Editorial

Extracellular vesicle-based *EGFR* genotyping in bronchoalveolar lavage fluid

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Lung cancer, the most common malignancy worldwide, is highly lethal with short survival especially in advanced stage disease (1). Recent development of targeted therapies such as epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) has improved the survival of subsets of patients with advanced stage non-small cell lung cancer (NSCLC) harboring driver mutations including *EGFR* sensitizing mutations (2). At present, *EGFR* genotyping is mostly performed on tissue biopsy specimens. However, the size and location of the lung tumor may pose a challenge to conventional tumor biopsy to determine the status of *EGFR* mutation. Liquid biopsy using plasma cell-free circulating tumor DNA (ctDNA) can be used to supplement tissue-based genotyping but the amount of ctDNA in plasma cell-free DNA (cfDNA) is only about 1% (3,4). Although genotyping using ctDNA is highly specific, the clinical utility of ctDNA genotyping is constrained by its variable and low sensitivity because of the low copy number of ctDNA detectable in the blood and its shorter than 2 hours half-life (4,5).

Extracellular vesicles (EVs) are submicrometer phospholipid bilayer-enclosed vesicles or membrane fragments ranging in size from 30 to 10,000 nm in diameter released by various cells into bodily fluids such as plasma, saliva, pleural effusions, bronchoalveolar lavage fluid and urine (6,7). EVs contain DNA, RNA and proteins from the cellular environment and cell-surface of the parent cell (7,8). EVs mediate intercellular communication in diverse physiological and pathological cellular processes by transferring membrane and cytosolic proteins, lipids and nucleic acids bearing genetic information between cells. EVs not only transfer functionally active biological materials to target cells in the surrounding environment but also to distant organs by the blood stream and lymphatics (9). Large amounts of EVs are shed by tumor cells into the tumor microenvironment (7). While cfDNA are short fragments of 200–400 base pairs, the length of EV-derived DNA (EV-DNA) are more than 1kb double-stranded DNA (7,10-12).

There is increasing evidence that EVs play a complex role in the development and metastasis of tumors (13). It has been demonstrated that EV-DNA is representative of the entire genome and mutational status of the parent cells from which the EV-DNA is released (12,14). Copy number variations of EV-DNA have been found to be the same as those of the parent cells. Furthermore, mutations of the *KRAS* and *EGFR* genes have been identified from plasma EVs of NSCLC patients (15).

Circulating EV-DNA has been shown to be superior to plasma cfDNA in the detection of *EGFR* mutations in early-stage NSCLC using amplification-refractory-mutation-system-based polymerase chain reaction (PCR) assays (ARMS-PCR) with a detection limit of 0.1% to interrogate the $EGFR^{E19del/T790M/L858R}$ mutation status (16) and in the detection of *KRAS* mutations in early-stage pancreatic

cancer with droplet digital PCR (17). However, there are technical difficulties associated with isolating and purifying EVs from the plasma and blood EV-DNA-based liquid biopsy suffers from a low sensitivity of 50–60% (8). Plasma lipoproteins, particularly low-density lipoproteins (LDLs) have characteristics very similar to that of EVs. The low sensitivity of genotyping by liquid biopsy using plasma EV-DNA is probably because of the contamination of samples in which EVs are isolated by LDLs which interfere with the analysis (18).

Earlier studies with small sample size have shown that EVs isolated from bronchoalveolar lavage fluid (BALF) (n=23) and pleural effusion (n=32) of NSCLC patients with tissue biopsy proven *EGFR* genotyping to be tissue-specific and contain abundant double-stranded DNA (8,19). Through bronchoalveolar lavage (BAL), cellular and non-cellular materials from the distal airways and alveoli can be obtained from the diseased site (20). BALF from the site of the lung tumor potentially enriches EVs derived from the tumor, thus enhancing the sensitivity of *EGFR* mutation detection (8). The sensitivity and specificity of BALF (n=23) cfDNA and EV-DNA *EGFR* genotyping were previously found to be significantly higher than those of plasma (n=20) ctDNA and EV-DNA genotyping, illustrating that cfDNA and EV-DNA in biofluids in close proximity to the lung cancer represent the tumor status better (8). In addition, BALF EV-DNA *EGFR* genotyping was found to be more sensitive and more specific than BALF cfDNA *EGFR* genotyping (8). The size of BALF EVs was identified to range from 20 to 250 nm while plasma EVs were smaller with size ranging from 5 to 15 nm (8).

In this issue of the journal, Hur *et al.* demonstrated in a prospective study involving 137 treatment naïve patients with NSCLC at Konkuk University Medical Center, Seoul, Republic of Korea the utility of detecting sensitizing *EGFR* mutations using EV-DNA from the supernatant of BALF compared to standard tumor tissue- or cytology-based genotyping at the initial diagnosis (21). The peptide nucleic acid (PNA)-mediated real-time PCR clamping method was used for *EGFR* genotyping. Fifty-four NSCLC patients (39.4%) were found to have sensitizing *EGFR* mutations based on tissue genotyping.

