Can quantifying free-circulating DNA in plasma be used to identify subjects with high-grade pre-invasive endobronchial lesions?

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ABSTRACT

Introduction
Increased concentrations of free circulating plasma DNA (cpDNA) are observed in patients with invasive cancer, including lung cancer. It is currently not known whether cpDNA levels are elevated in subjects with high-grade pre-invasive lesions of squamous cell lung carcinoma (lung SqCC), and whether its detection could potentially be of value for identifying subjects at highest risk of lung SqCC.

Materials and Methods
This study assessed cpDNA levels in subjects with high-grade and low-grade pre-invasive squamous endobronchial lesions relative to both patients with clinically overt lung SqCC and healthy controls using real-time quantitative PCR methodology.

Results
Median cpDNA levels of patients with invasive lung SqCC (n=16) were significantly higher compared with those of healthy controls (n=16) (p<0.01), whereas cpDNA levels in subjects with pre-invasive lesions (n=20) did not differ from those of controls (p=0.29). The cpDNA levels in subjects with high-grade pre-invasive lesions were highly comparable to those diagnosed with low-grade pre-invasive lesions (p=0.85).

Conclusions
Our data suggest that cpDNA levels are not increased during pre-invasive stages of squamous cell lung carcinogenesis.

INTRODUCTION

Early lung cancer detection represents an important approach for reducing disease mortality rates. Low-dose spiral computed tomography (CT) scanning has demonstrated effectiveness in detecting early-stage peripheral lung cancers. However, CT yield for early-stage central airway cancers is relatively poor and its costs seem to limit implementation at large. Autofluorescence bronchoscopy (AFB) has shown to be highly sensitive for the detection of early-stage (micro-) invasive carcinoma and pre-invasive lesions in the central airways. Unfortunately, its high false-positive rate and its rather invasive approach hamper a wider use as a cost-effective screening tool. The detection of molecular biomarkers that are representative of disease state in low- or non-invasively collected biological fluids, such as blood or sputum, might represent an alternative or addition to this imaging technique and might improve cost-effectiveness of screening.

Analysis of free circulating plasma DNA (cpDNA) levels is a non-invasive tool that has shown potential to detect patients with lung cancer. Significantly higher cpDNA levels have been demonstrated in patients with invasive lung cancer, independently of tumor stage, as compared to controls. However, it is currently not known whether cpDNA levels are increased in subjects with high-grade pre-invasive squamous endobronchial lesions or if cpDNA level quantification may be of value as a marker of invasive growth in these subjects. This information may contribute to more knowledge about cpDNA quantification as a potential non-invasive screening tool for identifying individuals at the highest risk of developing lung SqCC. Here, we evaluated cpDNA levels in a cohort of subjects with AFB-visualized pre-invasive endobronchial lesions comprising the full spectrum of premalignant squamous disease, relative to both patients with clinically overt lung SqCC and cancer-free, healthy controls. The current study was set out to assess whether cpDNA quantification is able to discriminate among these subgroups.

MATERIALS AND METHODS

Study subjects
Peripheral blood was collected with informed consent from (i) subjects with pre-invasive squamous endobronchial lesions, (ii) patients diagnosed with invasive lung SqCC (Stage I-IV), and (iii) cancer-free, healthy individuals. Characteristics of the study population are shown in Table 1. Pre-invasive endobronchial lesions were visualized by autofluorescence bronchoscopy (AFB) in subjects at risk of lung cancer on the basis of smoking habits (i.e., more than 20 pack years), chronic obstructive pulmonary disease (COPD), signs/symptoms, and/or history of lung or head and neck cancer, but without clinically overt lung cancer. For this study, subjects were selected in such a way that AFB-visualized lesions included the full spectrum of pre-invasive squamous endobronchial disease and that variables that could potentially confound the analysis such as gender, age, smoking status, number of pack years, COPD-
status (p=0.56) and storage time of plasma specimens (p=0.19), were kept similar to cancer cases. Staging of lung cancers was performed according to the 6th edition of the TNM classification system of malignant tumours\(^1\), and histology according to the 2004 IASLC/WHO histological classification system of pre-invasive and invasive squamous lesions of the bronchus\(^1\). This study followed the ethical guidelines of the Institutional Review Board.

