MOLECULAR DIAGNOSTICS

02

Circulating Fetal Nucleic Acids

Principal Investigators

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Team Members

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Research Progress Summary

As pioneers in molecular diagnostic, Professor Dennis Lo and Professor Rossa Chiu have been leading their team to investigate the mechanisms of how circulating cell-free DNA (cfDNA) was fragmented and to translate the findings into biomarkers for noninvasive diagnosis. Such a budding area of research is broadly named as plasma DNA fragmentomics and is becoming prominent in the field. This year, their research team further elucidated the fragmentation patterns of circulating cfDNA and pushed the boundaries to establish a model of cfDNA fragmentation.

The team has investigated the fragmentation patterns of plasma DNA through multiple facets, including the fragment sizes of cfDNA molecules, their relationships with nucleosome positioning and characteristics related to the fragment end points. The research team hypothesised that human plasma DNA ends might have a preponderance of certain nucleotide contexts, in other words, preferred fragment end motifs. They adopted the normalised Shannon entropy to arrive at a motif diversity score (MDS) by comparing frequencies across 256 motifs. The MDS value was indicative of the level of variety of plasma DNA molecules with different end motifs. In plasma samples from patients with hepatocellular carcinoma (HCC), the team observed a high MDS value reflecting a significant increase in the diversity of DNA end motif species. Moreover, compared with HCC patients, patients without HCC showed a preferential pattern of 4-mer end motifs. In particular, the abundance of plasma DNA motif CCCA was much lower in HCC patients than in subjects without HCC. The aberrant end motifs were also observed in patients with other cancer types, including colorectal cancer, lung cancer, nasopharyngeal carcinoma and head and neck squamous cell carcinoma.

Furthermore, by studying donors and recipients of liver transplantation, the profile of 4-mer end motifs among the DNA molecules in the recipient's plasma was studied. The MDS values were significantly lower (P-value = 0.0009, Wilcoxon signed-rank test) in liver-derived DNA molecules. The hierarchical clustering analysis showed that the patterns of the 256 4-mer end motifs for liver-specific and shared DNA molecules were clustered into two groups. These results provided evidence that plasma DNA end motifs carried information about the tissue of origin of cfDNA. By using

a similar analytic strategy, pregnancy was another attractive model for studying the biology of tissue-specific cfDNA molecules. The patterns of the 4-mer end motifs originating from fetal DNA molecules across different samples formed a cluster which was distinct from that of the maternally derived DNA molecules according to hierarchical clustering analysis. Six representative motifs showing significant differences in frequencies between fetal and maternal DNA molecules included three motifs (CCCA, CCAA, and CAAA) with significant enrichment in fetal DNA molecules and another three motifs (ACTT, ACCT, and CTGG) with significant decreases among fetal DNA molecules. The work further illustrated that the plasma DNA end motifs represented a distinct type of plasma DNA fragmentation signatures. The profile of end motifs carried tissue-of-origin information and had the potential to serve as a new class of biomarkers in the nascent field of fragmentomics for liquid biopsy in oncology, noninvasive prenatal testing and transplantation monitoring.

Apart from end motifs, the research team also wondered if double-stranded plasma DNA molecules might carry single-stranded ends, termed jagged ends. They developed two approaches for investigating the presence of jagged ends in a plasma DNA pool with the use of DNA end repair to introduce differential methylation signals between the original sequence and the jagged ends. The majority of plasma DNA molecules (87.8%) were found to bear jagged ends. The jaggedness varied according to plasma DNA fragment sizes and appeared to be in association with nucleosomal patterns. In the plasma of pregnant women, the jaggedness of fetal DNA molecules was higher than that of their maternal counterparts. The jaggedness of plasma DNA correlated with the fetal DNA fraction. Similarly, in the plasma of cancer patients, tumour-derived DNA molecules in patients with hepatocellular carcinoma showed an elevated jaggedness compared with nontumoral DNA. Moreover, in mouse models, knocking out of the Dnase1 gene reduced jaggedness, whereas knocking out of the deoxyribonuclease 1 like 3 (Dnase1l3) gene enhanced jaggedness. Hence, plasma DNA jagged ends represented an intrinsic property of plasma DNA and provided a link between nuclease activities and the fragmentation of plasma DNA.

To further elucidate the biology of cfDNA fragmentation and its association with nuclease activities, the research team explored the roles of deoxyribonuclease 1 (DNASE1), DNASE1L3, and DNA fragmentation factor subunit beta (DFFB) with mice deficient in each of these nucleases. By analysing the ends of cfDNA fragments in each type of nuclease-deficient mice with those in wild-type mice, it was demonstrated that each nuclease had a specific cutting preference that revealed the stepwise process of cfDNA fragmentation. Essentially, the team demonstrated that cfDNA was generated first intracellularly with DFFB, intracellular DNASE1L3, and other nucleases.

