



ELSEVIER

**LUNG
CANCER**



www.elsevier.com/locate/lungcan

REVIEW

Circulating tumour-derived DNA and RNA markers in blood: a tool for early detection, diagnostics, and follow-up?

Roy M. Bremnes^{a,*}, Rafael Sirera^b, Carlos Camps^b

^a Department of Oncology, University Hospital of Northern Norway, University of Tromsø, N-9038 Tromsø, Norway

^b Department of Medical Oncology, Hospital General Universitario de Valencia, Spain

Received 25 October 2004; received in revised form 16 December 2004; accepted 20 December 2004

KEYWORDS

DNA;
mRNA;
Plasma;
Serum;
Lung cancer;
NSCLC;
Circulating

Summary

Background: Lung cancer is the most common cause of cancer death in developed countries. The prognosis is poor with only 10–15% of patients surviving 5 years after diagnosis. This dismal prognosis is attributed to the lack of efficient diagnostic methods for early detection and lack of successful treatment for metastatic disease. Within the last decade, rapid advances in molecular biology and radiology have provided a rational basis for improving early detection and patients' outcome. A non-invasive blood test effective in detecting preneoplastic changes or early lung cancer in high risk individuals has been perceived as a holy grail by cancer researchers.

Methods: The introduction of polymerase chain reaction (PCR)-based technology in the late 1980s and its refinement over the last 10 years have allowed us to detect and quantify extremely small amounts of tumour-derived nucleic acids. This has led to an increased knowledge of the molecular pathogenesis of lung cancer and a basis for the use of DNA and RNA markers in blood for early cancer detection, diagnostics, and follow-up. Common genetic alterations in lung carcinogenesis are already well known. We reviewed published literature on DNA and RNA in plasma or serum in lung cancer patients up to 2004, with particular emphasis on reports published since 1995.

Results: Twenty-two clinical studies have evaluating the role of DNA and RNA aberrations in the blood of lung cancer patients. A total of 1618 (range 10–163/study) cases and 595 (range 10–120/study) control cases were evaluated, and overall plasma/serum abnormalities were found in 43% (range 0–78%) of cases and 0.8% of healthy controls. For (1) total DNA and gene expression levels, 61% (range 53–71%) of cases and 0.9% of controls; (2) oncogene mutations, 16% (range 0–30%) and 0%; (3) microsatellite alterations, 46% (range 24–71%) and 21% (controls with non-malignant pulmonary disease); (4) promoter methylation, 42% (range 5–73%) and 0%; (5) tumour-related RNAs, 54% (range 39–78%) and 6%. In general,

* Corresponding author. Tel.: +47 906 52753; fax: +47 776 26779.

E-mail address: roy.bremnes@yahoo.no (R.M. Bremnes).

the studies contain small series of lung cancer patients and even smaller or missing case control groups.

Conclusion: The analysis of circulating DNA or RNA in plasma is a promising non-invasive diagnostic tool, requiring only a limited blood sample. Its wide applicability and potential importance will possibly lead to increasing clinical impact in the near future. However, large prospective clinical studies are needed to validate and standardise any tests for DNA or RNA alteration in plasma or serum of high risk individuals or patients with established lung cancer.

© 2005 Elsevier Ireland Ltd. All rights reserved.

Contents

| | |
|--|----|
| 1. Introduction | 2 |
| 2. History of circulating nucleic acids | 3 |
| 3. Circulating tumour nucleic acids in plasma and serum | 4 |
| 3.1. Oncogene mutations and amplifications | 4 |
| 3.2. Microsatellite alterations | 4 |
| 3.3. Epigenetic changes | 5 |
| 3.4. Mitochondrial DNA | 5 |
| 3.5. Plasma RNA detection | 5 |
| 4. Summary of available data on circulating DNA and RNA in plasma or serum of lung cancer patients | 6 |
| 4.1. Total circulating DNA | 6 |
| 4.2. Circulating gene mutations | 7 |
| 4.3. Circulating microsatellite alterations | 7 |
| 4.4. Methylated promoter regions in plasma or serum | 8 |
| 4.5. Plasma RNA detection | 8 |
| 5. Future prospects and challenges | 9 |
| 6. Conclusions | 10 |
| Acknowledgement | 10 |
| References | 10 |

1. Introduction

In the western world, lung cancer is one of the most common cancers and the leading cause of cancer deaths. In the US, lung cancer accounts for more deaths than prostate cancer, breast cancer, and colorectal cancer combined [1]. The high mortality rate is related to the low cure rate (6–15%), which in turn is related to the lack of adequate screening and early detection measures. Only 15% of lung cancer patients are diagnosed at an early stage [2]. The lung cancer cure rates have been relatively unaltered for 40 years, and further substantial increase is not to be expected with the current treatment modalities. The most promising way to improve this dismal prognosis is by means of early detection.

About 90% of the lung cancer cases are caused by cigarette smoking [3–5]. Primary prevention

measures aimed at decreasing tobacco exposure are extremely important. But even if interventions reduced cigarette smoking dramatically, today's smokers will have a significantly increased lung cancer risk for the next 20–40 years. Thus, in addition to primary prevention, secondary prevention such as early detection measures will be important tools for reducing lung cancer deaths.

The aim for early detection is to identify lung cancer at a stage early enough to be curable by surgery. The problem has, hitherto, been lack of valid screening methods. There are no consensus or established methods to screen smokers or other high risk individuals for lung cancer. Previous studies of sputum cytology or annual X-rays showed no benefits in lung cancer mortality reduction [6–9]. Nevertheless, low resolution lung CT screening of high risk individuals (heavy smokers)

appears promising [10–13]. The first two large randomised trials are already on its way in France and the US (National lung screening trial).

Undoubtedly, the success in improving lung cancer outcomes lies along the path of improving the detection of early lung cancer. We know that 20–30 years of smoking are required before lung cancer rates significantly increase. Lung carcinogenesis is a multistep process characterised by the accumulation of successive molecular genetic and epigenetic abnormalities, resulting in selection of clonal cells with uncontrolled growth capacities throughout the respiratory tract. This concept is known as “field cancerisation”, whereby the gradual accrual of sequential genetic and morphological changes ultimately results in the formation of an invasive tumour. Molecular lesions far precede morphological transformation of preneoplastic bronchial or alveolar lesions [14]. Therefore, it can be inferred that this preinvasive phase of lung cancer may last for a period of years to decades. The length of this interval has lately been a window of opportunity for early detection of genetic and epigenetic abnormalities, in the genes involved in cell cycle, senescence, apoptosis, repair, differentiation, and cell migration control, in bronchial biopsies or sputum specimens.

