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Annals of Oncology 29: 2379–2383, 2018
doi:10.1093/annonc/mdy458
Published online 18 October 2018

ORIGINAL ARTICLE

Nanoscale extracellular vesicle-derived DNA is superior to circulating cell-free DNA for mutation detection in early-stage non-small-cell lung cancer

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Background: The comparison between relatively intact nanoscale extracellular vesicle-derived DNA (nEV-DNA) and fragmented circulating cell-free DNA (cfDNA) in mutation detection among patients with non-small-cell lung cancer (NSCLC) has not been carried out yet, and thus deserves investigation.

Patients and methods: Both nEV-DNA and cfDNA was obtained from 377 NSCLC patients with known *EGFR* mutation status and 69 controls. The respective *EGFR*$^{E19del/T790M/L858R}$ mutation status was interrogated with amplification-refractory-mutation-system-based PCR assays (ARMS-PCR).

Results: Neither nEV-DNA nor cfDNA levels show a strong correlation with tumor volumes. There is no correlation between cfDNA and nEV-DNA levels either. The detection sensitivity of nEV-DNA and cfDNA using ARMS-PCR in early-stage NSCLC was 25.7% and 14.2%, respectively, with 96.6% and 91.7% specificity, respectively. In late-stage NSCLC, both nEV-DNA and cfDNA show ~80% sensitivity and over 95% specificity.

Conclusions: nEV-DNA is superior to cfDNA for mutation detection in early-stage NSCLC using ARMS-PCR. However, the advantages vanish in late-stage NSCLC.

Key words: liquid biopsy, extracellular vesicles, circulating tumor DNA, non-small-cell lung cancer, epidermal growth factor receptor, PCR

Introduction

Extracellular vesicles (EVs) are cell-derived lipid bilayer-enclosed vesicles of sub-micrometer sizes that are secreted by various cells [1]. EVs can mediate intercellular communication through transferring donor cell derived proteins and nucleic acids. Currently, nanoscale EVs (nEVs, 30–220 nm) including exosomes are under intense investigation [2]. In tumors, growing evidence indicates that nEVs have a complicated relationship with tumor development and metastasis [3]. Particularly, nEVs enable liquid biopsy for cancer diagnostics and treatment monitoring [4]. Previous studies demonstrated that nEV-derived DNA (nEV-DNA) represents the entire genome and reflects

the mutational status of parental cells [5, 6]. We further found that copy number variations of nEV-DNA are identical to those of the original cells. Moreover, we identified *KRAS* and *EGFR* mutations from plasma nEVs isolated from patients with non-small-cell lung cancer (NSCLC) [7]. The above findings indicate nEV-DNA could be a new promising marker of cancer. Compared with circulating cell free DNA (cfDNA, average ~130 bp), nEV-DNA fragments are relatively intact (average ~15k bp) due to protection of the lipid envelop from degradation by DNase [7, 8]. In addition, only up to 1-ml plasma is required for extracting nEV-DNA, while cfDNA routinely needs to be isolated from 5-ml plasma [5, 7]. Nevertheless, it is not conclusive yet whether nEV-DNA is superior to cfDNA in a clinical setting. On the other hand, to translate nEV-DNA or cfDNA in clinical use, a sensitive and quantitative test platform for detection of mutations is required, given the median mutant allele frequency (MAF) of circulating tumor DNA or tumor cell-derived nEV-DNA typically is <1% in the plasma of cancer patients.

In this study, we developed amplification-refractory mutation system (ARMS)-based PCR assays (ARMS-PCR) [9, 10], which detect $EGFR^{E19del/T790M/L858R}$ with a detection limit (LoD) of 0.1% in a clinical setting. With the assays, we tested nEV-DNA and cfDNA isolated from patients with NSCLC and age-matched controls. We found in early-stage NSCLC neither nEV-DNA nor cfDNA levels show a strong linear correlation with tumor volumes. No association between nEV-DNA and cfDNA levels was found either. More importantly, our results indicate the sensitivity and specificity of nEV-DNA are better than that of cfDNA in patients with early-stage NSCLC. However, the advantages vanish in late-stage NSCLC.