Then, cfDNA fragmentation continued extracellularly with circulating DNASE1L3 and DNASE1. With the use of heparin to disrupt the nucleosomal structure, the team also showed that the 10 bp periodicity originated from the cutting of DNA within an intact nucleosomal structure. Altogether, the work established a model of cfDNA fragmentation.

The work on murine models illustrated that the extracellular DNase, DNASE1L3, played an important role in the fragmentation of plasma DNA. Taking another step forward, the research team studied the cfDNA fragmentation pattern in human plasma samples from patients with DNASE1L3 disease-associated gene variants. DNASE1L3 deficiency causes familial monogenic systemic lupus erythematosus with childhood-onset and anti-dsDNA reactivity. It was observed that human patients with DNASE1L3 disease-associated gene variations showed aberrations in size and a reduction of a "CC" end motif of plasma DNA. The analyses of the size distribution and end motifs of plasma DNA suggested that the cleavage of DNA by DNASE1L3 might be inter-nucleosomal and might exhibit an increased likelihood to be adjacent to cytosine dinucleotides (CC). To directly test the notion, the research team digested both cell nuclei and naked DNA by DNASE1 and DNASE1L3 and analysed the results by DNA sequencing. Interestingly, DNA from cell nuclei digested by DNASE1L3 exhibited a size profile similar to plasma cell-free DNA with a major peak at 153 bp followed by a series of smaller 10-bp periodic peaks with CC motif frequencies resembling plasma DNA from healthy individuals. In contrast, this pattern was not observed after digesting naked DNA with either DNASE1L3 or DNASE1. These results suggested that these two nucleases might have different substrate preferences and that DNA-bound proteins, e.g., histones, might be a factor influencing the fragmentation patterns of plasma DNA. Furthermore, adeno-associated virus-based transduction of Dnase1l3 into Dnase1l3-deficient mice restored the end motif profiles to those seen in the plasma DNA of wild-type mice. The findings demonstrated that DNASE1L3 was an important player in the fragmentation of plasma DNA, which appeared to act in a cell-extrinsic manner to regulate plasma DNA size and motif frequency.

Besides chromosomal DNA, there was also extrachromosomal DNA existing in human plasma, including mitochondrial DNA (mtDNA) and extrachromosomal circular DNA (eccDNA). Due to the maternally-inherited nature of mitochondrial DNA, there was always a bottleneck in studying fetal mtDNA in plasma of pregnant women. By studying surrogate pregnancies, in which there were genotypic differences between fetal and surrogate maternal mtDNA molecules, the research team explored the presence and topologic forms of circulating fetal and maternal mtDNA molecules in maternal plasma by using cleavage-end signatures of *BfaI* restriction enzyme. Fetal-derived mtDNA molecules were mainly linear (median: 88%; range: 80%-96%), whereas approximately half of the maternal-derived mtDNA molecules were circular (median: 51%; range: 42%-60%). The fetal DNA fraction of linear mtDNA was lower (median absolute difference: 9.8%; range: 1.1%-27%) than that of nuclear DNA (median: 20%; range: 9.7%-35%). The fetal-derived linear mtDNA molecules were shorter than the maternal-derived ones. The work demonstrated the existence of fetal mtDNA, which was mainly linear molecules, in maternal plasma.

The different topologic forms of mtDNA inspired the research team to explore the presence of plasma DNA molecules that originated from the genome that were of other topological forms. Through sequencing following either restriction enzyme or Tn5 transposase treatment, eccDNA molecules were identified in the plasma of pregnant women. The eccDNA molecules showed bimodal size distributions peaking at ~202 and ~338 bp with distinct 10-bp periodicity observed throughout the size ranges within both peaks, suggestive of their nucleosomal origin. Also, the predominance of the 338-bp peak of eccDNA indicated that eccDNA had a larger size distribution than linear DNA in human plasma. Moreover, eccDNA of fetal origin were shorter than the maternal eccDNA. Genomic annotation of the overall

population of eccDNA molecules revealed a preference of these molecules to be generated from 5'-untranslated regions (5'-UTRs), exonic regions, and CpG island regions. Two sets of trinucleotide repeat motifs flanking the junctional sites of eccDNA supported multiple possible models for eccDNA generation. The finding in the study highlighted the topologic analysis of plasma DNA, which was becoming an emerging direction for circulating nucleic acid research and applications.

