Molecular Typing of Lung Adenocarcinoma on Cytological Samples Using a Multigene Next Generation Sequencing Panel

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Abstract

Identification of driver mutations in lung adenocarcinoma has led to development of targeted agents that are already approved for clinical use or are in clinical trials. Therefore, the number of biomarkers that will be needed to assess is expected to rapidly increase. This calls for the implementation of methods probing the mutational status of multiple genes for inoperable cases, for which limited cytological or biopptic material is available. Cytology specimens from 38 lung adenocarcinomas were subjected to the simultaneous assessment of 504 mutational hotspots of 22 lung cancer-associated genes using 10 nanograms of DNA and Ion Torrent PGM next-generation sequencing. Thirty-six cases were successfully sequenced (95%). In 24/36 cases (67%) at least one mutated gene was observed, including EGFR, KRAS, PIK3CA, BRAF, TP53, PTEN, MET, SMAD4, FGFR3, STK11, MAP2K1. EGFR and KRAS mutations, respectively found in 6/36 (16%) and 10/36 (28%) cases, were mutually exclusive. Nine samples (25%) showed concurrent alterations in different genes. The next-generation sequencing test used is superior to current standard methodologies, as it interrogates multiple genes and requires limited amounts of DNA. Its applicability to routine cytology samples might allow a significant increase in the fraction of lung cancer patients eligible for personalized therapy.

Introduction

Lung cancer is the leading cause of cancer-related death worldwide [1,2,3]. It is classified as small cell or non-small cell lung cancer (NSCLC), the latter comprising three of the most common subtypes: adenocarcinoma, squamous cell carcinoma, and neuroendocrine tumors [4].

The majority of NSCLC are diagnosed at an advanced stage with inoperable disease [5]. Therefore, in more than 85% NSCLC minimally invasive procedures must be employed to obtain diagnostic material, which is consequently represented by either small biopsies or cytology samples [5,6]. This significantly affects the morphological and molecular characterization required for targeted therapies, whose efficacy is limited to patients with specific genetic alterations [5].

For lung adenocarcinomas, epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors have been approved for treatment of tumors carrying EGFR gene mutations, and crizotinib for tumors with anaplastic lymphoma kinase (ALK) gene rearrangements [7,8,9,10]. Clinical trials are ongoing in subgroups of patients harboring specific molecular alterations such as BRAF, PIK3CA or KRAS activating mutations [11,12,13]. Therefore, the number of predictive biomarkers to be assessed for novel targeted drugs entering into clinical practice is expected to rapidly increase [2,10,14].

Sanger sequencing is currently the most widely applied technique in the characterization of EGFR gene status in clinical practice [15]. Real-time PCR-based methods have been shown to efficiently detect EGFR mutations in samples containing 1% mutated cancer cells [16]. However, there is no sufficient information on the predictive ability of these techniques, since no clear correlation has been established up to now between the quantity of mutant alleles in the cancer and the extent and duration of response to therapy [16,17]. More importantly, most methods have been developed and validated to assess single gene alterations. Massive parallel sequencing, also known as next generation sequencing (NGS) or deep sequencing, has been
recently introduced and is the most sensitive approach to index multiple genes starting from a limited amount of DNA [18].

The Ion AmpliSeq Colon and Lung Cancer Panel (Life-technologies, Carlsbad, CA, USA) multi-gene next generation sequencing (NGS) allows assessment in a single analysis of hotspot mutations in 22 genes related to lung and colon tumorigenesis. The panel has been validated through a collaborative effort of 8 European institutions (http://tools.invitrogen.com/content/sfs/brochures/AmplicSeq-Colon-Lung-Cancer-Panel-Flyer.pdf).

With the present study, the performance of the Ion AmpliSeq Colon and Lung Cancer Panel was investigated in a series of lung adenocarcinoma cytological samples to define its diagnostic relevance.

**Materials and Methods**

**Ethic statement**

Written informed consent was obtained from all patients involved in the study, which was approved in the final form by the Ethics Committee of the Azienda Ospedaliera e Università degli Studi di Padova (N. 0002537 in January 16th, 2013). All the samples were received anonymously and processed at the Molecular Pathology Unit of the Department of Pathology and Diagnostics at the University of Verona.

