Circulating tumor cells (CTC) and Cell-free DNA (cfDNA): Liquid biopsy for cancer diagnostics

Ayaka Nakamura1,2, Minako Abe3, Yukie Saeki1, Fumika Kono3, Yasuha Ono1,2 and Hiroyuki Abe2,3

1) ImmunoGenex, Tokyo, Japan
2) Fukuro Clinic, Tokyo, Japan
3) Tokyo Cancer Clinic, Tokyo, Japan

Abstract:
Cancer has been the leading cause of death in Japan since 1981, and deaths due to cancer continue to rise. The early detection of cancer and determining therapeutic effect using noninvasive techniques are critical in the treatment of cancer. We conducted a study assaying both circulating tumor cells (CTCs) and cell-free DNA (cfDNA) using a small quantity of blood (5 mL) in order to explore the utility of using liquid biopsies to detect and monitor various types of cancers. Our results confirm that not only are CTCs detected in multiple types of cancers, but also that there is a clinical correlation between the number of CTCs and cancer progression and the presence or absence of tumor metastasis. Furthermore, a significant increase in cfDNA concentration levels between healthy volunteers and cancer patients was confirmed. The measurements of CTCs and cfDNA levels have clinical significance, and can be expected to play a larger role in the diagnosis and treatment of cancer in the near future.

Keywords:
circulating tumor cells (CTC), cell-free DNA (cfDNA), liquid biopsy, cancer

1. Introduction
With the aging population in Japan, the number of deaths due to cancer has been steadily rising and has become the leading cause of death since 1981. In 2018, cancer accounted for 27.4% of all fatalities, as approximately one in every 3.6 people died of cancer [1]. The early detection of cancer and determining therapeutic effect using noninvasive techniques are extremely important in the treatment of cancer.

In recent years the use of circulating tumor cells (CTCs) and cell-free DNA (cfDNA) have gained attention as liquid biopsy in oncology. CTCs are tumor cells that infiltrate into blood vessels from the primary or metastasized tumor and circulate in the bloodstream. CTCs are known to be released from the primary tumor site even in the early stages of cancer and include cells that have the ability to metastasize to other sites [2–5].

CFDNA are degraded DNA fragments that are released into the bloodstream. When cancer cells undergo apoptosis or are destroyed by immune cells, and when CTCs within the bloodstream are degraded, the genomic DNA of cancer cells in the circulation can be detected and the concentration of cfDNA levels found in plasma will rise. Elevated levels of cfDNA in cancer patients has been well documented and cfDNA analysis has been proposed as a promising future tool for detection and early cancer screening [6]. The use of a noninvasive liquid biopsy in early detection, progression, and serial monitoring of cancer treatments is rapidly gaining favor in personalized medicine and oncology. In this study, we evaluated the clinical correlation of CTCs and cfDNA in various types of cancers in varying stages of progression us-

*Corresponding author: Ayaka Nakamura, info@immunogenex.co.jp

Article history: Received 27 August 2020, Received in revised form 24 September 2020, Accepted 28 September 2020

https://doi.org/10.46459/pmu.2020011

Copyright © 2020 International Society of Personalized Medicine
ing a minimal amount (5 mL) of blood sampling.

2. Materials and methods

2.1 Study selection

Written informed consent was obtained from all participants included in the present study (from two clinical sites, the Tokyo Cancer Clinic and the Hanzomon Gastrointestinal Clinic in Tokyo, Japan), and was performed in accordance with the amended Declaration of Helsinki. Clinical data was collected from 29 patients with various cancer types, stages, and with the presence or absence of metastasis. The median age of the cancer patients (16 male and 13 female) was 59.0 years (range 19–74 years). The median age of 10 healthy donors (4 male and 6 female) was 32.5 years (range 27–73 years).

2.2 CTC number analysis

Peripheral blood was collected from each donor into a blood collection tube containing EDTA/2Na and 4 mL was used for analysis. The samples were hemolyzed using Lysing Buffer (BD, New Jersey, USA) within 24 hours of collection. The number of cells were counted using TC20 (Bio Rad, California, USA), and staining was performed using the appropriate concentration of antibodies correlating to the number of cells. FcR Blocking Reagent human (Miltenyi Biotec, Bergisch Gladbach, Germany), DAPI (BioLegend, California, USA), anti-human Cytokeratin Antibody (BioLegend) and anti-human CD45 Antibody (BioLegend) were used for staining.

