Plasma EGFR mutation detection using a combined exosomal RNA and circulating tumor DNA approach in patients with acquired resistance to first-generation EGFR-TKIs

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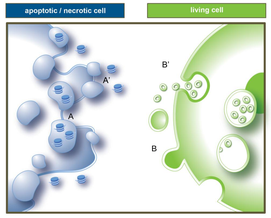
Background

After initial responses to tyrosine kinase inhibitors (TKIs), NSCLC patients harboring EGFR activating mutations inevitably progress, with the "gatekeeper" EGFR T790M resistance mutation accounting for approximately 60% of cases of acquired resistance (AR) to TKIs. EGFR activating and T790M resistance mutations can be found in plasma in both RNA contained within exosomes and in circulating free tumor DNA (ctDNA). While ctDNA is released by dying cells, exosomal RNA is actively released by many living cells (Schwarzenbach et al. Nat Rev Clin Oncol 2014; Jahr et al. Cancer Res 2001; Thery et al. Nat Rev Immunol 2009). Here we present matched tumor and plasma data from a subset of patients in TIGER-X (NCT01526928), a Ph1/2 study of rociletinib in previously treated mutant EGFR patients with advanced NSCLC. We demonstrate the detection of EGFR mutations in plasma using a-spin-column based method (ExoLution Plus) for combined isolation of exosomal RNA (exoRNA) and ctDNA in a single step. This approach improves sensitivity and demonstrates the ability to detect mutations using exosomal RNA in patients previously described as negative by an external high-sensitivity qPCR method relying on ctDNA alone.

Fig. 1 - Two distinct sources of cell-free nucleic acids in plasma



- **Extracellular vesicles**, mainly 50-200 nm in size, are actively secreted by the cell and carry a snapshot of the body's transcriptome (exoRNA).
- **Circulating cell-free DNA** (cfDNA) including circulating tumor DNA (ctDNA) is released by necrotic and apoptotic events in tumor and normal tissue.

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Extracellular RNA and DNA in plasma. Apoptotic or necrotic cells may release cell-free DNA (cfDNA) in apoptotic vesicles (A) or as circulating nucleosomes (A'). Exosomes are actively released by living cells directly from the plasma membrane (B) or via the multivesicular body pathway (B'), carrying RNA into circulation (exoRNA).

Fig. 2 - A single-step isolation platform for exoRNA and cfDNA from patient plasma samples

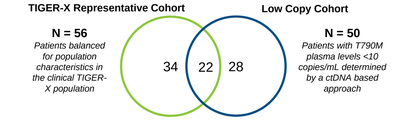
ExoLution Plus spin-column

- **Bind vesicles and cfDNA** to ExoLution Plus spin-column membrane & wash
- On-column lysis and release
- Mini spin-column purification
- Elution of **exoRNA and cfDNA**

Workflow for co-isolation of exoRNA and cfDNA from biofluids using the ExoLution Plus technology platform. The ExoLution Plus platform employs a proprietary capture mechanism in a disposable spin-column format to enable routine parallel co-extraction of exoRNA and cfDNA from biofluids.

Fig. 3 - Overview of study cohort and sub-cohorts of EXO1000 liquid biopsies on tissue-matched plasma of NSCLC patients

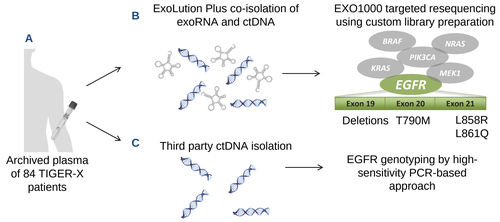
84 NSCLC patients
enrolled in TIGER-X
(Ph1/2 study of rociletinib in previously treated mutant EGFR patients with advanced NSCLC)



- N=21 patients in the low copy cohort were classified as M0/M1a (i.e. intrathoracic disease). These patients have been shown to be especially challenging for mutation detection in liquid biopsies (Tseng et al. J Thorac Oncol 2015).
- N=10 out of the 84 patients, had tissue biopsies that were T790M-negative or invalid/inadequate by central laboratory assessment

Overview of NSCLC samples analyzed with EXO1000: Among the 84 patients [all enrolled by Dec 31, 2014], two main sub-cohorts, the TIGER-X representative cohort and the low copy cohort, have been defined according to criteria specified next to the circles of the Venn diagram.

Fig. 4 - Experimental Setup



Overview of NSCLC samples analyzed with EXO1000: A) Matched pretreatment tumor tissue and plasma were collected from 84 NSCLC patients enrolled in TIGER-X. B) A column-based method (ExoLution Plus) was applied to isolate both exoRNA and ctDNA from up to 6 mL of patient plasma, where available, and mutations were analyzed with a custom procedure for next generation sequencing (EXO1000). The targeted EXO1000 sequencing panel covered EGFR mutations on exon 19, 20 and 21. C) Data were compared to a generic vendor who performed a PCR-based EGFR genotyping of ctDNA.

Fig. 5 - Patient baseline characteristics

Baseline characteristics	TIGER-X study	Representative population cohort
	N = 456	N = 56
Median age	63 years	61 years
Female	67%	79%
Asian ethnicity	20%	21%
ECOG PS grade 0	28%	27%
M1a or M0	29% *	25%

* N = 255

84 NSCLC patients enrolled in TIGER-X: Phase 1/2 trial of rociletinib
Key eligibility criteria:
- Advanced or recurrent NSCLC with a documented activating EGFR mutation.
- Prior treatment with EGFR-directed therapy.
- Recent biopsy available; plasma samples collected within 60 days of biopsy.