**Samples**

A series of 38 lung adenocarcinoma trans-thoracic fine needle aspiration (FNA) cytology specimens consecutively collected in 2012 at the Surgical Pathology and Cytopathology Unit of Padua University and the Pharmacogenomic Laboratory of the INT-Fondazione Pascale in Napoli, were studied (Table 1). In two cases a matched tumor biopsy was also available. The original routine slides were re-assessed by three pathologists (AS, MF and AF) according to current WHO criteria [4].

The series included 21 cytological smears and 17 fine needle aspirate (FNA) washings:

i) Routine smear cytological slides fixed with Cytofix (H, Bio-Fix 05-x200, Bio-Optica, Milano, Italy) and stained with Papanicolaou or Diff-Quick. Tumor cells were scraped from the original smear, by manually microdissection at the microscope in order to obtain at least 100 tumor cells. A mean number of 1,050±1,480 tumor cells per slide were retrieved (range 100–5,000).

ii) Cells obtained by needle washing of FNA fixed in FineFix (Milestone Medical Technologies Inc; Kalamazoo, MI). Half sample was processed for cell-block preparation for routine diagnosis [19], the other half was stored at −80°C and used for the analysis. The quantity of cancer cells present in each needle-washing sample was at least 1,000, as inferred from the histological analysis of the corresponding cell-block.

**DNA extraction**

i) Cells scraped from the original cytology slides: coverslips were removed by immersion in xylene for 72 hours and the slides were rinsed in 95% ethanol three times. Cells on the slides were scraped in 1.5 ml tubes by using sterile razors. DNA was isolated using the QIAamp DNA Mini kit (Qiagen, Milano, Italy).

ii) Cells recovered from washing of fine-needles: samples were centrifuged at 12,000 g for 10 min to discard FineFix and washed in PBS. DNA was isolated from the cell pellets using the QiAmp DNA Mini kit (Qiagen).

iii) Formalin-fixed paraffin-embedded tumor biopsies: four 10 µm paraffin sections were manually microdissected to ensure that each tumor sample contained at least 70% neoplastic cells. DNA was isolated using the QiAmp DNA FFPE tissue kit (Qiagen).

DNA was quantified and its quality assessed using NanoDrop® (Invitrogen Life Technologies; Milan, Italy) and Qubit® (Invitrogen Life Technologies) platforms according to the manufacturer’s instructions.

**Deep Sequencing of Multiplex PCR Amplicons**

Deep sequencing were performed using the Ion Torrent platform (Life Technologies), according to the manufacturer’s specifications. Briefly, 10 ng of purified genomic DNA were used for library construction with the Ion AmpliSeq Colon and Lung Cancer Panel v1 [Life Technologies] that targets 504 mutational hotspot regions of the following 22 cancer-associated genes, in alphabetical order: AKT1, ALK, BRAF, CTNNB1, DDR2, EGFR, ERBB2, ERBB4, FBXW7, FGFR1, FGFR2, FGFR3, KRAS, MAP2K1, MET, NOTCH1, NRAS, PIK3CA, PTEN, SMAD4, STK11, TP53.

Emulsion PCR was performed either manually or with the OneTouch DL system (Life Technologies). The quality of the obtained library was evaluated by the Agilent® 2100 Bioanalyzer on-chip electrophoresis (Agilent Technologies; Santa Clara, CA). Sequencing was run on the Ion Torrent Personal Genome Machine™ (PGM, Life Technologies) loaded with a 316 chip as per manufacturer’s protocol. Data analysis, including alignment to the hg19 human reference genome and variant calling, was done using the Torrent Suite Software v.3.2 (Life Technologies). Filtered variants were annotated using both the Ion Reporter software v1.2 (Life Technologies) and the SnpEff software v.3.0 [20] (alignments visually verified with the Integrative Genomics Viewer; IGV v.2.1, Broad Institute [21]).

DNA from normal human lymphocytes and from the carcinoma cell line AVG1 [22] were retrieved from the ARC-NET biobank at Verona University and respectively used as negative and positive control for assessment of sensitivity.

