



***EML4-ALK* translocation identification in RNA exosomal cargo (*ExoALK*) in NSCLC patients: a novel role for liquid biopsy**

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Abstract: The introduction of druggable targets has significantly improved the outcomes of non-small cell lung cancer patients (NSCLC). *EML4-ALK* translocation represents 4–6% of the druggable alterations in NSCLC. With the approval of Crizotinib, first discovered drug for the *EML4-ALK* translocation, on first line treatment for patients with detected mutation meant a complete change on the treatment landscape. The current standard method for *EML4-ALK* identification is immunohistochemistry or FISH in a tumor biopsy. However, a big number of NSCLC patients have not tissue available for analysis and others are not suitable for biopsy due to their physical condition or the location of the tumor. Liquid biopsy seems the best alternative for identification in these patients that have no tissue available. Circulating free RNA has not been validated for the identification of this mutation. As a complementary tool, exosomes might represent a good tool for predictive biomarkers study, and due to their stability, they preserve the genetic material contained in them. Our group has described for the first time the translocation *EML4-ALK* in RNA isolated from exosomes derived from NSCLC patients using next generation sequencing.

Keywords: Exosomes; liquid biopsy; ALK translocation; non-small cell lung cancer (NSCLC); biomarkers

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Introduction

Lung cancer treatment landscape has completely changed in the last decade. The introduction of druggable targets in the therapeutic scenario gave the opportunity to significantly improve the outcomes in the most lethal malignant disease. *EML4-ALK* translocation represents a story of success in drug development. The discovery of the aberration in tumors of patients with lung cancer in 2007 (1) soon translated into a rapid approval of the first ALK tyrosine kinase inhibitor (TKI), crizotinib (2), and the subsequent early arrival of second and third generation TKIs, all happening in roughly a decade. The

current standard for the identification of the target necessarily implies identification of rearrangement in the tumor tissue either by FISH or immunohistochemistry. Unfortunately, despite the efforts, a big number of non-small cell lung cancer (NSCLC) patients have no tissue available for determination. The isolation of ctDNA is a novel approach, but still not validated for *EML4-ALK* translocation (3,4). Exosomes, new members of the liquid biopsy family, carrying genetic material represent an important tool for biomarkers discovery (5). Our group has identified, for the first time, *EML4-ALK* translocation in exosomes (*ExoALK*), using a next generation sequencing technique.