The stained cells underwent flow cytometry analysis and fluorescence microscopy using Cell Sorter SH800S (Sony Biotechnology, California, USA). Cells positively staining for leukocyte markers (DAPI+/Cytokeratin+/CD45-) were counted as CTCs.

2.3 cfDNA concentration analysis

Peripheral blood was collected from each donor into a blood collection tube containing EDTA/2Na and 1 mL was used for analysis. Plasma collection by centrifugation occurred within 24 hours. cfDNA was extracted from 200 μL of this plasma, using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). The concentration of the extracted cfDNA was measured using Qubit (Thermo Fisher Scientific, Massachusetts, USA).

2.4 Determination of the reference value

In this study, the number of healthy donor controls was limited to 10, and it was not possible to determine statistical criteria for the range of normal for the number of CTCs and concentration of cfDNA. We therefore determined the reference range for healthy subjects by referring to the literature.

In numerous studies, the number of CTCs found in healthy subject included 1–5 CTCs/7.5 mL [7–10]. In our study, as our assessment used a smaller quantity of blood (4 mL), 2 or more CTCs were considered a positive result. In healthy subjects, a cfDNA concentration of 1–20 ng/mL is considered normal [11] with up to 30 ng/mL that may be detected [12]. In this study, we used the criteria of 20 ng/mL or more to be positive.

3. Results

3.1 Comparison of CTC numbers of cancer patients and healthy donors

The median number of CTCs in 4 mL of peripheral blood in cancer patients was 3.0 (range 0–14) and that of healthy donor control subjects was 0.0 (range 0–1). Samples containing 2 or more CTCs were considered to be a positive result. 86% of samples from cancer patients were found to be positive for CTCs, and 100% of healthy donors were found to be negative [Table 1].

The CTC numbers of cancer patients in comparison to healthy donors are shown in the following graph, where the number of CTCs in cancer patients was found to be significantly higher (p-value < 0.005) than that of healthy donors. [Fig. 1A]

In comparing cancer patients with and without tumor metastases, patients with metastases had higher CTC numbers with a smaller but significant difference (p-value < 0.05) [Fig. 1B].

3.2 Comparison of cfDNA concentrations of cancer patients and healthy donors

The median cfDNA concentration of cancer patients was 22.4 (range 7.3–58.9) and that of healthy subjects was 12.2 (range 8.1–18.5). cfDNA samples with a concentrations above 20 ng/mL are considered to be higher than normal levels. 59% of samples from cancer patients had cfDNA concentrations above normal levels, and 100% of healthy donors had concentrations within the standard value [Table 1].

The cfDNA concentration of cancer patients in comparison to healthy donors are shown in the following graph, where the cfDNA concentration of cancer patients was found to be significantly higher (p-value < 0.005) than in the healthy donors [Fig. 1C].

In comparing cancer patients with and without tumor metastasis, patients with metastases showed higher cfDNA concentration levels, however no statistically significant difference was observed [Fig. 1D].

3.3 Comparison between CTC numbers and cfDNA concentration

The relationship between the number of CTCs and cfDNA is shown on the distribution map. Although the correlation coefficient between these two values was slight at 0.41, the result shows that the correlation is statistically significant (p-value <0.05) [Fig. 1E].
### Table 1. Characteristics and results of CTCs and cfDNA concentrations in cancer patients and healthy donors.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Cancer Patients</th>
<th>Healthy donors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>% of total</td>
</tr>
<tr>
<td>Total Patients enrolled</td>
<td>29</td>
<td>-</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>59.0</td>
<td>-</td>
</tr>
<tr>
<td>Range</td>
<td>29–74</td>
<td>-</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>16</td>
<td>55%</td>
</tr>
<tr>
<td>Female</td>
<td>13</td>
<td>45%</td>
</tr>
<tr>
<td>Cancer type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colon</td>
<td>11</td>
<td>38%</td>
</tr>
<tr>
<td>Lung</td>
<td>5</td>
<td>17%</td>
</tr>
<tr>
<td>Stomach</td>
<td>3</td>
<td>10%</td>
</tr>
<tr>
<td>Breast</td>
<td>2</td>
<td>7%</td>
</tr>
<tr>
<td>Liver</td>
<td>2</td>
<td>7%</td>
</tr>
<tr>
<td>Other</td>
<td>6</td>
<td>21%</td>
</tr>
<tr>
<td>Metastasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>16</td>
<td>55%</td>
</tr>
<tr>
<td>Negative</td>
<td>13</td>
<td>45%</td>
</tr>
<tr>
<td>CTC number (in 4 mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>3.0</td>
<td>-</td>
</tr>
<tr>
<td>Range</td>
<td>0–14</td>
<td>-</td>
</tr>
<tr>
<td>Positive (&gt;2CTCs)</td>
<td>25</td>
<td>86%</td>
</tr>
<tr>
<td>cfDNA (ng/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>22.4</td>
<td>-</td>
</tr>
<tr>
<td>Range</td>
<td>7.3–58.9</td>
<td>-</td>
</tr>
<tr>
<td>Positive (&gt;20 ng/mL)</td>
<td>17</td>
<td>59%</td>
</tr>
</tbody>
</table>

