# 論　文　内　容　要　旨

Live single cell mass spectrometry reveals cancer-specific metabolic profiles of circulating tumor cells

（一生細胞質量分析法を用いた循環腫瘍細胞のがん特異的代謝プロファイルの解析）

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The proven heterogeneous behaviour of cancer cells on the single-cell level might be one of the factors contributing to tumor relapse and increased incidence of metastasis. However, studying cancer on the single cell level is a challenging task. Aside from imaging techniques for diagnostic purposes, the only reliable way of studying cancer cells in clinical setting is to perform surgical biopsy on the tumor site, which, due to its invasiveness, is unsuitable for large scale studies.

Liquid biopsy has been introduced as a possible method that can allow access to molecular information of single cancer cells without the need for invasive procedures. It is a simple, non-invasive technique that targets circulating free nucleic acids (cfDNA and cfRNA) or circulating tumor cells (CTCs) in the peripheral blood of cancer patients. CTCs in particular, present wide-ranging features of phenotypic and genotypic information depending on their primary tumor source. CTCs are cells that shed from the primary tumor into the vasculature and circulate through the blood stream and potentially metastasize to various body organs. Accordingly, they ought to have insightful and essential information on the primary tumor which will be of great importance for differential cancer diagnoses. However, due to their scarcity, their molecular characterization especially on the single cell level has been difficult to achieve.

This study attempted to overcome the aforementioned issue with CTC analysis by integrating microfluidics and live single-cell mass spectrometry (LSC-MS) to isolate, enrich, and characterize patient derived CTCs. Untargeted analysis of CTCs obtained from gastric cancer (GC) and colorectal cancer (CRC) patients was performed. The possibility of discriminating between CTCs and lymphocytes obtained from the same patients was explored, as well as the ability to discriminate between CTCs obtained from different cancer types and patients on the single cell level.

Blood samples were obtained from three patients diagnosed with GC, and seven patients diagnosed with CRC. CTCs enrichment was done by using the ClearCell® FX system. The enriched CTCs were stained with a fluorescent antibody (mouse anti-human CD45-FITC) where CD45 negative CTCs were individually sampled into a Cellomics tip by using a micromanipulator. Similarly, CD45 positive lymphocytes were also individually sampled and used as control. Prior to mass spectrometric analysis, 2 microliters of the organic solvent were introduced to the rear end of the tip by a micropipette. The organic solvent consisted of 80% methanol, 10% dimethyl sulfoxide, and 0.1% formic acid. Ultra-sonication was then applied to the tips containing the cells using a homogenizer rod. Sonicating the tips before mass spectrometry measurement enhances the extraction of metabolites and lipids in addition to improving robustness by reducing tip plugging. Mass spectrometry measurement was done using LTQ orbitrap Velos pro instrument equipped with a nanospray source. Positive mode was selected to ensure wider metabolite and lipid coverage as positive mode exhibits overall more exhaustiveness than negative mode due to the higher efficiency of protonation compared to deprotonation process. For untargeted analysis, 'SIM stitching'

method was used which provides higher dynamic range and lower overall signal to noise ratios without compromising mass accuracy, thus improving the number of metabolites and lipids detected. We focused on detecting lipids to explore their role in both cancer types. This was done by optimizing our mass spectrometry method.

To visualize the metabolomic differences across multiple samples in a reduced dimensional space, a supervised approach utilizing principle component analysis followed by discriminant analysis (PCA-DA) was performed using MarkerView® software. Furthermore, to discern the possible unique peaks to the different groups, Welch's t-test was done between CTCs and lymphocytes (control) and between GC CTCs and CRC CTCs, the test was done on R statistical software.

Our analyses revealed metabolomic difference between single CTCs and control cells (lymphocytes). This was evident by the clustering behavior between CTCs, lymphocytes and the organic solvent blank showed in the PCA-DA. This clustering behaviour was observed on the cancer level as well as the patient level, indicating differences in the metabolomic profile between each group. In addition, numerous metabolites/lipids were found to be common and unique to all CTC samples ($p < 0.05$). Specifically, several glycerophospholipids (GPLs) that are believed to have biological significance in cancer physiology were found including, PC(32:1), PC(34:1), PS(38:5), PE(38:6), PC(32:3) and PC(34:2). These GLPs are essential structural components in biological membranes in the cell, thus, their common presence in all CTCs can act as potential biomarkers in future studies.

The metabolomic differences between GC CTCs and CRC CTCs were depicted by PCA-DA. Despite the heterogeneity caused by samples obtained from different patients, significant clustering could still be observed where CTCs cluster into two distinct groups corresponding to their cancer type. The difference in phenotype was further confirmed by studying the frequency of peaks distribution across the *m/z* scale of different cancer types, in which, high *m/z* peaks were more frequent in GC CTCs. This Highlights the potential role of CTC metabolome as a feature for cancer characterization. The metabolic differences between the two cancer types were studied further where CRC CTCs exhibited elevated levels of sterol lipids (SLs) compared to other lipid classes. Previous studies showed the correlation between high levels of SLs and distant metastasis in CRC patients, which is consistent with our study subjects. Eicosanoids were also elevated in CRC CTCs. This could be due to the chronic inflammation associated with the development of CRC. Furthermore, low levels of fatty acyls (FAs) and GPLs were detected in CRC CTCs. On the other hand, FAs and GPLs were preeminent in GC CTCs. This could be explained by the increased de-novo synthesis and elongation of FAs and GPLs. In which, GPLs synthesis was proved to be a key factor in cancer proliferation as its responsible mainly for membrane and energy production. Our results showed that metabolite/lipid profiling of CTCs may be used as biomarkers for cancer differentiation from single CTCs. This presents an interesting future direction to study CTC molecular characterization on a larger scale and adapt it for future diagnostic applications .