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REVIEW ARTICLE

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Genomic studies of Hodgkin lymphoma using circulating cell-free DNA

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Abstract

The revolutionary finding of cell free DNA (cfDNA) circulating in the bloodstream had a huge impact on the development of non-invasive prenatal testing (NIPT) (obstetrics) and liquid biopsies (oncology). The latter, combined with the sequencing of tumor DNA-containing cfDNA, have been widely applied in cancer research, demonstrating the potential of these techniques to improve prognostication and guide individualized treatment strategies. During routine NIPT analysis of more than 88,000 pregnant women performed in our institution, 14 abnormal genomic profiles suggestive of maternal tumor have been identified. Interestingly, one patient was further diagnosed with classic Hodgkin lymphoma (cHL), a tumor characterized by a low content (<2%) of neoplastic cells in tumor mass. To examine whether circulating cfDNA can be informative about genomic imbalances in neoplastic Hodgkin/Reed-Sternberg (HRS) cells, we performed a pilot study of nine prospective cHL cases. This study showed that genomic profiles of cfDNA correspond to the profiles of HRS cells. To get further insights into the genome of cHL, a large study on cfDNA from 177 prospective cHL patients was subsequently established. Based on ultra-low pass sequencing of cfDNA from this cohort, we built a comprehensive catalog of genomic abnormalities, as well as their frequencies and patterns. Besides the known recurrent imbalances, such as gain/amplification of 2p16/*REL-BCL11A* and 9p24/*JAK2-CD274-PDCDLG2*, novel recurrent abnormalities were identified in cHL. Altogether, we have provided evidence that cHL is characterized by consistent and recurrent genomic imbalances and we have shown the potential of genomic profiling of cfDNA as a novel and non-invasive tool in the diagnosis and follow up of cHL patients.

Key words: circulating cell-free DNA, liquid biopsy, genomic profiling, DNA sequencing, Hodgkin lymphoma

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Introduction

Cancer is a genetic disorder driven by accumulating genetic abnormalities, including oncogenic DNA mutations and chromosomal defects in somatic cells [1]. Acquired aberrations result in deregulated expression of involved genes (oncogenes and tumor suppressor genes) and consequently lead to malignant transformation of affected cells. To date, more than 500 driver genes, activated or inactivated by genetic and epigenetic aberrations, are known to be involved in carcinogenesis.

The major source of neoplastic material used for cancer research have been traditional biopsies taken for diagnostic purposes. Sampling tumor tissue, however, is invasive, associated with procedural risks, sampling errors and the potential inability to capture special heterogeneity in the setting of multifocal disease. Over the last two decades, another fraction of tumor DNA known as circulating cell-free tumor DNA, has attracted attention of the scientific community. Circulating cell-free DNA (ccfDNA), first reported by Mandel and Metais in 1948 [2], comprises genome fragments that float freely through the bloodstream. This

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fraction of DNA became particularly interesting when it was discovered that fetal DNA, mainly originating from placental trophoblast cells, can cross the placenta and be identified in the plasma of pregnant women [3]. This revolutionary finding launched the era of non-invasive prenatal testing (NIPT), a screening of fetal genome in maternal ccfDNA using next generation sequencing [4, 5]. Interestingly, it appears that tumors also shed DNA to the circulation and that ccfDNA of patients with cancer contains circulating tumor DNA (ctDNA) which can serve as a 'liquid biopsy' [6].

The liquid biopsy' terms a minimally invasive technique of extraction of tumor-derived material (circulating tumor cells, as well as DNA, RNA, microRNA and proteins) from non-solid biological tissue like blood, saliva and urine, for further genomics and proteomics analysis in patients with cancer. Circulating cfDNA fragments are stable in circulation, but their half-life is relatively short. DNA is shed primarily through apoptosis and necrosis, but also through secretion. ccfDNA in the blood of healthy individuals is present at low levels, but is increased in oncological patients, being shed from both normal and malignant cells.

Given that ctDNA harbours cancer-specific modifications, including point mutations and chromosomal copy number abnormalities (CNA) (gains and losses), and sampling can be easily done at each stage of disease, interest of non-invasive diagnostic of tumors has grown enormously [7–12].

Detection of incipient tumors in pregnant women by screening of plasma cell-free DNA

NIPT has been recently widely applied in routine practice. Since then, occasional reports of discordant NIPT results indicating for detection of tumor-derived CNAs in plasma cfDNA of pregnant women have been published [13, 14]. In University Hospital Leuven, Belgium, NIPT was introduced in 2013. Over 6.5 years, more than 88,000 pregnant women underwent NIPTing. Application of the Genome-wide Imbalance Profile sequencing pipeline (GIPSeq) developed in our institution allows unbiased detection of CNA in cfDNA [5].

