Peer Review File

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Comment (1) There were similar reports (Cancer. 2020 Apr 15;126(8):1804-1809) and (J Clin Med. 2019 Jul 10;8(7):1011) in PubMed. What is the novelty in this paper? Please elaborate on this in the introduction.

Reply 1-1: Thank you for your comments. The suggested papers that used PCR-based assays for ctDNA detection reported the usefulness of ctDNA analysis in lung cancer treatment. Especially, the former paper (Cancer. 2020 Apr 15;126(8):1804-1809) is important since this paper evaluated ctDNA obtained before the surgery (n = 192) and reported the sensitivity and specificity of ctDNA analysis in early-stage lung cancers. However, our study is distinct from these studies by means of using an NGS-based panel test to detect ctDNA.
Following the comments by the reviewer, we modified the Introduction referring to the suggested paper.

Changes in the text (Page 4, Lines 65-72)
(Before)
Circulating tumor DNA (ctDNA) analysis is a useful technique for detecting mutations in tumor cells. Detecting mutations in *EGFR*, including the T790M resistant mutation, is already used for clinical decision-making in advanced non-small cell lung cancer patients (4).

(After)
Circulating tumor DNA (ctDNA) analysis, which detects mutations of circulating cell-free DNA (cfDNA) that is shed by tumor cells, is a useful technique for detecting mutations in tumor cells. Detecting mutations in *EGFR*, including the T790M resistant mutation, is already used for clinical decision-making in advanced non-small cell lung cancer patients (6). It has been reported that ctDNA analysis prior to the surgery in early-stage lung cancer patients can detect somatic mutations in tumors at high sensitivity and specificity (7), and that ctDNA analysis is able to detect mutations which will present even in a heterogeneous manner in tumor tissues (8, 9).

Changes in the text (Page 11, Lines 187-190)
(Before)
In this study, we found that detecting mutations in cancer-related genes from postoperative ctDNA (POD 3–12) predicted poor patient outcomes.

(After)
There are several techniques to detect ctDNA, including PCR-based (7, 23), NGS panel based, and bespoked NGS (10). In this study, using a next-generation sequencing (NGS)-based panel test for cfDNA analysis, we found that detecting mutations in cancer-related genes from postoperative ctDNA (POD 3–12) predicted poor patient outcomes.

Added references
7. Leung M, Freidin MB, Freydina DV, et al. Blood-based circulating tumor DNA mutations

as a diagnostic and prognostic biomarker for lung cancer. Cancer 2020; 126(8): 1804-1809.

8. Gerlinger M, Rowan AJ, Horswell S, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med 2012; 366(10): 883-892.

9. Murtaza M, Dawson SJ, Tsui DW, et al. Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. Nature 2013; 497(7447): 108-12.

23. Alama A, Coco S, Genova C, et al. Prognostic Relevance of Circulating Tumor Cells and Circulating Cell-Free DNA Association in Metastatic Non-Small Cell Lung Cancer Treated with Nivolumab. J Clin Med 2019; 8(7): 1011.

Reply 1-2: In addition, in this study, we found that the post-surgical ctDNA was the best predictor of post-surgical recurrence compared with pre-surgical ctDNA or pre- / post-surgical CEA levels. Our results also revealed that ctDNA in genes other than lung cancer-related genes (such as EGFR, KRAS, TP53) will also be a good biomarker to predict post-surgical recurrence. These findings have been described in the Discussion, and these sentences were partially modified to highlight the new findings in your study.

Changes in the text (Page 11, Lines 188-Page 12, 205)

(Before)

In this study, we found that detecting mutations in cancer-related genes from postoperative ctDNA (POD 3–12) predicted poor patient outcomes. This finding was compatible with a recent study by Chen et al. (18) that showed postoperative ctDNA detection (POD 3 or POD 1 month) was significantly correlated with RFS. Although the study by Chen et al. detected mutations in only nine cancer-related genes (*EGFR*, *KRAS*, *ERBB2*, *BRAF*, *PIK3CA*, *ALK*, *RET*, *MET* exon 14 skipping, and *TP53*), our study revealed that detecting other lung cancer-related genes (such as *PDGFRA*, *HCN1*, and *LRRTM4*) from ctDNA can also be prognostic.
It is noteworthy that three of the four patients who were positive for postoperative ctDNA relapsed within 6 months. This result was similar to the observation by Hu et al. that two patients with detectable *EGFR* mutations in ctDNA at 1 month after surgery both experienced recurrence within 4 months of surgery (17). Our results also suggested that postoperative ctDNA detection may more accurately predict poor patient outcomes than the pre- or postoperative tumor marker analysis and preoperative ctDNA detection.