The early tumour markers were primarily proteins, but only a small subset of tumours would secrete specific proteins that could be used as tumour markers. However, many of these early methods were subjected to major shortcomings such as limited sensitivity and specificity. The recent advances in molecular biology and genetics, and the accelerated knowledge within molecular pathology of cancer have led to a rapid technological development. Thus, today multiple new genetic and epigenetic alterations can be characterised in cancer cell lines and in malignant tumours. These abnormalities include genetic gains, losses, and loss of heterozygosity (LOH: deletion of one copy of allelic DNA sequences) and may affect several chromosomes. Upregulation of oncogenes and downregulation of tumour suppressor genes may result from chromosomal events, such as gene amplification, deletion, or modification of DNA transcription. Of diagnostic significance is the fact that many of these changes are also detectable in the plasma/serum of cancer patients [15].

Based on new molecular detection methods, mainly polymerase chain reaction (PCR)-based assays, circulating tumour DNA can now easily be extracted from serum, plasma, saliva, bronchoalveolar lavage fluid, and urine. Due to its high specificity and sensitivity, these PCR-based techniques can detect small amounts of circulating aber-

rant DNA among an excess background of normal genes.

2. History of circulating nucleic acids

The presence of DNA and RNA in plasma of cancer patients has been recognised since the 1970s [16,17]. But it was not until the late 1980s that this circulating DNA was shown to exhibit tumour-related alterations [18]. In 1994, two groups of investigators demonstrated that specific oncogene mutations could be detected in the plasma of patients suffering from pancreatic cancer [19] and myelodysplastic syndrome [20]. Two years later, LOH and microsatellite instability (MSI) were found in the primary tumours as well as in the plasma or serum of patients suffering of lung and head and neck cancers [21,22]. These findings suggested that high fractional concentrations of tumour DNA were present in the plasma/serum of these patients.

During the 1990s, a large number of tumour-associated genetic and epigenetic changes were detected in the plasma/serum of cancer patients: *Ras* and *p53* mutations, microsatellite alterations, aberrant promoter hypermethylation of several genes, rearranged immunoglobulin heavy chain DNA, mitochondrial DNA mutations, and tumour-related viral DNA [23,24]. Lately, work on mRNA, using essentially similar methodology, have appeared promising as the percentage of tumours (different malignancies) detectable with this assay appears to be higher than that found with DNA markers [25]. Cell-free mRNA can be detected in plasma as well [26]. Studies with RNA markers are particularly promising due to their close association with malignancy, and RNA markers may eventually improve the early detection rate in plasma/serum of lung cancer patients [27].

Over this period, it was demonstrated that tumour-related DNA was not confined to any specific cancer type, but appeared to be a common finding across different malignancies. Hence, mutant plasma DNA has been found in lung, head and neck, colorectal, gastric, pancreatic, liver, biliary tree, skin, breast, kidney, ovarian, cervical, bladder, and prostate cancers as well as haematological malignancies including lymphomas. In this review, we will emphasise different approaches for tumour-related nucleic acid detection in the circulation, the potential of circulating nucleic acids as tumour markers in early detection, monitoring and/or prognostication, and more specifically present results from different studies of DNA and RNA in plasma/serum from lung cancer patients.

3. Circulating tumour nucleic acids in plasma and serum

3.1. Oncogene mutations and amplifications

Oncogene mutations can be found in a wide variety of cancers. The detection of such mutations in plasma or serum provides solid evidence that nucleic acids are released into the circulation by tumours.

The *K-ras* family of proto-oncogenes is part of the downstream signalling pathway of several different molecules. Activated point mutations of the *K-ras* gene are one of the most common genetic alterations found in human malignancies, and it was the first tumour-specific mutation sequence detected in the blood of cancer patients [19,20]. *K-ras* mutations are rather frequently seen in lung cancer, but are largely limited to adenocarcinomas [28].

K-ras gene mutations in plasma are of particular interest and are the most extensively studied mutations in blood [29]. These gene mutations are: (1) frequently found in various types of tumours (colorectal, pancreatic, and lung cancers), (2) point mutations (codons 12, 13, and 61) identical in the patient's tumours and plasma [19,30,31], and (3) relatively easily detected due to a limited repertoire of mutations. Since the point mutations occur early in the development of cancer, are highly specific for cancer, have well-characterised sites, and has been detected in plasma up to several months prior to the clinical diagnosis in occasional cases [32] they offer interesting insights into the possibilities for use in serologic diagnosis. While the broad spectrum of tumours exhibiting *K-ras* mutations limits the specificity of *K-ras* mutation tests for particular cancers, any mutation detected in plasma will be specific for a malignant disorder in contrast to non-malignant conditions.

The *p53* gene, located on the short arm of chromosome 17, is one of the prototype tumour suppressor genes, involved in the regulation of cell proliferation. Mutations of this gene are also among the most common genetic changes in human cancers, and occur at an early stage of lung carcinogenesis. According to the study by Zienolddiny et al. [33], there is a highly significant correlation between *p53* mutations and LOH (deletions on 3p, 5q, 9p, and 17p). Mutated *p53* sequences have also been detected in the plasma of lung cancer patients [34–36]. The mutations in plasma correspond to those in the primary tumour, and the presence in plasma of these mutated genetic sequences is generally associated with more advanced staging and larger tumours [34].

3.2. Microsatellite alterations

Genomic instability is a hallmark of malignant cells and creates a permissive state allowing for the accumulation of genetic alterations, which lead to tumour development and progression. Microsatellites are repetitive DNA sequences (up to five nucleotides long) that form variable-length stretches of DNA. In mammalian DNA, the most common microsatellite is the nucleotide repeat of cytosine and adenine which occurs in tens of thousands locations. With appropriate primers it is possible to amplify DNA fragments that can be used as microsatellite markers, and with a "panel" of such markers, tumours can be profiled. Characteristic microsatellite alterations due to genomic instability, such as microsatellite instability and LOH are frequently demonstrated in tumour tissues and in plasma. The detection of LOH in plasma or serum of patients suffering from small cell lung cancer (SCLC) and head and neck cancer, was simultaneously reported by two groups in 1996 [21,22]. Microsatellite alterations were present in three-quarters of the tissues and in a similar fraction of plasma, and interestingly these changes were identical to those found in the primary tumours. These findings not only confirmed the presence of tumour DNA in plasma/serum, but moreover suggested that they constituted a major proportion of the circulating DNA. Similar results have also been shown for non-small cell lung cancer (NSCLC) [37–39].