Upon analysis of the 88,294 NIPT results, 14 women without a previous medical history of cancer were identified with a GIPSeq result suggesting an occult maternal malignancy [15, 16]. The patients underwent further clinical investigations, including whole-body diffusion-weighted magnetic resonance imaging (WB-DWI MRI), which led to identification of maternal tumors in 12 pregnant women. In one case, the cancer diagnosis (primary mediastinal large B-cell lymphoma) was made three years after the detection of an aberrant result by NIPT/genome-wide imbalance profile sequencing (GIPSeq). More than 66% of tumors were of hematological origin, mainly Hodgkin (75%) and non-Hodgkin lymphomas (25%). One-third (33.3%) of cancers were solid tumors (ovarian and breast carcinoma, high grade osteosarcoma). In several cases with confirmed cancer diagnoses, further genetic analyses, using array comparative genomic hybridization (aCGH), fluorescence *in situ* hybridization (FISH), or low-pass whole genome sequencing (0.1×) of biopsy DNA, evidenced that the ccfDNA-detected CNAs represent genomic modifications of tumor DNA. In two patients with an aberrant GIPSeq profile and lack of suggestive malignancy, clinical follow-up was advised. Although the detected maternal malignancies were occult, it is possible that the symptoms of cancer (such as fatigue, nausea, abdominal discomfort, and vaginal blood loss) easily could have been misinterpreted as physiological gestational symptoms.

Genome-wide studies of classic Hodgkin lymphoma (cHL) using plasma cfDNA

Initial discovery and pilot study of cHL

The identification of genomic abnormalities in plasma cfDNA of a pregnant woman further diagnosed with early-stage cHL (Ann Arbor stage IIA) [15] was unexpected, because cHL is hallmarked by a minority of neoplastic cells (0.1–2%) amidst an overwhelming majority of non-malignant immune cells [17]. For this reason, detection of HRS cell-derived DNA in plasma was intriguing. The rarity of neoplastic cells in cHL tumor mass complicates the analysis of somatic genetic alterations in this malignancy and hampers the elucidation of its pathogenesis, biology and diversity [18]. Genetic features of HRS cells has been initially studied in HL-derived cell lines or sporadically in original tumor samples by FISH [19–21]. More recently, challenging attempts were undertaken to investigate genomics of cHL using HRS cells isolated by laser microdissection or cell sorting after whole genome DNA amplification [22–28]. The discovery that HRS-derived DNA is present in a patient's plasma opened a new avenue to remotely sample the HRS genome in a minimally invasive way and to impact HL research.

To validate our initial observation of HRS cells-related chromosomal imbalances in patient's ccfDNA [15], we undertook a pilot study of nine prospective cases of biopsy-proven nodular sclerosis Hodgkin lymphoma, which is the most frequent subtype of cHL [29]. Eight cases were recruited at time of diagnosis and one at first relapse. The patients presented at stage IIA (7/9) and IVB (2/9) disease. The collected ccfDNA was subjected to low-coverage massive parallel sequencing. The downstream analysis detected genomic gains and losses in eight cases. The most frequent gains, found in ≥5 cases, affected 2p (n =7), 3q, 5q and 9p (n =5), while recurrent losses detected in 4–5 patients involved 1p, 6q, 7q, 9q, 10q, 11q, 13q and 22q. Some of these imbalances were extensively validated by FISH with probes representing affected chromosomal regions on HRS cells from either cytogenetic harvests or

biopsy samples. All patients, including the pregnant woman, underwent chemotherapy with or without the involvement of node radiotherapy. All responded, as shown by early clinical evaluation. Importantly, GIPSEq analysis performed in subsequent samples taken between days 15 and 43 after treatment initiation, showed normal genomic profiles in all cases, confirming clinical observation of complete metabolic remission. To gain insights into mechanisms underlying an abundant release of ccfDNA by scarce HRS cells, we analyzed the expression of cell cycle indicator Ki67 and cleaved caspase 3 by immunohistochemistry (IHC) in all nine cases. Coexpression of both molecules in HRS cells and the presence of necrosis in biopsy samples suggest a high turnover of neoplastic HRS cells in cHL. The finding of most complex genomic profiles in both patients with advanced disease (stage IVB) suggests an association between the HRS cell burden and the level of HRS cell derived DNA in ccfDNA. These pilot data indicates for potential of ccfDNA profiling as a novel non-invasive tool for diagnosis and monitoring of the disease.

Landscape of copy number variations in cHL

After the technical proof-of-principle study proving that circulating cfDNA from HL patients contains circulating tumor DNA (ctDNA) from HRS cells, and that the DNA can be profiled by massive parallel sequencing [29], we established a large collaborative project of KU Leuven and the Lymphoma Study group (LYSA) of cHL on cell-free DNA [30]. The project aimed at screening of genomic imbalances in HRS cells using ultra-low pass sequencing of cfDNA in a large series of cHL patients. Between 2014 and 2018, we prospectively collected plasma cfDNA from 177 new cHL patients (mostly early stage). To profile genomic imbalances, cfDNA samples were sequenced at low coverage (0.26×) and subjected to downstream bioinformatic analysis. In addition, we attempted to estimate the clonal fraction of cfDNA (presumably derived from HRS cells) and analyze a possible correlation of ctDNA with the known prognostic risk factors and tumor burden, and monitor the CNA evolution after treatment initiation.