Fig. 6 - High positive percent agreement for EXO1000 in comparison to ctDNA with central pathology results

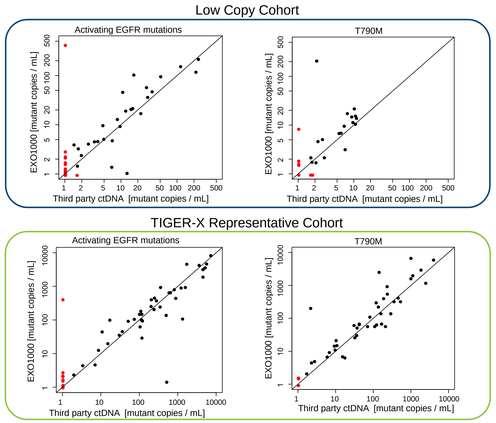
All patients (N = 84, hereof 4 w/o valid tissue status)		
Assay	**EGFR activating**	**EGFR T790M**
EXO1000 **exoRNA + ctDNA**	**88**% (70/80)	**77**% (53/69)
Third party liquid biopsy **ctDNA**	**74**% (59/80)	**74**% (51/69)

TIGER-X Representative Cohort (N = 56, hereof 2 w/o valid tissue status)		
Assay	**EGFR activating**	**EGFR T790M**
EXO1000 **exoRNA + ctDNA**	**96**% (52/54)	**88**% (43/49)
Third party liquid biopsy **ctDNA**	**82**% (44/54)	**84**% (41/49)

Low Copy Cohort (N = 50, hereof 2 w/o valid tissue status)		
Assay	**EGFR activating**	**EGFR T790M**
EXO1000 **exoRNA + ctDNA**	**81**% (39/48)	**58**% (22/38)
Third party liquid biopsy **ctDNA**	**58**% (28/48)	**53**% (20/38)

Concordance of EGFR mutations in NSCLC. EXO1000, the combined ctDNA and exoRNA approach, reaches a higher PPA over all patients analyzed in this study than a ctDNA-only test. This difference becomes more pronounced when the sub-cohort of T790M low copy samples is compared.

Fig. 7 - Higher copy numbers for EXO1000 in low copy samples in comparison to ctDNA only



Absolute mutant copy number comparison. The additional impact of RNA becomes evident when comparing absolute numbers of mutant copies of the low copy cohort. For samples with high loads of ctDNA, as seen in the TIGER-X Representative Cohort, the signal addition of RNA becomes less significant. Red dots = tissue-positive samples that were positive in one assay but negative in the other (set to 1 copy for plotting on log-scale).

Fig. 8 - Good sensitivity for activating EGFR mutations in challenging samples with EXO1000

M0/M1a (N = 21, 2 w/o valid tissue status)			
PPA with central pathology	L858R	del19	T790M
EXO1000 **exoRNA + ctDNA**	**74% (14/19)**		**25%** (4/16)
	75% (3/4)	**73%** (11/15)	
Third party liquid biopsy **ctDNA**	**26% (5/19)**		**19%** (3/16)
	25% (1/4)	**27%** (4/15)	

Liquid biopsy results of challenging samples. Patients with intrathoracic disease (M0/M1a) are challenging to diagnose using ctDNA alone (PPA for ctDNA-only for activating mutations is 26%). By combining the exoRNA and DNA we achieved a sensitivity of 74% for activating mutations.

Fig. 9 - Biopsy-negative patients carry circulating mutations detected by two different liquid biopsies

	EXO1000 exoRNA + ctDNA			Third party liquid biopsy ctDNA			Response	
	L858R	del19	T790M	L858R	del19	T790M	Best	% shrink
Invalid tissue status		✓	✓		✓	✓	PR	-84
		✓	✓		✓	✓	SD	-9
		✓			✓		SD	0
		✓					No therapeutic dose	
T790M negatives in tissue		✓	✓				SD	-11
	✓		✓	✓		✓	PD	0
	✓		✓	✓			SD	-36
		✓	✓		✓	✓	No scan	
		✓			✓	✓	N/A	N/A
				✓		✓	SD	3.4

Liquid biopsy results of patients tested negative by tissue. Two independent plasma testing approaches in patients that have been tested negative by tissue lead to consistent results for activating mutations and T790M, indicating that tissue-negatives/plasma-positives are likely not "false positives".

Conclusions

- **Detection of both activating and acquired resistance mutations to EGFR therapy in plasma offers a promising alternative to tissue-based biopsy.**
- **Combined plasma exoRNA + ctDNA isolation increases the number of gene copies available for low abundant somatic mutation detection, compared to ctDNA-only, an improvement in the clinical sensitivity of liquid biopsies.**
- **In challenging cases, such as M0/M1a, the combined exoRNA + ctDNA isolation offers improved sensitivity for EGFR mutations.**
- **Tissue-negative/plasma-positive cases identified by EXO1000 are likely not "false positives" because they were identified as plasma-positive by a second test methodology.**
- **EXO1000 provides excellent analytical performance for detection of actionable mutations in plasma of NSCLC with immediate potential for clinical application.**

