentie van *KRAS* mutaties in longkanker is echter niet hoog, omdat *KRAS* mutaties vooral worden gevonden in adenocarcinomen (~ 30%), die ongeveer een derde van de niet-kleincellige longtumoren bedragen. Zoals ook ander onderzoek toont, was in onze studie de toegevoegde waarde van *KRAS* mutatie analyse in spoelingen van een niet-diagnostische bronchoscopie beperkt.[23,24] Hoewel de diagnostische waarde van *KRAS* mutaties beperkt is, worden *KRAS* mutaties beschouwd als een ongunstige prognostische factor. Deze aanwezigheid heeft consequenties voor behandeling, omdat *KRAS* mutaties gepaard kunnen gaan met resistentie voor chemotherapie en doelgerichte therapie gericht op de epidermale groei factor receptor (EGFR).[25,26]

Circulerend DNA in sputum en plasma
Moleculaire veranderingen, zoals puntmutaties, microsatellite veranderingen en gehypermethyleerde sequenties, kunnen zowel in tumorcellen als in extracellulair DNA gevonden worden. Extracellulair, ook genoemd vrij of circulerend, DNA is vermoedelijk afkomstig van afgebroken cellen en is verhoogd in *plasma* van kankerpatiënten in vergelijking met gezonde controle patiënten of patiënten met een goedaardige ziekte, maar is niet specifiek voor kanker.[27-29] Verhoogde hoeveelheden circulerend DNA in plasma zijn sterk verdacht voor kanker, maar dit moet cytologisch of histologisch bevestigd worden. Hoewel in de dagelijkse

klinische praktijk de diagnostische waarde van circulerend plasma DNA voor longkanker patiënten nog beperkt is, is dit interessant om verder te onderzoeken in combinatie met andere klinische parameters en/of moleculaire markers.[14,30,31] Tot op heden is onbekend of extracellulair DNA in *sputum* aanwezig is en of er een relatie is met longkanker. Uit ons onderzoek bleek dat extracellulair DNA inderdaad aanwezig was in sputum monsters van kankerpatiënten. In vergelijking met controle patiënten werd bij kankerpatiënten verhoogde methylering van RASSF1A werd gevonden om aan te tonen dat dit DNA tumor gerelateerd is. Zowel bij kankerpatiënten als controle patiënten was de hoeveelheid DNA echter gerelateerd aan inflammatie en niet aan kanker. Sputum cytologie en/of moleculaire veranderingen spelen nog geen rol in de vroege opsporing van longkanker, maar staan in toenemende mate in de belangstelling in grote screeningsstudies in combinatie met lage dosis CT.[32-35] Als longkanker eerder kan worden opgespoord, kunnen de behandeling en prognose worden verbeterd. Ondanks de klinisch diagnostische beperkingen van circulerend DNA, biedt het mogelijkheden als *prognostische* biomarker. Circulerend DNA kan worden gebruikt als een non-invasieve biomarker om het prognostische profiel van patiënten met niet-kleincellig longkanker en mogelijk met kleincellig longkanker te verfijnen. De resultaten van dit proefschrift tonen dat een hoge circulerend plasma DNA concentratie correleert met slechte overleving van patiënten met niet-kleincellig longkanker and mogelijk ook met kleincellig longkanker. Er werd geen relatie gevonden tussen de DNA concentratie en klinische en tumor karakteristieken, en evenmin met inflammatoire condities in tegenstelling tot onze sputumanalyse. Recente studies tonen ook dat circulerend DNA een prognostische factor voor overleving is voor patiënten met niet-kleincellig longkanker, maar de waarde voor de klinische praktijk blijft controversieel.[30,36,37] Ten eerste heeft circulerend DNA als prognostische marker een ruime range aan waarden, het is geen aan-uit-fenomeen. De noodzaak van een valide drempelwaarde is duidelijk, maar nog niet vastgesteld. Aangezien circulerend DNA kan worden bepaald met verschillende technieken, zoals qPCR, pico green kwantificatie en radioimmunoassay, en met verschillende target genen, is het lastig een drempelwaarde te definiëren die kan gelden voor alle patiënten met niet-kleincellig longkanker wereldwijd.[38-40] Daarnaast is de betekenis van een DNA concentratie net boven de drempelwaarde onduidelijk. Hierdoor kan de DNA concentratie van waarde zijn voor een groep patiënten, maar nog niet voor de individuele patiënt. Met de toenemende verfijning in predictieve markers en behandeling op maat, kan de rol van circulerend DNA als predictieve marker verder worden uitgewerkt. Recent onderzoek laat zien dat circulerend DNA voorspellend was in de respons op chemotherapie, maar nog niet op doelgerichte therapie.[30,37,41,42]

Naast extracellulair DNA kunnen circulerende tumorcellen worden gevonden in plasma.[43] Studies naar borst-, prostaat- en colorectaal kanker hebben de prognostische waarde van circulerende tumorcellen aangetoond. In recent onderzoek werd een verhoogd aantal circulerende tumorcellen gevonden

bij stadium IV longkankerpatiënten en dit was gecorreleerd aan slechte overleving.[44,45] Door een variatie aan onderzoeks- en detectiemethoden in verschillende laboratoria is interpretatie van data nog lastig, overeenkomend met de extracellulaire DNA studies. Het blijft een uitdaging in toekomstig onderzoek om de grenzen van klinische mogelijkheden van zowel circulerend DNA als circulerende tumorcellen op te sporen.

