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Cancer Genetics and Cytogenetics 184 (2008) 31–37

Cancer Genetics and Cytogenetics

Gain of the EGFR gene located on 7p12 is a frequent and early event in squamous cell carcinoma of the lung

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Received 21 December 2007; received in revised form 27 February 2008; accepted 5 March 2008

Abstract Identification of molecular alterations in biological fluids has been proposed as a powerful tool for cancer diagnosis. The purpose of this study was to identify cells that carry chromosomal alterations indicative of malignancy—specifically, gains in the loci 5p15.2 (D5S23, D5S721), 6p11~q11, 7p12 (*EGFR*), and 8q24.12~q24.13 (*MYC*)—for the detection of lung cancer using induced sputum. The overall sensitivity of the multicolor fluorescence in situ hybridization (FISH) assay from 52 lung cancer patients was 71% and the specificity was 100% (15 of 15). The most frequently detected gains were at 7p12 (*EGFR*) in 17 of 24 completely resectable early-stage (II+IIIA) non-small cell lung cancers (NSCLC) (71%). There was a statistically significant increase in the proportion of cases with gains of *EGFR* in squamous cell carcinomas (SCC), compared with adenocarcinomas (AC) (82 vs. 43%, respectively; $P = 0.017$), and a higher average *EGFR* gene copy number in the SCCs than in the ACs (5.04 vs. 3.78, respectively; $P = 0.013$) in 41 NSCLCs. Conversely, a gain at the 6p11~q11 and 8q24.12~q24.13 (*MYC*) regions appears to have a higher frequency of gain in the ACs (71 and 86%, respectively) than in the SCCs (48 and 56%, respectively). The results of this study showed the potential utility of the LAVysion FISH assay for the detection of lung cancer by a noninvasive technique based on the analysis of genetic alterations of induced sputum. Defining abnormalities in sputum specimens as FISH aneusomy may be a possible diagnostic method for the early detection of lung cancer in screening of high-risk populations and monitoring for recurrence.

1. Introduction

Lung cancer is the main cause of cancer mortality in the world and has one of the lowest survival rates of any solid tumor. The mortality rate of lung carcinoma is almost 90%, and the 5-year survival rate in patients with advanced-stage lung carcinoma is 2.5% [1]. The high mortality rate for lung cancer probably results, at least in part, from the absence of standard clinical procedures for the diagnosis of this disease (unlike breast, prostate, and colon cancers) at early and more treatable stages. Although chest X-rays and sputum cytology have long been used in screening, bronchoscopy is the most commonly used diagnostic tool for obtaining a definite histological or cytological lung cancer diagnosis [2]. These tests, however, have not been sufficiently robust to be generally adopted for lung cancer screening at a stage early enough to improve survival. There is an urgent need to develop reliable early diagnostic biomarkers for lung cancer, and its precursors, that can be evaluated by noninvasive methods in patients at high risk [3].

Fluorescence in situ hybridization (FISH) has been considered one of the most promising diagnostic technologies because it can be directly applied to clinical samples and so does not require microdissection, chromosomal, DNA, or RNA preparation [4–8]. In addition, compared with other techniques, FISH is more quantitative because it detects genetic abnormalities by counting signal numbers, which directly represent changes in the gene copy number. Despite the high frequency of aneusomy in lung cancer, FISH has not yet been fully exploited for early detection and monitoring of this tumor type, in part because of the lack of validated probes with specific application to lung cancer.

In the present study, we used a four-probe multicolor FISH panel containing probes to the pericentromeric region of chromosome 6 (6p11~q11) and to the 5p15.2 (D5S23, D5S721), 7p12 (site of the *EGFR* gene), and 8q24.12~q24.13 (site of the *MYC* gene) loci, to assess cells

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0165-4608/08/$ – see front matter
doi:10.1016/j.cancergencyto.2008.03.002

on smears prepared from induced sputum specimens for chromosomal abnormalities indicative of lung carcinoma.

The effectiveness of cytogenetic methods as complementary diagnostic and prognostic tools for the evaluation of lung carcinomas has been difficult to demonstrate, mainly due to the difficulty in obtaining fresh tissue, the low proliferative rate of these lesions in vitro, and the lack of specificity of the multiple changes typically detected in the cancers. Sputum is an accessible and noninvasive source of biological material that could be useful for the early detection of lung cancer and monitoring of treatment [6]. Sputum usually contains exfoliated cells from the oropharynx and the larger respiratory passages; in patients with lung carcinomas, the sputum also may contain tumor cells. Sputum has therefore long been considered a potential source of material that could be used for screening information among lung cancer patients, but few studies have reported its application for these purposes [9,10].