As different groups of investigators have used different numbers and locations of microsatellite markers, the results are heterogeneous. In a larger follow-study of SCLC patients, Gonzalez et al. [34] found that 71% of the cases exhibited at least one molecular change in plasma precisely matching that of the primary tumour. This alteration was also a strong prognostic indicator. Positive results can also be seen in patients having small tumours or in situ carcinomas [40,41]. This indicates that the amount of DNA released can be detectable even in the early stages of disease. On the other hand, there is a trend that positive results correlates with the clinicopathological characteristics of the lung carcinoma as patients having LOH in their plasma/serum are more likely to have invasive tumours, regional spread, and distant metastases [34].

Microsatellite alterations in plasma DNA were detected in 43% of patients with stage I non-small cell lung cancer and in 45% of tumours up to 2 cm in diameter [42]. Sixty-one percent of the cases displayed the same abnormalities in the primary tumour as in the plasma DNA. None of the control subjects had genetic abnormalities in the plasma (specificity 100%). However, in five operated

cases these abnormalities were only observed in the plasma DNA, raising the hypothesis that in spite of complete resection, these abnormalities in circulating DNA may be micrometastases. Sozzi et al. [43] and Gonzalez et al. [34] further demonstrated that in lung cancer (both NSCLC and SCLC) patients whose plasma showed LOH, these changes disappeared from their plasma after successful therapy. In contrast, the patients with no treatment response showed no normalisation of the plasma DNA abnormalities. Interestingly, at recurrence of their lung carcinomas, these tumour specific changes reappeared. Thus, microsatellite markers appear valuable in early detection, prognostication, and monitoring treatment effects.

3.3. Epigenetic changes

Epigenetic mechanisms are the regulation of changes in gene expression by means which do not involve modifications in DNA sequence, and provide additional instructions of how, where, and when the genetic information should be used. In contrast to the former permanent genetic alterations, epigenetic changes do not need to be permanent. The major form of epigenetic information in mammalian cells is DNA methylation. Thus, epigenetic changes describe the inheritance of information by the modifications of DNA other than its sequence.

CpG islands are 0.5–2 kb regulatory regions, rich in cytosine–guanine dinucleotides and are present in the 5'-region of approximately half of human genes [44]. DNA methylation of these cytosine residues is an epigenetic characteristic associated with the silencing of gene expression. Alteration of DNA methylation patterns, including global genome hypomethylation and regional hypermethylation of tumour suppressor genes at the CpG islands in the promoter regions of these genes, are increasingly found in different types of tumours. This is a common phenomenon in lung cancer, as demonstrated by the analysis of the methylation status of more than 40 genes from lung cancer tumours, cell lines, patients' sputum and/or serum [45]. Determination of the methylation patterns of multiple genes to obtain complex DNA methylation signatures appears promising in order to provide a highly sensitive and specific tool for lung cancer diagnosis [45].

These changes are thought to be closely associated with tumorigenesis, and have aroused much interest since methylation should be amenable to therapeutic intervention. Genes, which are frequently hypermethylated in lung cancers, are *p16*, *death-associated protein kinase (DAPK)*, *glutathione S-transferase P1 (GSTP1)*, and *O⁶-methylguanine*

methyl-transferase (MGMT) [46]. These genes may play important roles either in the regulation of cell cycle or in the pathway of DNA repair [47]. Development of methods like methylation-specific polymerase (MSP) chain reaction [48] allows the detection of small amounts of hypermethylated sequences in a background of a wide range of sequences and thus makes assessment in plasma/serum of cancer patients possible. The rate of detecting such changes in plasma/serum depends on the sensitivity of the detection method. In cohorts of NSCLC patients, Esteller et al. [46] found hypermethylation of the *DAP* kinase gene in serum from 80% of the patients whereas in the study by Ramirez et al. [49] this hypermethylation was detected in the serum of 40% of the patients. The hypermethylation in serum and tumour tissues was similar [46].

3.4. Mitochondrial DNA

Human cells contain several hundred copies of mitochondrial DNA that encodes respiratory chain proteins, tRNAs, and rRNAs. Several mutations in the mitochondrial genome have been documented for colorectal, bladder, lung, and head and neck carcinomas [50]. In prostate cancer patients, Jeronimo et al. [51] reported in 2001 for the first time the finding of mutated mitochondrial DNA sequences in plasma. They reported that identical mutations of the mitochondrial DNA could be detected in both plasma and tumour tissues from the patients. This study showed for the first time the presence of tumour specific mitochondrial DNA in the circulation. As tumour tissues contain high copy numbers of mitochondrial DNA sequence, detection in plasma/serum of tumour mitochondrial DNA may offer a sensitive way to detect early disease. Until now, mutated mitochondrial DNA sequences in plasma have not been reported in lung cancer patients.

3.5. Plasma RNA detection

Messenger RNA (mRNA) is the intermediate between the coding gene and the final product, which is protein, and at the cellular level, the concentration of the respective protein is proportional to the mRNA level. As different tumour types typically express a different repertoire of protein, the respective mRNA can be used as a marker for cancer screening and monitoring. As circulating RNase is present in the blood of healthy individuals with an even higher level in cancer patients [52], one would expect all free RNA in the blood to be rapidly destroyed. In 1999, however, Lo et al. [53] found that

Table 1 Studies evaluating total DNA in the blood of lung cancer patients and controls

| References | Tumour type | Marker | No. of lung cancer cases | Serum/plasma positive | | No. of controls | % Positive |
|------------|-------------|------------|--------------------------|-----------------------|----|-----------------|------------|
| | | | | No. | % | | |
| [57] | NSC/SC | Total DNA | 68 | 36 | 53 | 26 | 0 |
| [55] | NSC/SC | Circ DNA | 45 | 32 | 71 | 59 | 0 |
| [43] | NSC | Circ DNA | 84 | 45 | 54 | 43 | 0 |
| [58] | NSC | QPCR hTERT | 100 | 69 | 69 | 100 | 2 |
| Total | | | 297 | 182 | 61 | 228 | 0.9 |