### TABLE 1 Study population characteristics

<table>
<thead>
<tr>
<th>STUDY GROUPINGS</th>
<th>(i)</th>
<th>(ii)</th>
<th>(iii)</th>
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<tbody>
<tr>
<td><strong>subjects with pre-invasive squamous endobronchial lesions</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Gender (male : female)</td>
<td>14 : 6</td>
<td>9 : 7</td>
<td>11 : 5</td>
</tr>
<tr>
<td>Age (in years, median (range))</td>
<td>62 (46-71)</td>
<td>63 (50-81)</td>
<td>56 (28-87)</td>
</tr>
<tr>
<td>Smoking status (C : F : N)</td>
<td>8 : 12 : 0</td>
<td>7 : 8 : 0*</td>
<td>2 : 4 : 9*</td>
</tr>
<tr>
<td>No. of pack years (median (range))</td>
<td>40 (22-120)</td>
<td>40 (15-60)*</td>
<td>39 (20-46)*</td>
</tr>
</tbody>
</table>

C, current smoker; F, former smoker; N, never smoker
* Data were not available for 1 SqCC patient and 1 healthy control subject
* Never-smokers were not taken into account

**Sample handling and DNA isolation**

Peripheral blood was processed within 1 hour of collection. The plasma component was carefully separated by two centrifugation rounds at 3000 rpm for 10 min at room temperature. The plasma specimens were stored in 1 ml aliquots at –80 °C until use. DNA isolation was performed as essentially described before\(^11\). In short, DNA was isolated from a plasma aliquot using the Qiagen QIAamp DNA mini kit (Westburg, Leusden, The Netherlands) according to manufacturer’s instructions for blood and body fluids. Specimens were spiked with plasmid DNA (pHPV16) prior to DNA isolation to control for DNA extraction efficiency.

**Plasma DNA quantification**

Circulating plasma DNA levels were determined in quadruplicate by means of quantitative real-time PCR amplification of the human Fra-1 gene on the ABI/Prism 7300 Real-Time PCR System (Applied BioSystems, Nieuwerkerk a/d IJssel, The Netherlands) and further quantified using a standard calibrator curve of human placental DNA\(^19\). HPV16-specific quantitative real-time PCR to specifically quantify the spiked plasmid DNA was also performed on the DNA isolates to assess DNA extraction efficiency\(^20\).

**Statistical analysis**

cpDNA quantification assays were performed in a blinded fashion, and afterwards the results were correlated to disease category. For analysis, pre-invasive lesions graded as hyperplasia, squamous metaplasia, mild dysplasia, and moderate dysplasia were categorized into low-grade pre-invasive disease (LGD), and severe dysplasia and carcinoma in situ (CIS) into high-grade pre-invasive disease (HGD). Statistical analyses were performed using SPSS Statistics v17.0 software package (Chicago, Illinois, USA). Differences between groups as for frequencies of patient characteristics were examined using Fisher’s exact and Mann-Whitney U testing. Median plasma DNA levels between groups were compared by means of non-parametrical Kruskal-Wallis testing.

**RESULTS**

A total of 52 plasma samples were compared for cpDNA levels comprising (i) 20 subjects with AFB-visualized pre-invasive endobronchial lesions (i.e., LGD, n=10; HGD, n=10), (ii) 16 patients with clinically overt, invasive lung SqCC (i.e., stage I, n=7; stage III, n=6; stage IV, n=3) and (iii) 16 cancer-free, healthy individuals (further referred to as controls). Patients with clinically overt lung cancers demonstrated significantly higher levels of cpDNA (median 6.2 ng/ml, range 0.8 – 77.6 ng/ml) as compared to controls (p<0.01) (Figure 1). In contrast, cpDNA levels could not discriminate at risk subjects with pre-invasive lesions (median 4.9 ng/ml, range 1.6 – 10.2 ng/ml) from controls (median 2.8 ng/ml, range 1.0 – 10.1 ng/ml), neither in case of LGD (p=0.10) nor HGD (p=0.29). Moreover, cpDNA levels in subjects that were diagnosed with HGD (median 4.9 ng/ml, range 1.6 – 10.2 ng/ml) were highly comparable to those diagnosed with LGD (4.8 ng/ml, range 1.7 – 9.0 ng/ml) (p=0.85). Of note, 3 of the ten individuals presenting with HGD at time of peripheral blood sampling were diagnosed with invasive lung cancer within a follow-up period of 6 months, and none of them had elevated cpDNA levels at pre-invasive stage, i.e., their cpDNA levels were 1.6 ng/ml, 2.2 ng/ml and 4.8 ng/ml, respectively.