Prof. Lo and his team are experts in translating scientific findings into clinical applications. One of the successful examples was the detection of Epstein-barr virus (EBV) DNA in a plasma sample, which not only allowed noninvasive diagnosis of nasopharyngeal carcinoma (NPC), but also made early NPC screening in asymptomatic persons possible as previously reported in *New England Journal of Medicine*. In screening, there were some individuals who did not have NPC but carried EBV DNA in plasma. However, it was not known if there might be any genotypic differences in EBV isolates from NPC and non-NPC subjects. To tackle this, the research team set up a training dataset comprised of plasma DNA sequencing data of NPC and non-NPC subjects and then studied the differences in the EBV single nucleotide variant (SNV) profiles between the two groups. They proposed an NPC risk score, which was derived from the genotypic patterns over the most differentiating SNV sites across the EBV genome, and subsequently analysed the NPC risk scores in a testing set. A total of 661 significant SNVs across the EBV genome were identified from the training set. In the testing set, NPC plasma samples were shown to have high NPC risk scores, which suggested the presence of NPC-associated EBV SNV profiles. Among the non-NPC samples, there was a wide range of NPC risk scores. These results supported the presence of diverse SNV profiles of EBV isolates from non-NPC subjects. EBV genotypic analysis was shown to be feasible through plasma DNA sequencing. The NPC risk score might be used to inform the cancer risk based on the EBV genome-wide SNV profile.

Cell-free DNA is a class of biomarkers with many current and potential applications in diagnosis, monitoring and prognosis of diseases. Prof. Lo, Prof. Chiu and their team's insightful work in the emerging area of plasma DNA fragmentomics had shed light on the future direction for circulating nucleic acid research and application. In the reporting period, the team published a total of 12 peer-reviewed articles and reviews in international journals. With the leading-edge discoveries in molecular diagnostics, Prof. Lo had been named the "Top 20 Translational Researchers of 2019" by the prestigious scientific journal *Nature Biotechnology* and it is the fourth consecutive year for Prof. Lo to receive this honour.

Research and Scholarship

Research Awards and Recognitions

Member's Name	Details	
	Award	Organisation
Dennis Lo	2nd National Award for Excellence in Innovation	China Association for Science and Technology
	Top 20 Translational Researchers of 2019	Nature Biotechnology
	The Association for Molecular Pathology (AMP) Award for Excellence in Molecular Diagnostics 2020	The Association for Molecular Pathology (AMP)
Rossa Chiu	Advance Awards 2020	Australian Government
	American Chamber of Commerce in Hong Kong Women of Influence Awards 2020 Leading Woman in STEM	The American Chamber of Commerce in Hong Kong

Fellowships

Member's Name	Details	
	Fellowship	Organisation
Dennis Lo	Doctor of Science, *honoris causa*	The Open University of Hong Kong

Academic Editorship

Member's Name	Details	
	Role	Journal
Dennis Lo	Editorial Board Member	Clinical Chemistry
		Disease Markers
		Prenatal Diagnosis
		BioScience Trends
		Fetal Diagnosis and Therapy
		Journal of Molecular Biotechnology
		Chimerism
		Journal of Pathology
		Philosophical Transactions of the Royal Society B
		Journal of Genomes and Exomes
		Reproductive BioMedicine Online
		Marrow
		The Journal of Precision Medicine
	Associate Editor	Expert Reviews in Molecular Medicine
		Clinical Chemistry
		npj Genomic Medicine
	Review Editor for Molecular Diagnostics and Therapeutics	Frontiers in Molecular Biosciences
Rossa Chiu	Editorial Board Member	Clinical Biochemistry
		Critical Reviews in Clinical Laboratory Sciences
		The Clinical Biochemist Reviews
	Associate Editor	Human Genetics and Genomics Advances
		Clinical Chemistry

Reviewer of Journal / Conference

Member's Name	Details	
	Role	Journal / Conference
Dennis Lo	Reviewer	Hong Kong Journal of Radiology
Rossa Chiu	Reviewer	Clinical Chemistry
		Clinical Chemistry and Laboratory Medicine
		American Journal of Obstetrics and Gynecology
		Obstetrics and Gynecology
		Prenatal Diagnosis
		The Journal of Obstetrics and Gynaecology Research
		Annals of Hematology