Methods

Study populations

From November 2016 to February 2018, 284 patients with early-stage NSCLC before cancer treatment and 69 age-matched controls were consented and enrolled. In addition, 93 archived 2-ml plasma samples from patients in late-stage NSCLC with acquired $EGFR^{T790M}$ mutation were received. These samples were collected and stored at −80°C since 2013, and the corresponding tissue specimens were not available. Patients consented to the protocol approved by an institutional review board of The First Affiliated Hospital of Soochow University. More information can be found in supplementary Material, available at *Annals of Oncology* online.

Primers design of ARMS-PCR

A forward primer, a TaqMan probe, and seven reverse primers were designed for detection 10 variations of $EGFR^{E19del}$. The point mutations of $EGFR^{T790M}$ and $EGFR^{L858R}$ were detected by respective primers and TaqMan probe (supplementary Tables S1 and S2, available at *Annals of Oncology* online). Additionally, a pair of primer and a probe for quality control of PCR were designed. The PCR conditions can be found in supplementary Materials, available at *Annals of Oncology* online.

Statistical analyses

Data analyses were carried out using SPSS 23 software program. The statistical significance was determined by chi-square test, Student's *t*-test, McNemar test, and Cochran–Mantel–Haenszel test. All tests were two-sided, and *P* value <0.05 were considered statistically significant.

Results

AMRS-PCR assays

All primers and PCR reaction conditions have been optimized. First, we investigated the efficiency, reproducibility, and LoD of PCR assays using diluted DNA samples in deionized water (supplementary Figure S1, available at *Annals of Oncology* online). On the basis of the slope value of standard curves (from −3.514 to −3.229), we determined the amplification efficiency of assays (from 92.6% to 104%) falls within the range between 90% and 110%, which are generally considered as a quality capable of generating reliable data [11]. Moreover, all R^2 value is larger than 0.99, indicating good linearity. The LoD of 0.01% can be achieved with samples containing more than ~30 000 copies. Next, we spiked mutant DNA in wt-DNA and inspected the assays in a practical setting (supplementary Figure S2, available at *Annals of Oncology* online). Due to the influence of wt-DNA and pipetting error, the efficiency ranges from 70% to 140% [12]. The LoD decreased to 0.1% with ~10 000 copies. Nevertheless, the assays still provide good confidence ($R^2 > 0.985$).

Patients

The prevalence of NSCLC *EGFR* mutations in China can reach 64.5% [13]. Based on the prevalence, we determined at least 277 patients should be recruited if we assume that the true sensitivity and specificity is ~90%, and the SE of the estimates is no more than 5%, with 95% confidence interval. In fact, a total of 284 consecutive patients with stage I and II NSCLC were enrolled (supplementary Table S3, available at *Annals of Oncology* online). Of the 284 patients, 148 (52.1%) had *EGFR* mutation in their surgical tumor tissues, including 78 (27.5%) with an $EGFR^{E19del}$, 68 (23.9%) with an $EGFR^{L858R}$, and 2 (0.7%) with an $EGFR^{T790M}$. The most common NSCLC subtype was adenocarcinoma (81.3%). The *EGFR* mutation rate in patients with adenocarcinoma is much higher than that in patients with non-adenocarcinoma (60.2% versus 17.0%, $P < 0.0001$); the mutation rate is higher in females than in males (60% versus 41.2%, $P < 0.01$); and mutation rate for smokers is lower than non-smokers (21.7% versus 66.7%, $P < 0.00001$). Altogether, the characteristics of enrolled patients are consistent with China's epidemiological data [14].