Abbreviations: NSC, non-small lung cancer; SC, small cell lung cancer; hTERT, human telomerase catalytic component.

cell-free Epstein–Barr virus associated RNA could be detected in the blood of patients with nasopharyngeal carcinoma, using a similar methodology as for DNA analysis. At the same time, Kopreski et al. [26] demonstrated the presence of tyrosinase mRNA in malignant melanoma patients while no tyrosinase mRNA could be detected in the healthy individuals, suggesting the existence of extracellular tumour-derived RNA in the circulation, protected from RNase. This distinction between cancer patients and healthy individuals may allow plasma-based RNA expression profiling for cancer screening and monitoring in the future.

Several groups have confirmed these findings by detecting different mRNA types in plasma or serum of cancer patients [15]. In lung cancer patients, several potentially useful mRNAs have been investigated. In 18 lung cancer cases, Fleischacker et al. [27] found that hnRNP mRNA and Her2/neu-specific mRNA were detected in plasma in 14 and 7 cases, respectively, and that either of the two was selectively detectable in all cases with a malignant lung tumour. In a material of five patients with breast cancer and 14 with non-small cell lung cancer, Kopreski et al. [54] reported detection of tumour-derived tyrosinase mRNA in the serum of 43% of lung cancer patients and in only 12% of normal control sera.

4. Summary of available data on circulating DNA and RNA in plasma or serum of lung cancer patients

Hitherto, there are 22 clinical studies evaluating the role of circulating nucleic acids in blood as possible markers in lung cancer patients. These can be divided into studies measuring total DNA, gene expression levels, mutations in specific oncogenes, tumour suppressor genes, microsatellite alterations, promoter methylation of various tumour suppressor genes, and tumour-related RNAs. In general the case numbers are limited, the number of control

cases is smaller or controls are missing, and the methods have not been validated or standardised. For all 22 studies, a total of 1618 cases and 595 control cases were evaluated, and plasma/serum abnormalities were found in 43% (range 0–78%) cases and 9.9% among control cases. When estimations are based solely on healthy controls in this review, plasma/serum abnormalities were virtually none (0.8%) among these cases. In the study by Khan et al. [39], the control cases consisted of blood samples from patients with non-malignant respiratory diseases (Table 3), and these accounted for 54 of 59 (92%) positive control. In several studies, the plasma DNA concentrations have been reported to be substantially higher in patients with pulmonary diseases compared to healthy controls [55,56]. Except for the study of Khan et al. [39], there are no consistent reports on different frequencies of specific DNA alterations between healthy controls and patients with non-malignant pulmonary diseases.

4.1. Total circulating DNA

Four of the studies [43,55,57,58] measured circulating DNA quantitatively by various methods (Table 1). They reported increased (above cut-off) circulating DNA levels in 61% (range 53–71%) of cases and in only 0.9% (range 0–2%) of controls. In a case-control study of 330 subjects including healthy controls, patients with various cancers and patients with non-neoplastic diseases, the investigators found that earlier quantitative methods for assessments of plasma DNA were not adequately sensitive or specific for cancer screening or diagnosis [59]. These data were consistent with a recent European multicentre study in healthy controls, chronic obstructive pulmonary disease patients, and in patients with a variety of cancers [56]. The latest study by Sozzi et al. [58] is the largest series reported, and their employed real-time quantitative PCR method clearly has a more favourable sensitivity (78%) and specificity (95%). The control group ($n = 100$) in this study consisted of

Table 2 Studies evaluating circulating *K-ras* and *p53* mutations in the blood of lung cancer patients and controls

| References | Tumour type | Marker | No. of lung cancer cases | Serum/plasma positive | | No. of controls | % Positive |
|------------|-------------|-----------------------|--------------------------|-----------------------|----|-----------------|------------|
| | | | | No. | % | | |
| [49] | NSC | <i>K-ras</i> mutation | 50 | 12 | 24 | 0 | |
| [60] | NSC | <i>K-ras</i> mutation | 35 | 0 | 0 | 15 | 0 |
| [31] | NSC | <i>K-ras</i> mutation | 163 | 19 | 12 | 0 | |
| [35] | NSC | <i>p53</i> mutation | 64 | 19 | 30 | 0 | |
| [36] | SC | <i>p53</i> mutation | 10 | 1 | 10 | 0 | |
| Total | | | 322 | 51 | 16 | 15 | 0 |

Abbreviations: NSC, non-small lung cancer; SC, small cell lung cancer.

heavy smokers with minimum 20 pack years. Based on their data, the authors conclude that higher levels of free circulating DNA can be detected in patients with lung cancer compared with disease-free heavy smokers, and that this suggests a new non-invasive approach for early detection of lung cancer in high risk individuals [58].

4.2. Circulating gene mutations

Table 2 presents data from all available studies on circulating *K-ras* and *p53* mutations [31,35,36,49,60]. *K-ras* mutations were detected in the plasma of 13% of cases (range 0–34%) and *p53* mutations in 25% (range 12–30%). Kimura et al. [31] have recently reported the largest study ($n=163$) in this group. They found that *K-ras* point mutations in plasma DNA were identical to the mutations found in the tumours, confirming the tumour as the source. The only control case study was presented by Bearzatto et al. [60]. None of the con-

trols or the lung cancer cases in their study had *K-ras* mutations in their blood, though *K-ras* mutations were detected in 31 of 35 (89%) tumours. It has also been reported that that *K-ras* mutations in serum significantly correlate with survival [49]. In another recent study on 64 stages I–III non-small cell lung cancer patients, Andriani et al. [35] found *p53* mutations in 41% ($n=26$) of the lung tumours, and detected the identical mutation in the plasma of 73% ($n=19$) of the tumour positive cases.

4.3. Circulating microsatellite alterations

LOH and the presence of allele shifts, indicating genomic instability, have been studied in 11 reports (Table 3) [21,34,35,37–39,42,43,60–62]. In all series, there were used between 2 and 9 markers to increase the percentage of abnormal findings. Microsatellite alterations in serum or plasma were found in 46% (range 24–71) of 502 cases. In fact, in 5 of 11 studies, alterations were observed in 61–71%.