The subsequent genome-wide analysis of cfDNA from this cohort allows the construction of a comprehensive catalog of the types of CNA, their frequencies and patterns in cHL. More than 90% of patients (164/177) exhibited CNA in cfDNA. The remaining cases displayed balanced CN profiles at diagnosis, likely due to a low content of tumor fractions, below the detection threshold of the applied assay. CNA was detectable in 94% (140/149) and 92% (22/24) of cases with stage I–II disease and III–IV disease, respectively. Most cases (152/164) revealed complex profiles with five or more CNAs. The most prevalent gains affected 2p16 (69%), 5p14 (50%), 12q13 (50%), 9p24 (50%), 5q (44%), 17q (43%) and2q (41%). Genomic gain or amplification usually impacts expression of harbored genes, as show in the case of 2p16 and 9p24 amplification targeting the known lymphoma-related driver genes, *REL/BCL11A* and *JAK2/ /CD274/PDCDLG2,* respectively. Other candidate genes (unmutated or mutated) deregulated by genomic gains in cHL include *IL-10* (1q32), *XPO1* (2p15), *NFKB1* (4q24), *CASP6* (4q25), *CSF1R* (5q34) and *STAT6* (12q13). Genomic losses most frequently targeted 13q (57%), 6q25q27 (55%), 4q35 (50%), 11q23 (44%) and 8p21 (43%). The known tumor suppressor genes affected by losses of 6q and 13q include *TNFAIP3* and *FOXO1*, respectively. In addition, we identified loss of 3p13p26 and 12q21q24, and gain of 15q2-q26 as novel recurrent CNA in cHL.

FISH was used to validate genomic gains or losses detected in cfDNA. Using DNA probes representing the gained/lost regions of interest which were applied on stored cytogenetic harvests from 10 biopsies, we confirmed the imbalances in HRS cells of 9/10 cases. Notably, most CNAs detected in our series have been already reported in smaller studies on purified HRS cells or on tumor sections, but their occurrence and frequencies varied in different studies [19, 20, 22–25].

Our study, the largest study on CNA in cHL to date, provides a comprehensive landscape of CNAs with a reliable rating of their relative frequency. We found that occurrence of gain of 2p16, 5p14 and 12q13 and loss of 6q25 and 13q is similar to gain/amplification of 9p24 affecting *CD274/PD-L1* and *PDCDLG2/PD-L2*. The latter aberration is an important predictor for favorable outcome after anti-PD-1 driven immune checkpoint blockade, e.g. nivolumab [15, 31]. Significantly, we found loss of 3p13-p26 and 12q21-q24 and gain of 15q21-q26 as three novel non-random CNAs in cHL. Postulated candidate oncogenes harbored by chromosome 15q include several genes promoting cell proliferation and acting downstream of NF-κB, JAK-STAT and cytokine receptor signaling (*AKAP13, FES, STRA6* and *PIAS1*) and genes showing anti-apoptotic activity (*MAP2K1* and *MAP2K5*). Candidate tumor suppressor genes mapped on 3p and 12q include negative regulators of cell proliferation (*RHOA, VHL* and *TLR9*/3p, *SOCS2*/12q) and apoptosis (*TRAIP*/3p, *APAF1*/12q). Further investigations studying whether and how these novel CNAs are implicated in the pathogenesis of cHL are required.

In addition, we found that ctDNA concentration at diagnosis was associated with HRS cell burden and tumor mass volume. Notably, ctDNA and related CNA rapidly diminished upon treatment initiation, while persistence of CNA correlated with an increased probability of relapse.

Our study showed that cfDNA could be a gateway to the genome of HRS cells and serve as substrate for the monitoring of early disease response.

Altogether, we have provided evidence that cHL is characterized by consistent and recurrent genomic imbalances. The aberrations were detected by massive parallel sequencing of ccfDNA from 93% of patients with early and

advanced stages of newly diagnosed tumors. Besides the known recurrent gains and losses, loss of 3p13p26 and 12q21q24 and gain of 15q21q26 were identified as novel recurrent CNA in cHL. Genomic aberrations were correlated with disease burden and evolution. The rapid normalization of ccfDNA profiles on therapy initiation suggests a potential role for ccfDNA profiling in early response monitoring. These findings confirmed our pilot studies, and showed the potential of genomic profiling of cfDNA as novel and non-invasive tool at diagnosis and follow up of cHL patients. Moreover, our discoveries and those by other groups show several new possibilities for exploring the molecular pathogenesis of HL, as illustrated by recent publications [32–35] and have potential implications for the prospective clinical development of biomarkers and precision therapy for this tumor.

Authors' contributions

All authors contributed in the discussed research and writing of the article

Conflict of interest

The authors have no competing interests to declare.

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Ethics

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments; Uniform Requirements for manuscripts submitted to Biomedical journals.

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