Characterization of nEVs

Both nEVs isolated from a patient and a control exhibit saucer-shaped morphology under TEM (supplementary Figure S3A, available at *Annals of Oncology* online). Three commonly used EV markers CD9, CD81, and TSG101 were identified (supplementary Figure S3B, available at *Annals of Oncology* online). The average size of nEVs isolated from randomly selected 54 patients with stage-I and 90 patients with stage-II NSCLC was 114 ± 8 nm and 124 ± 18 nm, respectively, in comparison with nEVs isolated from 35 controls with 107 ± 7 nm. A significant difference in size was found between each other ($P < 0.0001$). There was a significantly higher level of nEVs in patients with stage-II NSCLC (range from 2.1×10^8/ml to 2.7×10^9/ml) compared with stage-I

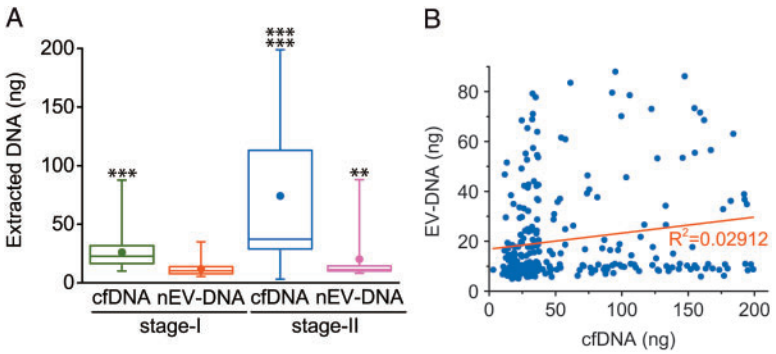

Figure 1. Measurement and analyses of nanoscale extracellular vesicle-derived DNA (nEV-DNA) and cell-free DNA (cfDNA). (A) nEV-DNA levels and cfDNA levels in two stages (cfDNA versus EV-DNA, ***$P < 0.0001$; stage II cfDNA versus stage I cfDNA, ***$P < 0.0001$; stage II nEV-DNA versus stage I nEV-DNA, **$P < 0.001$). (B) The correlation between nEV-DNA levels and cfDNA levels.

NSCLC (range from 1.4×10^8/ml to 1.2×10^9/ml) and controls (range from 1.3×10^8/ml to 4.8×10^8/ml) ($p < 0.001$).

Detection of *EGFR*$^{E19del/T790M/L858R}$

We extracted cfDNA and nEV-DNA in 1-ml plasma sample from patients and controls, and compared their level. In both stages, the average cfDNA level is significantly higher than that of nEV-DNA (stage-I: 33.1 ng versus 13.7 ng; stage-II: 74.0 ng versus 24.8 ng; $P < 0.0001$). In comparison, the average cfDNA and nEV-DNA level in 1-ml plasma of controls is 9.7 and 3.4 ng, respectively (Figure 1A). Moreover, both cfDNA level and nEV-DNA level are higher in stage-II than in stage-I (cfDNA: $P < 0.0001$; nEV-DNA: $P < 0.001$). However, we did not find linear association between nEV-DNA and cfDNA levels (Figure 1B). Neither nEV-DNA nor cfDNA levels show a strong linear correlation with tumor volumes (supplementary Figure S4A and B, available at *Annals of Oncology* online).

Then, *EGFR*$^{E19del/T790M/L858R}$ mutations were identified in 38 nEV-DNA samples (MAF ranges from 0.1% to 1.3%) and 21 cfDNA samples (MAF ranges from 0.1% to 0.6%), respectively (supplementary Tables S4 and S5, available at *Annals of Oncology* online). *EGFR* mutations were detected in additional 6 nEV-DNA and 14 cfDNA samples, respectively; however, wild-type *EGFR* was found in corresponding tumor tissue. In 69 controls, 1 and 3 false-positives were detected with nEV-DNA and cfDNA, respectively. Therefore, the sensitivity and specificity of nEV-DNA in the detection of *EGFR* mutation from early-stage NSCLC thus were determined to be 25.7% and 96.6%, respectively, with an accuracy of 66.9% [95% confidence interval (CI) 61.7% to 71.8%]. In comparison, the sensitivity and specificity of cfDNA are 14.2% and 91.7%, respectively, with an accuracy of 59.2% (95% CI 53.9% to 64.4%). The Youden index of nEV-DNA and cfDNA was 0.22 and 0.06, respectively. McNemar test on two sides shows a significant difference between nEV-DNA and cfDNA ($P < 0.01$), indicating nEV-DNA might be superior to cfDNA in *EGFR*$^{E19del/T790M/L858R}$ mutation detection in early-stage NSCLC.