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**Table 1.** Clinico-pathological features of the considered series.

| Characteristic | # |
|---------------|---|
| Gender        |   |
| Male          | 24 (63.2%) |
| Female        | 14 (36.8%) |
| Age           |   |
| -             | 69±9 (median 68; range 48–85) |
| G1            | 7 (18.4%) |
| Grading       |   |
| G2            | 27 (71.1%) |
| G3            | 4 (10.5%) |
| Stage IIIA    | 7 (18.4%) |
| Staging       |   |
| Stage IIB     | 4 (10.5%) |
| Stage IV      | 11 (28.9%) |
| missing       | 16 (42.1%) |
| Sources       |   |
| Cytological smears | 21 (55.3%) |
| FNA washings  | 17 (44.7%) |

[Link to data citation]
DNA Sanger Sequencing

To validate the mutations detected by deep sequencing, **EGFR** (exons 18, 19, 20 and 21) and **KRAS** (exon 2) specific PCR fragments were analyzed by conventional Sanger sequencing [23]. PCR products were purified using Agencourt AMPure XP magnetic beads (Beckman Coulter) and labelled with Big Dye Terminator v3.1 (Applied Biosystems, Monza, Italy). Agencourt CleanSEQ magnetic beads (Beckman Coulter) were used for post-labeling DNA fragment purification, and sequence analysis was performed on an Applied Biosystems 3130xl Genetic Analyser.

High resolution melting analysis

DNA was amplified for human **EGFR** (exons 19 and 21) and **KRAS** (exon 2) genes via real-time PCR, as previously described [24], in the presence of a proprietary saturating DNA dye contained in the LightCycler 480 High Resolution Melting Master (Roche Diagnostics, Milano, Italy) on the LightCycler 480 platform. A melting curve was produced using high data acquisition rates, and data were analyzed with the LightCycler 480 Gene Scanning Software Module for deletion and mutation identification.

Results

Patient Characteristics

The male/female ratio was 24/14 and mean age was 69±9 years (median = 68; range = 48–85). Tumor grading ranged from well (n = 7) to moderately (n = 27) or poorly differentiated (n = 4). In 22 cases a clinical TNM was available, 7 were Stage IIIA, 4 Stage IIIB and 11 Stage IV.

Deep sequencing of multiplex PCR products is sensitive in mutation assessment

The sensitivity of our experimental setup was tested by progressively diluting DNA from AVC1 cancer cells with DNA from normal human lymphocytes, to obtain samples with decreasing relative tumor DNA content: 50%, 25%, 20%, 15%, 10%, 7.5%, 5%, 2.5%, 1%, and 0%. Ten ng of each dilution point were subjected to Ion AmpliSeq Colon and Lung Cancer Panel analysis v1 (Life Technologies).

A known **KRAS** mutation of the AVC1 cell line [22] was used to assess the assay sensitivity; a novel **CTNNB1** S45F mutation was also found and served to further confirm the assay sensitivity at a second genomic location. The two mutations were identified in all samples containing tumor DNA, and were absent in the sample containing only non-tumor DNA from lymphocytes (data not shown).

Prevalence of driver genes mutations in lung adenocarcinoma cytology specimens

Ten nanograms of DNA were processed according to the manufacturers’ protocol. In 36/38 (95%) samples, an adequate library for subsequent sequencing was obtained. No library amplification was observed in two scraped slides-derived samples.

In 24/36 (67%) samples at least one mutation was observed among the 22 lung cancer-related genes (Table 2, Figure 1). **EGFR** and **KRAS** mutations were 6/36 (16%) and 10/36 (29%), respectively. Seven mutations were identified in the **TP53** gene (18%), three in **PIK3CA** (8%), two in **BRAF** (5%), one each in **SMAD4** (3%), **STK11** (3%), and **MAP2K1** (3%). Germline variants in **MET** (10%) were observed in two cases and in **FGFR3** (3%) in one case. Two cases (5%) harbored an A to T nucleotide substitution in **STK11** gene at the intronic position chr19:g.1221210.

All **EGFR** and **KRAS** mutations were confirmed at Sanger sequencing or high resolution melting analysis. **EGFR**, **KRAS** and **PIK3CA** mutations were mutually exclusive.