(After)

In this study, using a next-generation sequencing (NGS)-based panel test for cfDNA analysis, we found that detecting mutations in cancer-related genes from postoperative ctDNA (POD 3–12) predicted poor patient outcomes. This finding was compatible with a recent study by Chen et al. (18) that showed postoperative ctDNA detection (POD 3 or POD 1 month) was significantly correlated with RFS. Although the study by Chen et al. detected mutations in only nine cancer-related genes (*EGFR*, *KRAS*, *ERBB2*, *BRAF*, *PIK3CA*, *ALK*, *RET*, *MET* exon 14 skipping, and *TP53*), using an NGS-based detection platform, our study revealed that detecting other lung cancer-related genes (such as *PDGFRA*, *HCN1*, and *LRRTM4*) from ctDNA can also be prognostic. Therefore, we consider that postoperative cfDNA analysis with a comprehensive genetic panel will help to identify patients who may have high risk of post-

surgical recurrence.
It is noteworthy that three of the four patients who were positive for postoperative ctDNA relapsed within 6 months. This result was similar to the observation by Hu et al. that two patients with detectable *EGFR* mutations in ctDNA at 1 month after surgery both experienced recurrence within 4 months of surgery (17). Although this study was a pilot study with a small cohort, our results also suggested that postoperative ctDNA detection may more accurately predict poor patient outcomes than the pre- or postoperative tumor marker analysis and preoperative ctDNA detection.

Comment (2) The case samples were too small in the paper. How do you look at this issue?

Reply 2-1: We agree with the reviewer that the study sample size was small. However, we were able to find that post-surgical ctDNA could predict post-surgical recurrence better than pre-surgical ctDNA or tumor marker (CEA). To clarify the limitation of this study with a small sample size, we briefly modified our Discussion as below.

Changes in the text (Page 12, Lines 200-205):
(Before)
This result was similar to the observation by Hu et al. that two patients with detectable *EGFR* mutations in ctDNA at 1 month after surgery both experienced recurrence within 4 months of surgery [11]. our results also suggested that postoperative ctDNA detection may more accurately predict poor patient outcomes than the pre- or postoperative tumor marker analysis and preoperative ctDNA detection.

(After)
This result was similar to the observation by Hu et al. that two patients with detectable *EGFR* mutations in ctDNA at 1 month after surgery both experienced recurrence within 4 months of surgery [11]. Although this study was a pilot study with a small cohort, our results also suggested that postoperative ctDNA detection may more accurately predict poor patient outcomes than the pre- or postoperative tumor marker analysis and preoperative ctDNA detection.

Reply 2-2: In addition, we revised our title to indicate that our study was a pilot study.

Changes of the title (Page 1, Lines 4):
(Before)
Title: Prognostic implications of preoperative versus postoperative circulating tumor DNA in surgically resected lung cancer patients

(After)
Title: Prognostic implications of preoperative versus postoperative circulating tumor DNA in surgically resected lung cancer patients: A pilot study

Comment (3) Please supplement the introduction with ctDNA and the research progress of ctDNA.

Reply 3: We added several sentences to describe the recent progress of ctDNA for early stage lung cancer patients as a response to the Comment (1). In addition, we modified the Introduction to describe the research progress in this field.

Changes in the text (Page 4, Lines 72-Page 5, 74):
(Before)
Additionally, recent studies have shown that ctDNA can detect disease progression 5 months prior to radiological modalities (8),

(After)
Additionally, recent studies have suggested that ctDNA can be a potential biomarker for the assessment of post-surgical MRD (10), as well as a potential predictor for the disease progression prior to radiological modalities (11).

Added references
10. Abbosh C, Birkbak NJ, Swanton C. Early stage NSCLC - challenges to implementing ctDNA-based screening and MRD detection. Nat Rev Clin Oncol 2018; 15(9): 577-586.

Comment (4) The authors could enrich the introduction of the progress of the treatment for NSCLC.

Reply 4: We added several sentences describing recent progress in the treatment of resectable NSCLCs in the introduction.

Changes in the text (Page 4, Lines 55-64):
(Added sentences in the Introduction)
The risk of post-surgical recurrence is still problematic even when locoregional control is thought to have been achieved by complete surgical resection. For example, a Japanese lung cancer registry study (n = 18,973) reported that the disease-free survival rate at 5 years after pulmonary resection was 67.8% (1) To improve the outcomes of surgically resected non-small cell lung cancer (NSCLC) patients, several clinical trials employing tyrosine kinase inhibitors (2) or new chemotherapeutic regimen (3) have been performed. In addition to these efforts to develop novel adjuvant therapies, evaluation of the personal risks of recurrence is an important issue for a better post-surgical care (4), including the detection of post-surgical minimal residual disease (MRD) (5). If we are able to exclude patients who do not relapse from the candidates of adjuvant therapy, it will eliminate unnecessary adverse events or costs.