Later, we detected *EGFR*^{T790M} mutation in nEV-DNA and cfDNA, respectively, from NSCLC patients in late-stage after EGFR tyrosine kinase inhibitor (TKI) therapy. Sanger sequencing (supplementary Figure S5, available at *Annals of Oncology* online) confirmed the acquired *EGFR*^{T790M} mutation from 93 patients. The average size of nEVs isolated from 63 patients is 111 ± 15 nm. The amount of nEVs ranges from 2.2×10^{10}/ml to 4.7×10^{12}/ml, which is significantly higher than that of patients with early-stage NSCLC. The average cfDNA and nEV-DNA level in 1-ml plasma is 194.7 ng and 118.4 ng, respectively. There is no correlation between cfDNA and nEV-DNA levels. We identified *EGFR*^{T790M} mutation in nEV-DNA from 73 (MAF ranges from 0.1% to 20.2%) and in cfDNA from 76 patients (MAF ranges from 0.1% to 42.2%), respectively. The sensitivity and specificity of nEV-DNA in detection of *EGFR*^{T790M} from patients with late-stage NSCLC were 78.5% and 98.6%, respectively, with an accuracy of 87% (95% CI 80.9% to 91.2%). Correspondingly, the sensitivity and specificity of cfDNA were 81.7% and 95.7%, respectively, with an accuracy of 87.7% (95% CI 81.6% to 92.3%). The Youden index of nEV-DNA and cfDNA were both 0.77. McNemar test on two sides shows no significant difference between nEV-DNA and cfDNA ($P > 0.05$), indicating both nEV-DNA and cfDNA can efficiently identify *EGFR*^{T790M} mutation in advanced NSCLC.

Discussion

Hotspot-mutation analysis of *EGFR* can genotype patients as candidates who may respond favorably to TKI treatment and predict clinical outcomes of *EGFR*-targeted therapies [15]. The ARMS-PCR assays we developed satisfy a high degree of specificity and reproducibility in detection of *EGFR* mutations using liquid biopsy samples. Although in clinical setting LoD is ~0.1%, the assays still successfully detect *EGFR* mutations from samples. Of note, the sensitivity of these assays could be further improved by using chemically modified primers.

In the first cohort study, we only recruited patients with early-stage NSCLC for two reasons. First, patients were newly diagnosed with lung cancer and did not take any prior cancer treatment. All patients underwent surgery, and thus we were able to obtain tumor tissue for molecular analyses and used it to inspect nEV-DNA and cfDNA. Second, we hypothesize that the relatively intact nEV-DNA preserves mutation information and would be more attractive than degraded cfDNA for mutation detection, especially in the early stage in which only a few copies of tumor-

derived DNA might be available. We found the average size and concentration of the nEVs correlate with stage I/II and significantly higher than that of controls. Previous studies have similar findings and further reveal that higher nEVs concentration may indicate worse survival [16].

We did not find correlation between levels of nEV-DNA or cfDNA and tumor volumes. It was reported that high cfDNA levels are strongly associated with the number of metastatic sites and tumor volume at diagnosis [17]. However, in other studies the correlation was not identified [18]. These discrepancies may be attributed to differences in the methods employed to determine tumor volume and DNA level. Moreover, the undetectable micro-metastases could contribute to the level of tumor-derived DNA in plasma, causing errors on the measurement of tumor volume and data analysis [19]. Hence, it is not conclusive yet that whether nEV-DNA or cfDNA can be used to predict tumor burden. In addition, the levels of nEV-DNA and cfDNA do not correlate with each other either. This finding may indicate the origins of these two DNA types are different, which is supported by their fragment difference.