### 4. Discussion

Liquid biopsy, assessing CTCs and cfDNA, is emerging as a promising new tool in personalized oncology. This non-invasive approach for the detection and monitoring of cancer biomarkers provides information about disease progression and therapy in real-time without the burden of conventional tissue biopsies.

In our study, we assessed the value of the CTC number and cfDNA concentration in cancer patients and healthy subjects using a small amount of peripheral blood (5 mL) to evaluate its correlation with disease progression. With such noninvasive testing using minimal blood sampling, much information can be gathered with little burden to the patient. Currently there are still technological limitations and challenges as to how these biomarkers should be interpreted across various disease stages and tumor types while taking into consideration tumor heterogeneity [7]. As more data is gathered, liquid biopsies will be taken a step further to include genomic and epigenetic analysis, and provide a wealth of information in order to provide early detection and help guide oncologic patient care.

In this study, we confirmed that there is a statistically significant difference in the CTC number and cfDNA concentration between cancer patients and healthy subjects. There was also a significant difference in the number of CTCs in patients with and without tumor metastasis. Furthermore, we were able to show a slight correlation between the number of CTCs and cfDNA in both cancer patients and healthy subjects. As each test measures a different aspect of tumor pathology, the extravasation of CTCs into the bloodstream versus the degradation and release of cfDNA from tumor cells, this result may be unsurprising. As one would expect an increased number of CTCs and cfDNA with tumor progression, with an increased number of subjects a stronger correlation may also be expected to be seen.

It has been reported that CTCs and cfDNA can be detected in various types cancers [7–15]. In our study, as the number of patients tested were few, we did not see a difference in results of CTC number and cfDNA concentration depending on the type of cancer. It is presumed that there are differences in detection rates with various types of cancers depending on the expression of nuclear and epithelial markers. Further research on this subject should help to further elucidate such differences between cancer types and help with use for clinical diagnostics.

Although both CTC and cfDNA have been proposed to have comparable utilities, further investigation is required to determine if one biomarker is superior to the other and until further information is available, using both of these tests to-
Fig. 1.
A. Comparison of CTC numbers of cancer patients and healthy donors
B. Comparison of CTC numbers of cancer patients with and without metastasis
C. Comparison of cfDNA concentrations of cancer patients and healthy donors
D. Comparison of cfDNA concentrations of cancer patients with and without metastasis
E. Comparison between CTC numbers and cfDNA concentrations of cancer patients and healthy donors

5. Conclusion

In this study, we succeeded in detecting both CTCs and cfDNA concentrations taken from a small amount of peripheral blood (about 5 mL) from patients with various cancer types in different stages of progression. We confirmed that there is a statistically significant difference in the CTC number and cfDNA concentration between cancer patients and healthy subjects. We also found a significant difference in the number of CTCs in patients with and without tumor metastasis, as well as a correlation between the number of CTCs and cfDNA in both cancer patients and healthy subjects. It is imperative that further evidence is gathered, and that with the advancement of this technology including genomic and epigenetic information, we anticipate liquid biopsy to become the standard of care in personalized oncology.
Declarations of interest

None.

Acknowledgements

We would like to thank Dr. Kazutoshi Kaketani for his kind collaboration in providing part of the data and blood samples for this study from healthy donors and patients from the Hanzomon Gastrointestinal Clinic.

References