Table 3 Studies evaluating circulating microsatellite alterations (MSA) in the blood of lung cancer patients and controls

| References | Tumour type | MSA No. of markers | No. of lung cancer cases | Serum/plasma positive | | No. of controls | % Positive |
|------------|-------------|-----------------------|--------------------------|-----------------------|----|-----------------|-----------------|
| | | | | No. | % | | |
| [42] | NSC | 2 | 87 | 35 | 40 | 14 | 0 |
| [37] | NSC/SC | 3 | 43 | 14 | 33 | 10 | 0 |
| [61] | NSC/SC | 3 | 28 | 17 | 61 | 31 | 0 |
| [21] | SC | 3 | 21 | 15 | 71 | 0 | |
| [39] | NSC/SC | 3 | 86 | 59 | 69 | 120 | 42 ^a |
| [34] | SC | 4 | 35 | 25 | 71 | 0 | |
| [38] | NSC | 4 | 22 | 6 | 28 | 0 | |
| [35] | NSC | 5 | 64 | 23 | 36 | 0 | |
| [60] | NSC | 5 | 34 | 11 | 32 | 0 | |
| [43] | NSC | 5 | 38 | 9 | 24 | 43 | 0 |
| [62] | NSC/SC | 9 | 24 | 17 | 71 | 20 | 0 |
| Total | | | 502 | 231 | 46 | 238 | 21 |

Abbreviations: NSC, non-small lung cancer; SC, small cell lung cancer.

^a Patients with non-malignant respiratory diseases.

Table 4 Studies evaluating circulating methylated genes in the blood of lung cancer patients and controls

| References | Tumour type | Methylation markers | No. of lung cancer cases | Serum/plasma positive | | No. of controls | % Positive |
|------------|-------------|---------------------|--------------------------|-----------------------|----|-----------------|------------|
| | | | | No. | % | | |
| [63] | NSC | <i>APC</i> | 89 | 42 | 47 | 50 | 0 |
| [49] | NSC | <i>TMS1</i> | 50 | 17 | 34 | 0 | |
| | | <i>DAPK</i> | 50 | 20 | 40 | 0 | |
| | | <i>RASSFS1A</i> | 50 | 17 | 34 | 0 | |
| [60] | NSC | <i>p16</i> | 35 | 12 | 34 | 15 | 0 |
| [46] | NSC | <i>p16</i> | 22 | 3 | 14 | 0 | |
| | | <i>DAKP</i> | 22 | 4 | 18 | 0 | |
| | | <i>GSTP1</i> | 22 | 1 | 5 | 0 | |
| | | <i>MGMT</i> | 22 | 4 | 18 | 0 | |
| [64] | NSC | <i>p16</i> | 105 | 77 | 73 | 0 | |
| Total | | | 467 | 197 | 42 | 65 | 0 |

Abbreviations: NSC, non-small lung cancer; SC, small cell lung cancer; *APC*, adenomatous polyposis coli; *TMS1*, target of methylation induced silencing; *RASSFS1A*, Ras effector homologue; *DAPK*, death-associated protein kinase; *p16*, suppressor gene *p16*; *GSTP1*, glutathione S-transferase P1; *MGMT*, O⁶ methylguanine methyl-transferase.

In five of six studies that included control cases, all controls were negative for alterations in the blood. However, the one study by Khan et al. [39], representing about half of the control cases in this group of cases, revealed microsatellite alterations in the plasma of 42% of their control cases. In their study, however, patients with non-malignant respiratory diseases constituted their group of controls.

There was a relatively good correlation in prevalence for microsatellite alterations in tumour tissue and in blood. About 67% (range 56–83%) of the tumour tissues were positive for at least one microsatellite alteration. Of these cases, 77% (range 58–94%) also displayed identical alterations in the blood [21,35,42,60–62], providing proof that the plasma DNA alterations are tumour-specific in most cases. However, the studies were limited in size, and no significant associations were found between the frequencies of microsatellite alterations in plasma and tumour stage, histology or other clinical factors. As a matter of fact, plasma alterations have been detected at a similar frequency in stage I patients when compared to patients in all stages [35], indicating that DNA alterations are early events in lung cancer.

4.4. Methylated promoter regions in plasma or serum

Methylation-specific PCR techniques have been used to quantify methylation of the promoter region of a number of tumour suppressor genes [46,49,60,63,64]. Summarising the five studies (Table 4), methylation of the examined gene in

blood was detected on average in 42% (range 5–73%) of cases and in none of 65 controls. Assessing the methylation of three genes, Ramirez et al. [49] found that methylation in tumour and serum was closely correlated. Their ratio of gene methylation in plasma versus tumour was 93%. In another study, 88% of the cases with methylated *p16* sequences in tumour samples also demonstrated this epigenetic alteration in the corresponding plasma DNA. And only patients whose tumour cells had hypermethylated *p16* gene exhibited aberrant methylation in their plasma samples. Esteller et al. [46], searched for promoter hypermethylation of the tumour suppressor gene *p16*, the putative metastasis suppressor gene *DAPK*, the detoxification gene *GSTP1*, and the DNA repair gene *MGMT* in 22 non-small cell lung cancer patients. Despite low frequencies of methylation in plasma for each of the genes, using all for as markers they could detect 50% of patients with hypermethylations in plasma.