We also noticed that in patients with late-stage NSCLC, the mean size of nEVs is smaller than that in early stage. Due to extremely few reports, it is unclear whether the size of nEVs is associated with cancer stages. Therefore, additional clinical trials enrolling adequate patients must be carried out in future. Nevertheless, we admit that long-term cryopreservation of plasma samples may influence size distribution of EVs. The size of nEVs measured from less than 17 patients in stage IV is inadequate in statistical analysis either.

In processing DNA samples of 284 patients, nEV-DNA and cfDNA have mutual false-positives of $EGFR^{E19del}$ in four patients. In nEV-DNA group, there are two additional false-positives of $EGFR^{E19del}$; in cfDNA group, there are additional 10 false-positives (seven $EGFR^{E19del}$ and three $EGFR^{L858R}$). On the contrary, only wild-type *EGFR* was found in the respective tumor specimen. In controls, we noticed frequent but not mutual false-positives from $EGFR^{E19del}$ and a false-positive of $EGFR^{L858R}$. Given the proportion of detected $EGFR^{E19del}$ and $EGFR^{L858R}$ in 284 patients is very close (27.5% versus 23.9%), the disproportionate high false-positives of $EGFR^{E19del}$ indicate the primers for $EGFR^{E19del}$ deserve further optimization. Of note, the $EGFR^{E19del}$ mutation has more than 20 variants [20], which inherently posts greater challenges in primer design. Moreover, a low amount of nEV-DNA or cfDNA can be associated with some artefactual mutations leading to false-positives, causing a prominent problem in detecting mutations in from early-stage cancers [21]. On the other hand, we suspect these $EGFR^{E19del/L858R}$ positives might be true as previous studies suggest that genomic heterogeneity in the tumor may not have been identified in tissue biopsy but could be reflected in the plasma DNA [22]. Of noting, in this study the false-positive was defined as detection of the mutation in the cfDNA/nEV-DNA but absent in the tumor. If the speculation is valid it hints that ARMS-PCR detects true-positives, which were not identified by sequencing. Therefore, the result of 'false-positive' in ARMS-PCR must be interpreted with caution.

In patients with stage-I/II NSCLC, the detection sensitivity of the assays is ~14% (21 out of 148) using cfDNA. It is relatively low, but rational as a few studies reported ARMS-PCR could detect mutations in cfDNA from ~30% patients with advanced cancers [23, 24]. In contrast, using nEV-DNA, the detection sensitivity can be improved to ~26% (38 out of 148). Lipid membrane protection of wrapped dsDNA from DNase could contribute to this. It was found during the cell translocation, the nuclear deformation damages integrity of nuclear envelope and chromosomal DNA, potentially leading to DNA fragments flow into the cytoplasm [25, 26]. Recent reports further indicate that in cancer dsDNA can enter into cytoplasm due to chromosomal instability, inflammation, and cell senescence [27–29], and thus potentially can be wrapped into nEVs. Accordingly, nEV-DNA could benefit mutation detection.

This study had several potential limitations. First, it is a retrospective study with all its inherent defects. Second, we only investigate three hotspot mutations in *EGFR*, while there are hundreds of potential mutation loci in NSCLC. Third, the plasma samples of additional 93 patients with acquired T790M mutation have been stored for few years, and thus the quality and quantity of nEV-DNA and cfDNA might be affected. Fourth, few genomic DNA fragments of blood cells attaching on to protein or vesicles may avoid nuclease digestion and probably contaminate EV-DNA and cfDNA samples. Fifth, we were unable to keep tracking patients' prognosis and overall survival time. Therefore, the correlation between mutations in plasma DNA and survival and response to therapy cannot be investigated. Finally, this is a single-center study.

In conclusion, we found average nEV size and concentration increase as disease progression. The strong correlation was not found between nEV-DNA/cfDNA levels and tumor burden, and neither was found between nEV-DNA and cfDNA levels. In early-stage NSCLC, nEV-DNA is superior to cfDNA in clinical detection sensitivity and specificity using ARMS-PCR.

Funding

Grants LCZX201402, BE2015638 and NNSF81672281 (HM) and NIH DP2CA174508 (S-YZ).

Disclosure

S-YZ is the founder of Captis Diagnostics, LLC. All remaining authors have declared no conflicts of interest.

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