Grants and Consultancy

Name	Project Title	Funding Source	Start Date (dd/mm/yyyy)	End Date (dd/mm/yyyy)	Amount (HK$)
Dennis Lo	Genomic Approaches for Predicting Severity of Organ Dysfunction and Outcomes in Sepsis: A Prospective Cohort Study in Adult Critically Ill Patients with Sepsis	The Chinese University of Hong Kong Faculty of Medicine – Faculty Innovation Award	01/01/2020	31/12/2022	750,000
Dennis Lo Rossa Chiu	Plasma DNA as a Platform Technology for Cancer Detection	The Chinese University of Hong Kong Focused Innovations Scheme C	01/12/2016	30/11/2021	2,093,500
	Plasma DNA as a Platform Technology for Cancer Detection	Research Grants Council – Theme-based Research Scheme	01/12/2016	30/11/2021	28,570,000
	Centre for Research into Circulating Fetal Nucleic Acids	Research Grants Council – Theme-based Research Scheme	01/01/2016	31/12/2020	37,286,000
	Centre for Research into Circulating Fetal Nucleic Acids	The Chinese University of Hong Kong Focused Innovations Scheme C	01/01/2016	31/12/2020	5,217,900

Publications

A. Journal Papers

1. Sin STK, Jiang P, Deng J, Ji L, Cheng SH, Dutta A, Leung TY, Chan KCA, Chiu RWK, Lo YMD. Identification and characterization of extrachromosomal circular DNA in maternal plasma. *Proceedings of the National Academy of Sciences*. 2020;117(3):1658-1665. doi:10.1073/pnas.1914949117.

2. Han DSC, Ni M, Chan RWY, Chan VWH, Lui KO, Chiu RWK, Lo YMD. The biology of cell-free DNA fragmentation and the roles of DNASE1, DNASE1L3, and DFFB. *The American Journal of Human Genetics*. 2020;106(2):202-214. doi:10.1016/j.ajhg.2020.01.008.

3. King AD, Woo JKS, Ai QY, Mo FKF, So TY, Lam WKJ, Tse IOL, Vlantis AC, Yip KWN, Hui EP, Ma BBY, Chiu RWK, Chan ATC, Lo YMD, Chan KCA. Early detection of cancer: Evaluation of MR imaging grading systems in patients with suspected nasopharyngeal carcinoma. *American Journal of Neuroradiology*. 2020;41(3):515-521. doi:10.3174/ajnr.A6444.

4. Lam WKJ, Ji L, Tse OYO, Cheng SH, Jiang P, Lee PHP, Lin SV, Hui EP, Ma BBY, Chan ATC, Chan KCA, Chiu RWK, Lo YMD. Sequencing analysis of plasma Epstein-barr virus DNA reveals nasopharyngeal carcinoma-associated single nucleotide variant profiles. *Clinical Chemistry*. 2020;66(4):598-605. doi:10.1093/clinchem/hvaa027.

5. Lo YMD, Chiu RWK. Racing towards the development of diagnostics for a novel Coronavirus (2019-nCoV). *Clinical Chemistry*. 2020;66(4):503-504. doi:10.1093/clinchem/hvaa038. (Editorial)

6. Jiang P, Sun K, Peng W, Cheng SH, Ni M, Yeung PC, Heung MMS, Xie T, Shang H, Zhou Z, Chan RWY, Wong J, Wong VWS, Poon LC, Leung TY, Lam WKJ, Chan JYK, Chan HLY, Chan KCA, Chiu RWK, Lo YMD. Plasma DNA end motif profiling as a fragmentomic marker in cancer, pregnancy and transplantation. *Cancer Discovery*. 2020;10(5):CD-19-0622. doi:10.1158/2159-8290.CD-19-0622.

7. Chiu RWK, Dutta A, Henssen AG, Lo YMD, Mischel P, Regenberg B. What is extrachromosomal circular DNA and what does it do? *Clinical Chemistry*. 2020;66(6):754-759. doi:10.1093/clinchem/hvaa096. (Editorial)