4.5. Plasma RNA detection

Summarised in Table 5 are data from the two published studies on circulating tumour-related mRNA in lung cancer patients. Control cases were included in both studies. Overall, plasma was positive for tumour-related mRNA in 54% of the cases and 6% of the controls. Using the mRNA markers hnRNP-B1 and Her2/neu, Fleischhacker et al. [27] were able to detect all patients with lung cancer by serum examinations. In contrast, other markers either lacked sensitivity (*MAGE-2*, *PGP*, *TTF-1*) or specificity (*CK19*). Investigating tyrosine mRNA, Ko-

Table 5 Studies evaluating circulating tumour-related mRNA in the blood of lung cancer patients and controls

| References | Tumour type | mRNA markers | No. of lung cancer cases | Serum/plasma positive | | No. of controls | % Positive |
|------------|-------------|--------------|--------------------------|-----------------------|----|-----------------|------------|
| | | | | No. | % | | |
| [54] | NSC | 5T4 | 14 | 6 | 43 | 25 | 12 |
| [27] | NSC/SC | hnRNP-B1 | 18 | 14 | 78 | 12 | 0 |
| | | Her2/neu | 18 | 7 | 39 | 12 | 0 |
| Total | | | 50 | 27 | 54 | 49 | 6 |

Abbreviations: NSC, non-small lung cancer; SC, small cell lung cancer; 5T4, thyrosinase mRNA; hnRNP-B1, hnRNP1 mRNA; Her2/neu; Her2/neu specific mRNA.

preski et al. [54] detected 5T4 in the sera of 43% of lung cancer patients, but also in 12% ($n=3$) of the healthy controls.

5. Future prospects and challenges

According to the available data, it may be possible to detect more than 80% of lung cancer cases using a combination of appropriate DNA and RNA markers, indicating the possibility to develop a simple blood test to help diagnose lung cancer. In this regard, circulating tumour-derived DNA and RNA may possibly be among the most clinically useful molecular markers of cancer during the next decade, due to the possibility to retrieve these markers in a non-invasive fashion.

As has been demonstrated in this review, the analyses of circulating nucleic acids allows the possibility of finding tumour-cell specific alterations circulating in blood at a premalignant phase or an early stage of lung cancer. Among individuals at high risk of lung cancer, Allan et al. [65] found that 33% (13/40) had microsatellite alterations in plasma. Twelve of these (92%) were subsequently diagnosed with lung cancer. Two patients were positive for LOH in plasma samples that pre-dated the cancer diagnosis by several months, indicating that assays of genetic alterations in circulating plasma DNA may be useful in early detection of the disease.

Furthermore, analyses of circulating nucleic acids may in the future be used for selection of treatment and monitoring of treatment effect in NSCLC [66]. In 2003, Clarke et al. [67] reported that epidermal growth factor receptor (EGFR) mRNA in the blood was found elevated in 30% of patients with NSCLC. Considering the EGFR mutation data published recently [68,69], the measurement in blood of activating mutations of the EGFR mRNA may be clinically relevant in the near future with respect to treatment with drugs targeting EGFR.

Most studies have found no correlations between tumour size, stage of disease, tumour site, prognosis, and amount of plasma DNA [43,59,62,70]. However, in a recently published study of 185 NSCLC patients, Gautschi et al. [71] found that plasma DNA concentrations correlated to elevated serum lactate dehydrogenase levels, advanced tumour stage, and poor survival. Anyway, data reviewed above indicate that nucleic acid alterations in plasma or serum are as frequent in early as advanced stage NSCLC.

Still, further work is clearly needed to refine present semiquantitative and quantitative assay techniques, identify additional DNA and RNA tumour markers, and refine present techniques for extracting high-quality nucleic acids from blood. At a technical level, a variety of analytical and preanalytical issues remain to be addressed. Further, standardisation and validation of the analytical methods have to be performed. This will make comparisons between different series more feasible. Regarding the data presented in this review, the frequency of nucleic acid alterations in plasma differs significantly between various studies: Assays for hTERT varied from 25 to 69% positive markers, *K-ras* mutations varied from 0 to 24%, and microsatellite alterations also varied considerably. This disparity may reflect variations in sample source (plasma or serum), collection and preparation of blood specimens, DNA/RNA extraction processes, and analytical methods [71,72]. Thus, validation and standardisation of each of these analytical steps are critical.

Nevertheless, the available data are promising and should instigate large-scale prospective clinical trials in high risk individuals, comparing molecular assays with the best conventional clinical tests. Such clinical trials should be based on the integration of conventional clinical and pathohistological information with molecular approaches. In addition to answering questions regarding molecular aspects of screening, diagnosis, and follow-up, such trials

may also provide new pathophysiological insights into the genesis and progression of this highly lethal disease.

6. Conclusions

There is an apparent need for novel markers and for the development of sensitive and specific non-invasive diagnostic assays for early detection, prognostic evaluation, and surveillance for recurrence. As circulating DNA and RNA are easily accessible in the blood and appear to be relevant surrogate materials for genetic alterations present in the primary tumour, tests for DNA and RNA alterations in plasma may have great potential especially for early detection, diagnostics, and monitoring for relapse during follow-up. These tests need, however to be validated in large prospective clinical lung cancer trials before they are ready for prime time.

Acknowledgement

The financial support from the Norwegian Cancer Society is greatly appreciated.