While *EGFR* copy number correlated with the EV-DNA concentration and EV concentration, *EGFR* copy number did not correlate with EV size (21). *EGFR* copy number also increased as T stage (according to the 8th edition of TNM classification) (22) advanced but EV concentration and size did not correlate with T stage. Similarly, EV-DNA concentration did not correlate with T stage.

The sensitivity and specificity of BALF EV-based *EGFR* genotyping averaged 76% and 87%, respectively with a significant increase in the sensitivity as the TNM stage advanced (21). The authors also showed the sensitivity of BALF EV-based *EGFR* genotyping increased as the T descriptor advanced with sensitivities of 40%, 75%, 100% and 100% in T1, T2, T3 and T4 stage, respectively. A similar increasing sensitivity was also noted for N staging with the sensitivity being 63.3%, 75%, and 100% at N0, N1/N2 and N3 stage, respectively. A 100% sensitivity was noted when metastasis was present irrespective of whether it was intrathoracic (M1a) or extrathoracic (M1b and M1c). The authors suggested that the increased sensitivity with more advanced disease to be related to a greater shedding of tumor EVs with *EGFR*-mutant DNA as the tumor progresses and metastasis occurs (21). The greater effectiveness of BAL as the cancer progresses could also be related to peribronchial thickening which increases stiffness around the tumor site, allowing smoother flow of BALF and increased retrieval of tumor-derived EVs. The lung of early-stage cancer may be more compliant and the negative suction pressure during collection of BALF may cause dynamic collapse of the distal bronchus, thereby compromising the retrieval of EV-containing BALF.

EGFR genotyping on BALF EV samples and on tissue/cytology samples had a concordance or agreement of 79% for stage I, 100% for stage II, 74% for stage III, and 92% for stage IV disease (21). All 31 patients with tissue-proven *EGFR*-mutated stage IV disease were positively identified by genotyping of BALF EV specimens. In addition, BALF EV-based genotyping identified six patients with stage IV *EGFR*-mutant disease (all with deletion mutation in exon 19) who were not identified by tissue/cytology-based genotyping. Compared to conventional tissue/cytology-based genotyping, the detection of *EGFR*-mutant cases was significantly increased with BALF EV-based genotyping, especially in the late disease stages.

Overall, BALF EV-based genotyping detected an additional 11 cases with *EGFR* mutation but failed to detect 13 tissue-proven *EGFR*-mutant cases vis-a-vis conventional tissue/cytology-based genotyping (21). All 13 patients in whom BALF EV-based genotyping failed to detect *EGFR* mutation were having stage I disease clinically. The exact location of the lung tumor and the presence or absence of the open bronchus sign did not seem to affect the performance of *EGFR* genotyping on BALF EV specimens.

The utility of BALF EV-based *EGFR* genotyping in

patients with early-stage NSCLC was demonstrated in 36 patients with stage I disease [solid nodules in 17 patients and ground glass nodules (GGNs) in 19 patients] in whom the sensitivity was 30% and specificity was 88.9% (21). In one series from Japan, of 104 GGNs with ground-glass component ≥50% on a thin-section computed tomography scan that were resected in 96 patients, *EGFR* mutations were detected in 64% of resected specimens (23). Compared to *EGFR* mutation-negative GGNs, there was correlation between *EGFR*-mutant GGN tumors and more malignant histology including minimally invasive adenocarcinoma or invasive adenocarcinoma rather than non-malignant histology of atypical adenomatous hyperplasia or adenocarcinoma in-situ. *EGFR* mutation-positive GGNs were also associated with growth rather than remaining unchanged for 2 years or longer (23). In the earlier report, Hur and colleagues demonstrated the utility of BALF EV-DNA-based genotyping to detect T790M mutation in nine patients who had developed resistance to EGFR-TKI treatment with a sensitivity that is higher than that of re-biopsy tissue-based genotyping (8).

The potential of tumor-derived EVs and EV-DNA as biomarkers in personalized cancer medicine is great with increasing new discoveries in a variety of tumors. There is a possibility of genotyping using BALF EV-DNA-based liquid biopsy which is sensitive, accurate, cheaper and faster to complement or even replace the current tissue biopsy in the initial diagnostic workup of advanced stage NSCLC and for monitoring the disease during targeted therapy. Liquid biopsy using BALF EV-DNA reduces turn-around time to 2 working days compared to about 10 working days for tissue-based *EGFR* genotyping (24). Although BAL is not totally non-invasive, BALF EV-based *EGFR* genotyping may replace tissue biopsy for patients in whom tumor biopsy is not feasible because of the patient's poor performance status or when the tumor is in a location which is difficult or hazardous for transthoracic needle biopsy. Genotyping using enriched tumor-derived EV-DNA in BALF obtained from the tumor site produced results which were highly accurate and sensitive.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tlcr.2020.03.06). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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