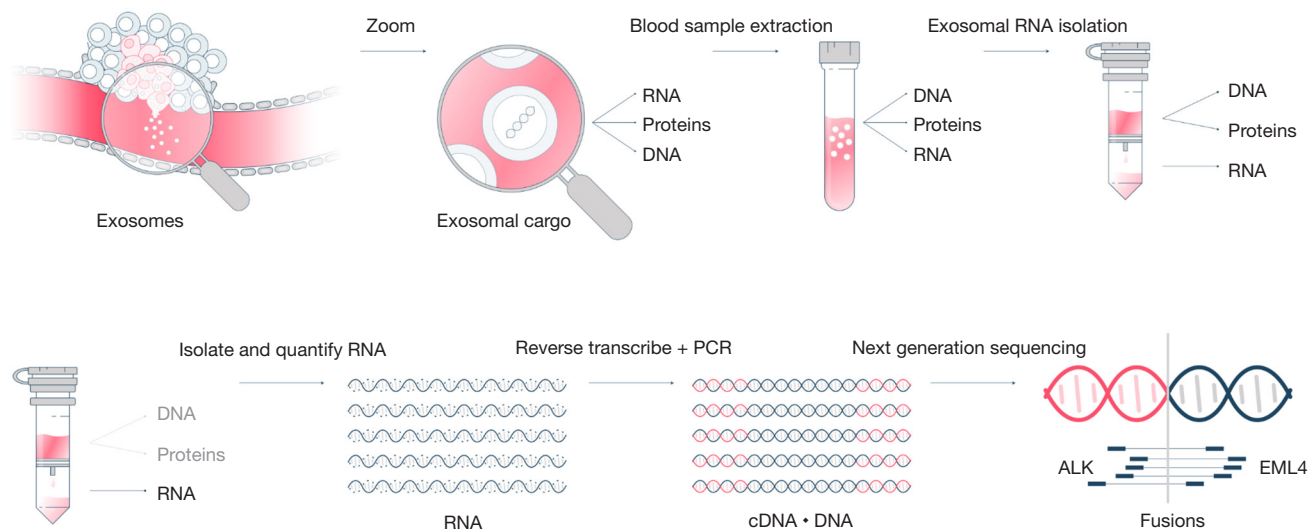


Figure 1 Schematic representation of *ExoALK* proof of concept study.

Material and methods

Patients selection

Patients with confirmed diagnosis of stage IIIB–IV NSCLC, naïve or under treatment with known *ALK* status were eligible for this proof of concept study. *EML4-ALK* translocation in tumor tissue was performed by FISH according to the clinical records of the patients. Patients gave their informed consent before the sample collection according to the institution regulation. Blood samples were collected only at a single point. Clinical data were collected from the electronic medical records. Samples and data from patients included in the study were provided by the Biobank of the University of Navarra (Reg#: B_0000612) and were processed following standard operating procedures approved by the Ethical and Scientific Committees. The trial was approved by the Antwerp University Hospital ethical committee n. 14/17/206.

Exosomal RNA isolation

The exosomal RNA cargo was extracted from 1 mL of plasma using ExoRNeasy Kit from QIAGEN with a modification of the original protocol to avoid loss of material and volume. This includes 3 centrifugations of $5' \times 500$ g, $15' \times 3,000$ g and $30' \times 10,000$ g instead of the filtration in $0.8 \mu\text{M}$ (Figure 1).

Next generation sequencing

The fusions were analyzed using the Ion Ampliseq RNA Fusion Lung Cancer panel (Thermo Fisher Scientific, Waltham, USA). The primers used for amplification were then partially digested by the Pfu enzyme. The product of digestion was ligated with corresponding barcoded adapters and purified using Ampure Beads (Agilent Genomics Inc). The product of purification was amplified for 5 more cycles and subsequently purified using Ampure Beads. Ten pM of each library was loaded into the IonChef system (Thermo Fisher Scientific, Waltham, USA) for the emulsion polymerase chain reaction (PCR) and then loaded in the chip.

The quality of the data was assessed using the Torrent suite software (Thermo Fisher Scientific, Waltham, USA) associated with the sequencing machine. The minimum read length for fusion detection was set to 50 bp (base pair). We consider that under this value the reads were too short to overlap targeted fusion breakpoint. In addition, to assess the detection quality, at least 20,000 reads were required per sample (Figure 1).

Results

A total of 19 patients with plasma samples were included in the study, 16 of them harboring *EML4-ALK* translocations in tissue. In 1 mL of plasma, the RNA concentration ranged

Table 1 Concordance between tissue and *ExoALK*

Exosomal RNA (<i>ExoALK</i>) (n=17)	Tissue (patients n=19)	
	Positive	Negative
Positive	9	0
Negative	5	3
Degraded RNA	2	–

Sensitivity =64%; Specificity =100%.

from 0.5 to 121 mg/mL. In total, 17 patients were finally eligible for extracellular vesicles analysis. In nine patients, we were able to identify the translocation in the exosomal RNA. The concordance between tissue and exosomes was 64% (9 out of 14 patients). All three patients being negative for the fusion gene in tissue resulted also negative in the *ExoALK* analysis, representing a specificity of 100% (Table 1). No correlation was found between the RNA yield and the type of sample or its clinical variables.

Discussion

Here we demonstrate for the first time that *EML4-ALK* translocation detection in exosomes of NSCLC patients is feasible, with a high sensitivity and specificity. The exosomal analysis represents an opportunity for patients therapeutic selection with a minimal invasive procedure, easy to perform and with an affordable cost. The development of new techniques with a direct detection of the fusion by capture instead of by amplification would be the key to improve the sensitivity of *ExoALK* in the future.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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