References

- [1] Wingo PA, Tong T, Bolden S. Cancer statistics. *CA Cancer J Clin* 1995;45:8–30.
- [2] American Cancer Society, ACS. Facts and figures. Atlanta, GA, USA: American Cancer Society; 2002.
- [3] Samet JM, Wiggins CL, Humble CG, Pathak DR. Cigarette smoking and lung cancer in New Mexico. *Am Rev Resp Dis* 1988;137:1110–3.
- [4] Janerich DT, Thompson WD, Varela LR, et al. Lung cancer and exposure to tobacco smoke in the household. *N Engl J Med* 1990;323:632–6.
- [5] Samet JM, Nero Jr AV. Indoor radon and lung cancer. *N Engl J Med* 1989;320:591–4.
- [6] Smart CR. Annual screening using chest X-ray examination for the diagnosis of lung cancer. *Cancer* 1993;72:2295–8.
- [7] Saccomanno G, Archer VE, Auerbach O, et al. Cancer of the lung: the cytology of sputum prior to the development of carcinoma. *Acta Cytol* 1965;9:413–23.
- [8] Lilienfeld KM, Archer PG, Burnett CH, et al. An evaluation of radiographic and cytologic screening for the early detection of lung cancer: a cooperative pilot study of the American Cancer Society and Veterans Administration. *Cancer Res* 1966;26:2083–121.
- [9] Qin D-X, Li L-Y, Sun L-Q, et al. Sputum occult blood screening for lung cancer. *Cancer* 1991;67:1960–3.
- [10] Henschke CI, McCauley DI, Yankelevitz DF, et al. Early lung cancer action project: overall design and findings from baseline screening. *Lancet* 1999;354:99–105.
- [11] Swensen SJ, Jett JR, Hartman TE, et al. Lung cancer screening with CT: Mayo clinic experience. *Radiology* 2003;226:56–61.
- [12] Bach PB, Kelley MJ, Tate RC, McCrory DC. Screening for lung cancer. A review of the current literature. *Chest* 2003;123:2S–82S.
- [13] Garg K, Keith RL, Byers T, et al. Randomized controlled trial with low-dose spiral CT for lung cancer screening: feasibility study and preliminary results. *Radiology* 2002;225:506–10.
- [14] Brambilla C, Fievet F, Jeanmart M, et al. Early detection of lung cancer: role of biomarkers. *Eur Respir J* 2003;39(Suppl.):36–44.
- [15] Chan KCA, Lo YMD. Circulating nucleic acids as a tumor marker. *Histol Histopathol* 2002;17:937–43.
- [16] Leon SA, Shapiro B, Sklaroff DM, Yaros MJ. Free DNA in the serum of cancer patients and the effect of therapy. *Cancer Res* 1977;37:646–50.
- [17] Shapiro B, Chakrabarty M, Cohn EM, Leon SA. Determination of circulating DNA levels in patients with benign or malignant gastrointestinal disease. *Cancer* 1983;51:2116–20.
- [18] Stroun M, Anker P, Maurice P, et al. Neoplastic characteristics of the DNA found in the plasma of cancer patients. *Oncology* 1989;46:318–22.
- [19] Sorenson GD, Pribish DM, Valone FH, et al. Soluble normal and mutated DNA sequences from single-copy genes in human blood. *Cancer Epidemiol Biomarkers Prev* 1994;3:67–71.
- [20] Vasioukhin V, Anker P, Maurice P, et al. Point mutations of the *N-ras* gene in the blood plasma DNA of patients with myelodysplastic syndrome or acute myelogenous leukaemia. *Br J Haematol* 1994;86:774–9.
- [21] Chen XQ, Stroun M, Magnenat JL, et al. Microsatellite alterations in plasma DNA of small cell lung cancer patients. *Nat Med* 1996;2:1033–5.
- [22] Nawroz H, Koch W, Anker P, et al. Microsatellite alterations in serum DNA of head and neck cancer patients. *Nat Med* 1996;2:1035–7.
- [23] Anker P, Mulcahy H, Chen XQ, Stroun M. Detection of circulating tumour DNA in the blood (plasma/serum) of cancer patients. *Cancer Metastasis Rev* 1999;18:65–73.
- [24] Lo YM. Circulating nucleic acids in plasma and serum: an overview. *Ann N Y Acad Sci* 2001;945:1–7.
- [25] Anker P, Mulcahy H, Stroun M. Circulating nucleic acids in plasma and serum as a noninvasive investigation: time for large scale clinical studies? *Int J Cancer* 2003;103:149–52.
- [26] Kopreski MS, Benko FA, Keak LW, Gocke CD. Detection of tumor messenger RNA in the serum of patients with malignant melanoma. *Clin Cancer Res* 1999;5:1961–5.
- [27] Fleischhacker M, Beinert T, Ermitsch M, et al. Detection of amplifiable messenger RNA in the serum of patients with lung cancer. *Ann N Y Acad Sci* 2001;945:179–88.
- [28] Kovalchuk O, Chyczewska E, Niklinska W, Niklinski J, Naumnik W, Chyczewski L. *K-ras* codon 12 mutations detected with enriched PCR method in operable non-small cell lung cancer. *Folia Histochem Cytobiol* 2001;39(Suppl. 2):68–9.
- [29] Sorenson GD. A review of studies on the detection of mutated *K-ras2* sequences as tumor markers in plasma/serum of patients with gastrointestinal cancer. *Ann N Y Acad Sci* 2000;906:13–6.
- [30] Anker P, Lefort F, Vasioukhin V, et al. *K-ras* mutations are found in DNA extracted from the plasma of patients with colorectal cancer. *Gastroenterology* 1997;112:1114–20.
- [31] Kimura T, Holland WS, Kawaguchi T, et al. Mutant DNA in plasma of lung cancer patients: potential for monitoring response to chemotherapy. *Ann N Y Acad Sci* 2004;1022:55–60.
- [32] Mulcahy HE, Lyautey J, Lederrey C, et al. A prospective study of *K-ras* mutations in the plasma of pancreatic cancer patients. *Clin Cancer Res* 1998;4:271–5.