Nine cancers (25%) were found to have multiple driver gene alterations (Table 2). In these cases, significant differences were observed in the proportion of alleles affected for distinct genes, supporting the presence of intra-tumor molecular heterogeneity. For example, case #23 had 73.2% of alleles with an **EGFR** exon 19 deletion coexisting with a 16.3% of **TP53** E285K and a 4.1% of **MAP2K1** Y130C. No significant association between type and number of mutations and clinicopathological data was observed.

Technology reproducibility

In two FNA-washing cases (#1 and #18), two different cell sample aliquots were available. To test the Ion Torrent technology intra-sample reproducibility, 10 ng of DNA obtained from each aliquot were deep sequenced with the Ion AmpliSeq Colon and Lung Cancer Panel. In both cases the mutations identified in the first aliquot were confirmed in the second one with comparable mutation frequencies (Table 3). In two cases (#8 and #21) a matched tumor biopsy, collected after cytological examination, was available and processed for deep sequencing with the Ion AmpliSeq Colon and Lung Cancer Panel. Case #8 showed an **EGFR** exon 21 L858R mutation in...
Table 2. Mutations found in 24 lung adenocarcinoma cytology specimens sequenced for 22 cancer-related genes using the Ion AmpliSeq Colon and Lung Cancer Panel v1.

| Sample | Type | # cells | Gene mutations |
|--------|------|---------|----------------|
|        |      |         | EGFR | KRAS | PIK3CA | BRAF | TPS3 | PTEN | MET | SMAD4 | FGFR3 | STK11 | MAP2K1 |
| #8     | F    | >100    |      | L858R (62.9%) | - | - | - | - | - | - | - | - | - |
| #18    | F    | >100    |      | delK745-A750 (64.8%) | - | - | E224D (70.8%) | - | - | - | - | - | - |
| #23    | S    | 400     |      | delL747-P753insS (73.2%) | - | - | E285K (16.3%) | - | - | - | - | - | Y130C (4.1%) |
| #30    | S    | 1,000   |      | L858R (17.3%) | - | - | G469R (2.5%) | - | - | - | - | - | - |
| #31    | S    | 300     |      | delE746-A750 (27.5%) | - | - | - | - | E285K (16.3%) | - | - | - |
| #34    | S    | 1,000   |      | delE746-A750 (74%) | - | - | - | - | - | - | - | - | - |
| #3     | F    | >100    |      | G12A (40.7%) | - | - | - | - | - | Q224/stop (6.4%) | - | - | E70/stop (21.9%) |
| #4     | F    | >100    |      | G12V (22.6%) | - | - | - | - | - | - | - | - | - |
| #9     | F    | >100    |      | G12/C (8.2%) | - | - | - | - | - | Q171stop (26.1%) | T1010I (40.8%) | - | chr19g.1221210A>T (46.3%) |
| #11    | F    | >100    |      | G12/C (8.2%) | - | - | - | - | - | - | - | - | - |
| #14    | F    | >100    |      | G13C (31.5%) | - | - | - | - | - | - | - | - | - |
| #22    | F    | >100    |      | G13C (48.4%) | - | - | - | - | - | - | - | - | - |
| #28    | F    | >100    |      | G12D (29.6%) | - | - | - | - | - | - | - | - | - |
| #29    | S    | 500     |      | G12V (74.9%) | - | - | - | - | - | - | - | - | - |
| #32    | S    | 750     |      | G12C (43.8%) | - | - | Q165/stop (71.2%) | - | - | - | - | - | - |
| #35    | S    | 250     |      | G12D (30.6%) | - | - | R273L (12.8%) | - | - | - | - | - | - |
| #38    | S    | 5,000   |      | G12V (52.7%) | - | - | - | - | - | - | - | - | - |
| #1     | F    | >100    |      | M1043V (6.5%) | - | - | G105C (5.1%) | - | - | - | F384L (55.2%) | - | - |
| #7     | F    | >100    |      | H1047R (6.7%) | - | - | - | - | - | - | - | - | - |
| #26    | S    | 150     |      | D1029Y (6.0%) | D594E (83.0%) | - | - | - | - | - | - | - | - |
| #13    | F    | >100    |      | - | - | Y220C (41.0%) | - | - | - | - | - | - | - | - |
| #17    | S    | 250     |      | - | - | S241Y (17.0%) | - | - | - | - | - | - | - | - |
| #21    | F    | >100    |      | - | - | - | T1010I (40.3%) | - | - | - | - | - | - | - | - |
| #10    | S    | 1,000   |      | - | - | - | - | - | - | - | - | - | Chr19g.1221210A>T (24.5%) | - | - |
| Total  |      | 6/36    | 10/36 | 3/36 | 2/36 | 7/36 | 1/36 | 2/36 | 1/36 | 1/36 | 3/36 | 1/36 |
| %      |      | 15.8    | 27.8 | 7.9 | 5.3 | 18.4 | 2.6 | 5.3 | 2.6 | 2.6 | 7.9 | 2.6 |