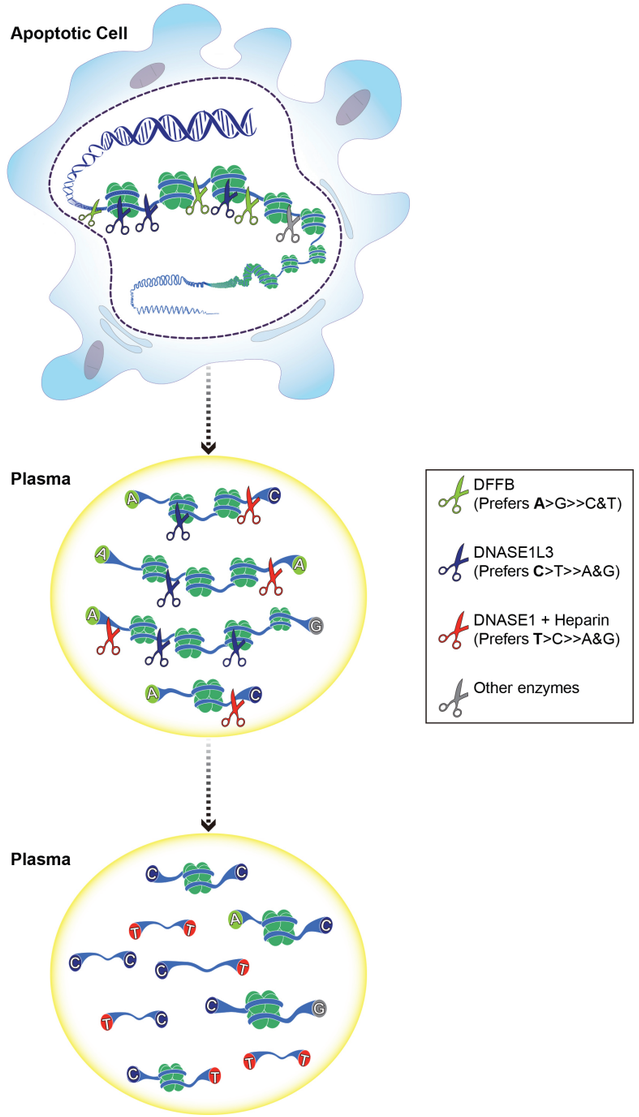
8. Chiu RWK. Fastidious detection of circulating tumor DNA mutations in residual breast cancer disease for ultimate analytical sensitivity and specificity. *Clinical Chemistry*. 2020;66(7):866-867. doi:10.1093/clinchem/hvaa068. (Editorial)

9. Jiang P, Xie T, Ding SC, Zhou Z, Cheng SH, Chan RWY, Lee WS, Peng W, Wong J, Wong VWS, Chan HLY, Chan SL, Poon LCY, Leung TY, Chan KCA, Chiu RWK, Lo YMD. Detection and characterization of jagged ends of double-stranded DNA in plasma. *Genome Research*. 2020;30(8):1144-1153. doi:10.1101/gr.261396.120.

10. Lo YMD, Lam WKJ. Towards multi-cancer screening using liquid biopsies. *Nature Reviews Clinical Oncology*. 2020;17(9):525-526. doi:10.1038/s41571-020-0404-0. (Editorial)

11. Ma ML, Yakovenko S, Zhang H, Cheng SH, Apryshko V, Zhavoronkov A, Jiang P, Chan KCA, Chiu RWK, Lo YMD. Fetal mitochondrial DNA in maternal plasma in surrogate pregnancies: Detection and topology. *Prenatal Diagnosis*. Published online November 12, 2020:pd.5860. doi:10.1002/pd.5860. (Epub ahead of print)

12. Chan RWY, Serpas L, Ni M, Volpi S, Hiraki LT, Tam LS, Rashidfarrokhi A, Wong PCH, Tam LHP, Wang Y, Jiang P, Cheng ASH, Peng W, Han DSC, Tse PPP, Lau PK, Lee WS, Magnasco A, Buti E, Sisirak V, AlMutairi N, Chan KCA, Chiu RWK, Reizis B, Lo YMD. Plasma DNA profile associated with DNASE1l3 gene mutations: Clinical observations, relationships to nuclease substrate preference, and in vivo correction. *The American Journal of Human Genetics*. 2020;107(5):882-894. doi:10.1016/j.ajhg.2020.09.006.



Model of Cell-free DNA Fragmentation

DNA fragmentation factor subunit beta (DFFB), deoxyribonuclease 1 like 3 (DNASE1L3) and other intracellular enzymes form newly released cell-free DNA (cfDNA) that is A-end enriched. In plasma, DNASE1L3 generates the predominantly C-end enriched cfDNA seen in the typical profile via its extracellular activity. Deoxyribonuclease 1 (DNASE1) with the help of heparin and endogenous proteases can further digest cfDNA into T-end fragments in plasma.

Source: Han DSC, Ni M, Chan RWY, Chan VWH, Lui KO, Chiu RWK, Lo YMD. The biology of cell-free DNA fragmentation and the roles of DNASE1, DNASE1L3, and DFFB. The American Journal of Human Genetics. 2020;106(2):202-214. doi:10.1016/j.ajhg.2020.01.008.