- [33] Zienolddiny S, Ryberg D, Arab MO, Skaug V, Haugen A. Loss of heterozygosity is related to *p53* mutations and smoking in lung cancer. *Br J Cancer* 2001;84:226–31.
- [34] Gonzalez R, Silva JM, Sanchez A, et al. Microsatellite alterations and TP53 mutations in plasma DNA of small-cell lung cancer patients: follow-up study and prognostic significance. *Ann Oncol* 2000;11:1097–104.
- [35] Andriani F, Conte D, Mastrangelo T, et al. Detecting lung cancer in plasma with the use of multiple genetic markers. *Int J Cancer* 2004;108:91–6.
- [36] Silva JM, Gonzalez R, Dominguez G, Garcia JM, Espana P, Bonilla F. TP53 gene mutations in plasma DNA of cancer patients. *Genes Chromosomes Cancer* 1999;24:160–1.
- [37] Bruhn N, Beinert T, Oehm C, et al. Detection of microsatellite alterations in the DNA isolated from tumor cells and from plasma DNA of patients with lung cancer. *Ann N Y Acad Sci* 2000;906:72–82.
- [38] Sanchez-Cespedes M, Monzo M, Rosell R, et al. Detection of chromosome 3p alterations in serum DNA of non-small-cell lung cancer patients. *Ann Oncol* 1998;9:113–6.
- [39] Khan S, Coulson JM, Woll PJ. Genetic abnormalities in plasma DNA of patients with lung cancer and other respiratory diseases. *Int J Cancer* 2004;110:891–5.
- [40] Chen X, Bonnefoi H, Diebold-Berger S, et al. Detecting tumor-related alterations in plasma and serum DNA of patients diagnosed with breast cancer. *Clin Cancer Res* 1999;5:2297–303.
- [41] Nunes DN, Kowalski LP, Simpson AJ. Circulating tumor-derived DNA may permit the early diagnosis of head and neck Squamous cell carcinomas. *Int J Cancer* 2001;92:214–9.
- [42] Sozzi G, Musso K, Ratcliffe C, et al. Detection of microsatellite alterations in plasma DNA of non-small cell lung cancer patients: a prospect of early diagnosis. *Clin Cancer Res* 1999;5:2689–92.
- [43] Sozzi G, Conte D, Mariani L, et al. Analysis of circulating tumor DNA in plasma at diagnosis and during follow-up of lung cancer patients. *Cancer Res* 2001;61:4675–8.
- [44] Bird AP. CpG-rich islands and the function of DNA methylation. *Nature* 1986;321:209–13.
- [45] Tsou JA, Hagen JA, Carpenter CL, Laird-Offringa IA. DNA methylation analysis: a powerful new tool for lung cancer diagnosis. *Oncogene* 2002;21:5450–61.
- [46] Esteller M, Sanchez-Cespedes M, Rosell R, Sidransky D, Baylin SB, Herman JG. Detection of aberrant promoter hypermethylation of tumor suppressor genes in serum DNA from non-small cell lung cancer patients. *Cancer Res* 1999;59:67–70.
- [47] Baylin SB, Herman JG. DNA hypermethylation in tumorigenesis: epigenetics joins genetics. *Trends Genet* 2000;16:168–72.
- [48] Herman JG, Graff JR, Myohanen S, et al. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci* 1996;93:9821–6.
- [49] Ramirez JL, Sarries C, de Castro PL, et al. Methylation patterns and *K-ras* mutations in tumor and paired serum of resected non-small cell lung cancer patients. *Cancer Lett* 2003;193:207–16.
- [50] Penta JS, Johnson FM, Wachsman JT, Copeland WC. Mitochondrial DNA in human malignancy. *Mutat Res* 2001;488:119–33.
- [51] Jeronimo C, Nomoto S, Caballero OL, et al. Mitochondrial mutations in early stage prostate cancer and bodily fluids. *Oncogene* 2001;20:5195–8.
- [52] Reddi KK, Holland JF. Elevated serum ribonuclease in patients with pancreatic cancer. *Proc Natl Acad Sci* 1976;1:2308–10.
- [53] Lo KW, Lo YM, Leung SF, et al. Analysis of cell-free Epstein–Barr virus associated with RNA in the plasma of patients with nasopharyngeal carcinoma. *Clin Chem* 1999;45:1292–4.
- [54] Kopreski MS, Benko FA, Gocke CD. Circulating RNA as a tumor marker: detection of 5T4 mRNA in breast and lung cancer patient serum. *Ann N Y Acad Sci* 2001;945:12–8.
- [55] Maebo A. Plasma DNA level as a tumor marker in primary lung cancer. *Nihon Kyobu Shikkan Gakkai Zasshi* 1990;28:1085–91.
- [56] Gormally E, Hainaut P, Caboux E, et al. Amount of DNA in plasma and cancer risk: a prospective study. *Int J Cancer* 2004;111:746–9.
- [57] Fournie GJ, Courtin JP, Laval F, et al. Plasma DNA as a marker of cancerous cell death: investigations in patients suffering from lung cancer and in nude mice bearing human tumours. *Cancer Lett* 1995;91:221–7.
- [58] Sozzi G, Conte D, Leon M, et al. Quantification of free circulating DNA as a diagnostic marker in lung cancer. *J Clin Oncol* 2003;21:3902–8.
- [59] Chang HW, Lee SM, Goodman SN, et al. Assessments of plasma DNA levels, allelic imbalance, and CA125 as diagnostic tests for cancer. *J Natl Cancer Inst* 2002;94:1697–703.
- [60] Bearzatto A, Conte D, Frattini M, et al. *p16*(INK4A) hypermethylation detected by fluorescent methylation-specific PCR in plasma from non-small cell lung cancer. *Clin Cancer Res* 2002;8:3782–7.
- [61] Cuda G, Gallelli A, Nistico A, et al. Detection of microsatellite instability and loss of heterozygosity in serum DNA of small and non-small cell lung cancer patients: a tool for early diagnosis? *Lung Cancer* 2000;30:211–4.
- [62] Beau-Faller M, Gaub MP, Schneider A, et al. Plasma DNA microsatellite panel as sensitive and tumor-specific marker in lung cancer patients. *Int J Cancer* 2003;105:361–70.
- [63] Usadel H, Brabender J, Danenberg KD, et al. Quantitative adenomatous polyposis coli promoter methylation analysis in tumor tissue, serum, and plasma DNA of patients with lung cancer. *Cancer Res* 2002;62:371–5.
- [64] An Q, Liu Y, Gao Y, et al. Detection of *p16* hypermethylation in circulating plasma DNA of non-small cell lung cancer patients. *Cancer Lett* 2002;188:109–14.
- [65] Allan JM, Hardie LJ, Briggs J, et al. Genetic alterations in bronchial mucosa and plasma DNA from individuals at high risk for lung cancer. *Int J Cancer* 2001;91:359–65.
- [66] Rosell R, Fosella F, Milas Luka. Molecular markers and targeted therapy with novel agents: prospects in the treatment of non-small cell lung cancer. *Lung Cancer* 2002;38:543–9.
- [67] Clarke LE, Leitzel K, Smith J, Ali SM, Lipton A. Epidermal growth factor receptor mRNA in peripheral blood of patients with pancreatic, lung, and colon carcinomas detected by RT-PCR. *Int J Oncol* 2003;22:425–30.
- [68] Lynch TJ, Bell DW, Sordilla R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small cell lung cancer to gefitinib. *N Engl J Med* 2004;350:2129–39.
- [69] Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004;304:1497–500.
- [70] Holdenrieder S, Stieber P, Forg T, et al. Apoptosis in serum of patients with solid tumours. *Anticancer Res* 1999;19:2721–4.

- [71] Gautschi O, Bigosch C, Huegli, et al. Circulating deoxyribonucleic acid as prognostic marker in non-small cell lung cancer patients undergoing chemotherapy. *J Clin Oncol* 2004;22:4157–64.
- [72] Gal S, Fidler C, Lo YMD, et al. Quantitation of circulating DNA in the serum of breast cancer patients by real-time PCR. *Br J Cancer* 2004;90:1211–5.

Available online at www.sciencedirect.com