This study was designed to investigate the correlation between chromosomal alterations and clinicopathological variables and to clarify the potential utility of the multitarget FISH assay in sputum specimens for the early detection and surveillance of lung carcinoma.

2. Materials and methods

2.1. Patients and sample treatment

Sputum specimens were obtained from 52 lung cancer patients who did not undergo surgical resection of their primary tumor. The majority of histologically confirmed cancer-positive specimens were non-small cell lung cancer (NSCLC; 41 of 52); of these 41 specimens, 27 were squamous cell carcinoma (SCC) and 14 were adenocarcinoma (AC). The remaining 11 specimens were from patients with SCLC. Sputum specimens from 15 exsmokers without lung cancer were used as a control.

Sputum was collected in the early morning for 3 days; spontaneous cough sputum specimens were collected in jars containing Saccomanno's fixative solution and stored at room temperature until analysis. Papanicolaou staining was performed on several sputum smears for confirmation of abnormal cells. Approximately 1 mL of sputum suspension was diluted with 5 mL of 1× phosphate-buffered saline and centrifuged; the cell pellet was resuspended at room temperature in 2.5 mL of Hanks' buffered saline solution containing 10 mmol/L ethylenediamine tetraacetic acid and 50 μL of dithiothreitol for 15 minutes. After another centrifugation, the cell pellet was fixed in Carnoy's fixative and the suspension was dropped onto glass slides with adjustments for proper cellularity when necessary.

2.2. FISH

A LAVysion kit (Abbott Molecular–Vysis, Des Plaines, IL) was used for the FISH. The probe set included one pericentromeric sequence (6p11~q11) labeled with SpectrumAqua and three single-copy DNA sequences that recognize 450-kb sequences mapped at 5p15.2 (D5S23, D5S721; SpectrumGreen), 7p12 (including the *EGFR* gene; SpectrumRed), and 8q24.12~q24.13 (including the *MYC* gene; SpectrumGold).

The laboratory process was performed according to the recommendations of the manufacturer with minor modifications. In brief, the slides were denatured by incubation with formamide (70% in 2× saline sodium citrate [SSC]) at 75°C for 7 minutes in a water bath. The slides were then dehydrated through a graded ethanol system (70% for 1 minute, 85% for 1 minute, and 100% for 1 minute). Hybridization solution (10 μL) was applied to each slide, which was then coverslipped and sealed with rubber cement. After hybridization for at least 20 hours at 37°C in a humidified chamber, slides were washed with 0.4× SSC–0.3% NP-40 for 2 minutes at 73°C. The slides were placed in 2× SSC–0.1% NP-40 detergent for 1 minute at room temperature. Then, 10 μL 4′,6-diamidino-2-phenylindole (DAPI) antifade solution was applied to each spot and the slides were coverslipped.

The slides were observed under a fluorescence microscope connected to a cooled charge-coupled device camera and an image analyzer system (CytoVision; Applied Imaging, Newcastle, UK).

The signals were scored on a cell-by-cell basis after hybridization and washing. The slides were assessed by scanning for morphologically abnormal cells with nuclear enlargement, irregular nuclear contour, patchy or lighter nuclear DAPI staining, and cell clusters, all of which were indicative of malignancy. A minimum of 25 morphologically abnormal cells were assessed. Slides that contained <6 cells were considered inadequate for evaluation. Overlapping cells or cells with blurred signals were not assessed. Signals that were located very close to each other were interpreted as split signals and were counted as one signal. Slides showing suspicious signals were re-examined by another technician to verify the results. A test result was defined based on previous studies [10,11]. The identification of 6 or more cells with polysomy, defined as gains of 2 or more chromosomes in a cell, was considered a cancer-positive result.

2.3. Statistical analysis

A range of descriptive and comparative statistical analyses was made using SPSS version 13.0 statistical software (SPSS, Chicago, IL). The χ^2-test was used to compare the positive FISH rates in each chromosome, and for the *EGFR* gene, by histological types (SCC vs. AC), and TNM tumor stages (II+IIIA vs. IIIB+IV) [12–17] in the 41 NSCLC patients. Possible correlations between the histological types (SCC and AC) and the mean chromosome copy number in each chromosome were evaluated via an