Note: F = fine needle aspirate washing; S = smear cytology.
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Discussion

Subgroups of lung adenocarcinomas are characterized by specific driver molecular alterations that also represent potential therapeutic targets. Mutations in the tyrosine kinase domain of the EGFR gene are driver alterations and predictive biomarkers of response to treatment with specific inhibitors that are already in clinical practice [13,25]. Several other driver molecular alterations have been identified in lung adenocarcinoma, including somatic mutations of KRAS, BRAF, STK11, DDR2 and members of the FGFR family, as well as ALK rearrangements [10,26,27,28,29,30,31]. Clinical trials are ongoing in lung cancers carrying PIK3CA, BRAF or KRAS mutations [32,33,34,35,36]. Therefore, a comprehensive molecular characterization of lung tumors is needed for patients to benefit from novel therapeutics in either clinical practice or trials.

In addition, since some driver mutations are mutually exclusive, detection of specific molecular alterations might also predict resistance to specific drugs. In contrast, the limited diagnostic material available in most NSCLC cases is incompatible with a comprehensive molecular characterization by conventional techniques [5,39].

In the present study, we show that targeted NGS using the Ion Torrent technology provides information about multiple genes starting from a very limited amount of DNA. In fact, in spite of a low amount of DNA input necessary for the analysis (i.e., 10 ng), Ion AmpliSeq Colon and Lung Cancer Panel can simultaneously interrogate 504 hotspot mutations in 22 lung cancer-associated oncogenes and tumor suppressor genes.

The prevalence and type of mutations detected in our series of cytology samples are comparable to those reported by The National Cancer Institute Lung Cancer Mutation Consortium in 1,000 lung adenocarcinomas [40], which were: KRAS 25%, EGFR 23%, BRAF 3%, PIK3CA 3%, MET amplifications 2%, ERBB2 1%, MAP2K1 0.4%, and NRAS 0.2% [40]. ALK rearrangements, which are not detected by our assay, were found by FISH analysis in 6% of cases [40].

NGS analyzing the exome, i.e. the portion of genome coding for proteins, or the entire genome has already been demonstrated to provide comprehensive molecular characterization of NSCLC [41,42,43], however this approach is not clinically applicable as of today. NGS technology has also recently shown to be high sensitive in EGFR single gene testing in cytology samples obtained from bronchoalveolar lavage and pleural fluid of lung adenocarcinoma patients [44]. Moreover, specific multiplex PCR assays targeting fusion genes (i.e. ALK and ROS1) are under development in the framework of the Onconetwork Consortium.

The application of the Ion AmpliSeq Colon and Lung Cancer Panel in routine pathology molecular diagnostics needs validation in larger series of cases. However, its performances in detecting a wide range of genetic alterations with an extremely high sensitivity and specificity can help to assess tumor-specific therapeutic susceptibility and individual prognosis. The upcoming challenge lies in the reliable identification of an ultimate NSCLC-specific muligen panel to significantly improve the care of lung cancer patients.

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Author Contributions

Conceived and designed the experiments: AS MF G. Torrella AF NN. Performed the experiments: KS AMR RC DA EA ML CE FS MS G. Turri. Analyzed the data: AS MF AM EB MC NN. Contributed reagents/materials/analysis tools: AS MC AF NN. Wrote the paper: AS MF KS NN.
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