**DISCUSSION**

The results of our study suggest that cpDNA levels are not increased during pre-invasive stages of squamous cell lung carcinogenesis. Whereas cpDNA levels were significantly higher in clinically overt lung cancer, a figure consistent with previous data\(^12\)\(^\text{13}\), none of the subjects with pre-
invasive lesions, neither LGD nor HGD, could be discriminated from controls on the basis of their cpDNA level. Our findings suggest that quantification of free-circulating DNA in plasma may not be a useful parameter for identifying subjects with high-grade pre-invasive lesions of lung SqCC, nor for prognostication in potentially malignant conditions.

Sozzi and co-workers\textsuperscript{21;22} suggested that the release of DNA in plasma is related to the establishment of a relatively advanced grade of interaction between the tumor and the microenvironment. The authors demonstrated that plasma DNA levels in patients with CT-detected lung cancer, which particularly consist of small, early-stage adenocarcinomas, were still comparable with those of disease-free subjects, and that only patients with clinically overt lung cancers were observed to have markedly higher levels of cpDNA. Studies on the quantification of cpDNA in subjects with precancerous lesions are scarce. Shukla and co-workers\textsuperscript{23} evaluated cpDNA levels in subjects with mucosal precancerous lesions (i.e., epithelial dysplasia) of oral squamous cell carcinoma. The authors concluded that levels of cpDNA in subjects with oral epithelial dysplasia were not higher compared with healthy controls, which is consistent with our data of subjects with pre-invasive lesions of lung SqCC. In the study by Shukla et al.\textsuperscript{23}, however, cpDNA levels were also not elevated in patients with oral SqCC, an observation that does not agree with our data and those of previous studies\textsuperscript{12-15}. The authors suggested this to be a property inherent to the type of neoplasm and its dissemination characteristics, which is different for lung cancer and oral cancer. The possibility that cpDNA quantification could provide a fingerprint of different aggressive behavior of tumors will require further testing. This will also be of interest within screening trials in the effort to improve the clinical management of CT-detected lung cancer. Furthermore, in patients with overt cancer, the abundance of cpDNA that probably originates from the cancerous cells offers the possibility to use this source material as a non-invasive monitoring system for applying companion diagnostics to determine an appropriate lung cancer treatment, as was recently shown for the detection of epidermal growth factor receptor (EGFR) mutations for EGFR-tyrosine kinase inhibitors\textsuperscript{24}.

The strength of the current study lies in the confirmatory results on cpDNA levels in patients with squamous (pre)cancerous lesions that well complement previous results from studies mainly including the adenocarcinoma histotype\textsuperscript{21;22}. A limitation of this study may be the number of pre-invasive lesions included, particularly in light of the fact that only a small fraction will progress to lung SqCC\textsuperscript{25}. However, it should be noted that the analyzed series of subjects with pre-invasive squamous endobronchial lesions form a unique study population that had only become available by close AFB surveillance of large numbers of individuals at risk of lung cancer during the past years. This reflects the important difficulties faced in studying pre-invasive endobronchial disease given, on one hand, the low prevalence of high-grade lesions in asymptomatic individuals within an at risk population, and secondly, the low progression rate to lung SqCC\textsuperscript{25}. Collecting sufficient biomaterials representing the full spectrum of pre-invasive squamous endobronchial disease to investigate the study concept requires an extensive period of close surveillance. This underscores the uniqueness of the series examined herein.

In conclusion, although median cpDNA levels of patients with invasive lung SqCC were significantly higher as compared to healthy controls, no elevated levels of cpDNA were observed in subjects with either LGD or HGD. cpDNA quantification does not appear to be a prognostic marker in potentially malignant conditions and is unlikely to be of value as an alternative or addition to the diagnostic algorithm of AFB for identifying patients at highest risk of developing lung cancer. Our study highlights the need to continue research efforts aiming to identify biomarkers that can be applied to non- or less invasively collected biomaterials for the early prediction of invasive cancer in the respiratory airways to improve screening and/or diagnostic algorithms.
REFERENCES