Bevolkingsonderzoek naar overleving van longkanker

Naast de prognostische waarde van circulerend plasma DNA voor de overleving van longkankerpatiënten, waren veranderingen in overleving ten gevolge van veranderingen in behandeling in de laatste 20 jaar onderdeel van dit proefschrift. De relatieve 5-jaarsoverleving van alle patiënten met niet-kleincellig longkanker in Nederland is licht verbeterd sinds 2004/5. Bij kleincellig longkanker verbeterde de relatieve 1-jaars overleving van patiënten tussen 45-59 jaar, maar niet voor de andere leeftijdsgroepen. Stadiummigratie heeft bijgedragen aan de kleine veranderingen in overleving in alle stadia.[46] Daarnaast heeft betere naleving van behandelingen volgens klinische standaarden zoals richtlijnen bijgedragen aan verbeterde overleving op bevolkingsnivo.[47] Hoewel de toepassing van standaard behandeling in de laatste jaren is toegenomen, bleef dit significant achter voor ouderen. Zowel uitkomsten van behandeling als overleving in klinische studies, die vooral jongere patiënten met een goede performance status includeren, staan nog ver af van de toepassing van behandeling en overlevingsresultaten van ongeselecteerde patiënten in de dagelijkse praktijk. We bevinden ons hier op het grensgebied van evidence-based medicine en patiënt-based medicine. De individuele therapiekeuze van ouderen, onder andere gebaseerd op comorbiditeit en kwaliteit van leven, begrenst bij hen de overleving. Daarentegen wordt de optimale overleving van jongeren meer geleid door strikte behandelprotocollen.

Naast het onderzoek naar een veelvoud van moleculaire biomarkers als prognostische en predictieve factoren voor overleving, worden nieuwe behandelstrategiëen op maat ontwikkeld en handelen artsen in toenemende mate volgens de laatste richtlijnen. Bovendien zijn artsen en onderzoekers zich toenemend bewust van het positieve effect van psychosociale en palliatieve zorg op de overleving.[48] Gemetastaseerd longkanker leidt tot een hoge symptoomlast en slechte kwaliteit van leven. In de palliatieve zorg, met het focus op het management van symptomen, psychosociale ondersteuning, en hulp in de besluitvorming, kan de kwaliteit van zorg worden verbeterd resulterend in minder depressieve symptomen en een lager gebruik van medische diensten. Optimale integratie van psychosociale ondersteuning en palliatieve zorg met standaard oncologische zorg bij patiënten met longkanker moet het focus zijn van de klinisch oncologische praktijk en toekomstig onderzoek.

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Nawoord: een weg van dankbaarheid

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Curriculum Vitae

Miep van der Drift is geboren op 3 mei 1969 in Leiden. Na de middelbare school in Voorburg (‘t Loo) en Leiden (‘t Visser het Hooft Lyceum) studeerde zij een jaar psychologie in Leiden omdat zij was uitgeloot voor de studie geneeskunde. In 1989 begon zij in dezelfde stad haar studie geneeskunde. Miep heeft tijdens haar studie ondermeer als student-assistent geneeskunde studenten begeleid en diverse wetenschappelijke onderzoeken ondersteund. Haar afstudeerscriptie bestond uit onderzoek naar oogafwijkingen bij hypofysetumoren. Zij was vervolgens vijf maanden verbonden aan de onderzoeksafdeling Neurologie van het Johns Hopkins Hospital in Baltimore, Amerika. In 1996 rondde zij haar studie cum laude af.
Vanaf 1996 werkte ze als arts-assistent niet in opleiding op de afdeling interne geneeskunde van het Deventer ziekenhuis waar zij in 1997 startte met de opleiding tot internist. Vanaf 2000 besloot zij zich verder te specialiseren tot longarts, in het Canisius-Wilhelmina Ziekenhuis in Nijmegen.
Vanaf 2004 werkt zij als longarts in het Universitair Medisch Centrum Nijmegen St. Radboud en het Universitair Centrum voor Chronische Ziekten Dekkerswald. Het aandachtsgebied bestaat uit de behandeling en begeleiding van patiënten met longkanker. Onder leiding van prof. dr. P.N.R. Dekhuijzen startte zij in samenwerking met de longartsen en de patholoog van het Canisius-Wilhelmina Ziekenhuis met het onderzoek dat de basis zou vormen voor dit proefschrift.
Zij is als keteneigenaar van de keten thoracale oncologie sterk betrokken bij de optimalisatie van kwaliteit, psychosociale zorg en logistiek. Zij heeft sinds 2010 mindfulness trainingen (training in aandacht) voor longkankerpatiënten en partners ontwikkeld. In september 2011 startte zij met de opleiding tot mindfulnesstrainer in het Radboud Centrum voor Mindfulness in Nijmegen.
Ze is getrouwd met Jan Douwes en samen hebben ze 2 kinderen, Bas (2003) en Julia (2005).