Added references
1. Okami J, Shintani Y, Okumura M, et al. Demographics, Safety and Quality, and Prognostic Information in Both the Seventh and Eighth Editions of the TNM Classification in 18,973 Surgical Cases of the Japanese Joint Committee of Lung Cancer Registry Database in 2010. J Thorac Oncol 2019, 14(2):212-222.

2. Herbst RS, Tsuboi M, John T, et al. Osimertinib as adjuvant therapy in patients (pts) with stage IB–IIIA EGFR mutation positive (EGFRm) NSCLC after complete tumor resection: ADAURA. J Clin Oncol 2020; 38 (suppl; abstr LBA5).
3. Kenmotsu H, Yamamoto N, Yamanaka T, et al. Randomized Phase III Study of Pemetrexed Plus Cisplatin Versus Vinorelbine Plus Cisplatin for Completely Resected Stage II to IIIA Nonsquamous Non-Small-Cell Lung Cancer. J Clin Oncol 2020; 38(19): 2187-2196.
4. Suda K. Personalized post-surgical care?-possible strategies for NSCLCs with EGFR mutation. Transl Lung Cancer Res 2020; 9(3): 441-445.

Comment (5) What are the inclusion and exclusion of enrolled patients?

Reply 5: The inclusion criteria have been described in the Patients and Methods. We added the details of patients who were excluded from the study.

Changes in the text (Page 6, Lines 92-98):
(Before)
Between January 2018 and May 2019, 20 lung cancer patients with clinical stage IIA–IIIA disease who underwent complete surgical resection were included in this study. Patients who received neoadjuvant chemotherapy or had advanced malignancies other than lung cancer within the past 5 years were excluded.

(After)
Between January 2018 and May 2019, 2~~0~~3 lung cancer patients with clinical stage IIA–IIIA disease who underwent complete surgical resection (inclusion criteria) were intended to be included in this study. Patients who received neoadjuvant chemotherapy or had advanced malignancies other than lung cancer within the past 5 years were excluded (exclusion criteria). Among these 23 patients, one patient without enough plasma sample and two patients who refused the enrollment were excluded from the study. Finally, the data of 20 patients were analyzed in this study.

Comment (6) Are there any advantages of using ctDNA as prognostic implications in NSCLC? They should be mentioned in the discussion.

Reply 6: We found that positive post-surgical ctDNA was a significant poor prognostic factor in our cohort. We believe that post-surgical cfDNA analysis will help to identify patients who may have high risk of post-surgical recurrence. We added a few sentences in the Discussion.

Changes in the text (Page 11, Lines 194-198):
(Before)
our study revealed that detecting other lung cancer-related genes (such as *PDGFRA*, *HCN1*, and *LRRTM4*) from ctDNA can also be prognostic.

(After)
our study revealed that detecting other lung cancer-related genes (such as *PDGFRA*, *HCN1*,

and *LRRTM4*) from ctDNA can also be prognostic. Therefore, we consider that postoperative cfDNA analysis with a comprehensive genetic panel will help to identify patients who may have high risk of post-surgical recurrence.

Comment (7) Please supplement the discussion with the mechanism analysis of ctDNA.

Reply 7: We added a few descriptions about the detection technique of ctDNA in the Discussion.

Changes in the text (Page 11, Lines 187-196):
(Before)
In this study, we found that detecting mutations in cancer-related genes from postoperative ctDNA (POD 3–12) predicted poor patient outcomes. This finding was compatible with a recent study by Chen et al. [12] that showed postoperative ctDNA detection (POD 3 or POD 1 month) was significantly correlated with RFS. Although the study by Chen et al. detected mutations in only nine cancer-related genes (*EGFR*, *KRAS*, *ERBB2*, *BRAF*, *PIK3CA*, *ALK*, *RET*, *MET* exon 14 skipping, and *TP53*), our study revealed that detecting other lung cancer-related genes (such as *PDGFRA*, *HCN1*, and *LRRTM4*) from ctDNA can also be prognostic.

(After)
There are several techniques to detect ctDNA, including PCR-based (7, 23), NGS panel based, and bespoked NGS (10). In this study, using a next-generation sequencing (NGS)-based panel test for cfDNA analysis, we found that detecting mutations in cancer-related genes from postoperative ctDNA (POD 3–12) predicted poor patient outcomes. This finding was compatible with a recent study by Chen et al. (20) that showed postoperative ctDNA detection (POD 3 or POD 1 month) was significantly correlated with RFS. Although the study by Chen et al. detected mutations in only nine cancer-related genes (*EGFR*, *KRAS*, *ERBB2*, *BRAF*, *PIK3CA*, *ALK*, *RET*, *MET* exon 14 skipping, and *TP53*), using an NGS-based detection platform, our study revealed that detecting other lung cancer-related genes (such as *PDGFRA*, *HCN1*, and *LRRTM4*) from ctDNA can also be prognostic.