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ORIGINAL ARTICLE

Genomewide copy number alteration screening of circulating plasma DNA: potential for the detection of incipient tumors

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Background: Early cancer diagnosis might improve survival rates. As circulating tumor DNA (ctDNA) carries cancer-specific modifications, it has great potential as a noninvasive biomarker for detection of incipient tumors.

Patients and methods: We collected cell-free DNA (cfDNA) samples of 1002 elderly without a prior malignancy, carried out whole-genome massive parallel sequencing and scrutinized the mapped sequences for the presence of (sub)chromosomal copy number alterations (CNAs) predictive for a malignancy. When imbalances were detected, 6-monthly clinical follow-up was carried out.

Results: In 3% of participants chromosomal imbalances were detected. Follow-up analyses, including whole-body MRI screening, confirmed the presence of five hematologic malignancies: one Hodgkin lymphoma (HL), stage II; three non-HL (type chronic lymphocytic leukemia, Rai I–Binet A; type SLL, stage III; type mucosa-associated lymphoid tissue, stage I) and one myelodysplastic syndrome with excess blasts, stage II. The CNAs detected in cfDNA were tumor-specific. Furthermore, one case was identified with monoclonal B-cell lymphocytosis, a potential precursor of B-cell malignancy. In 24 additional individuals, CNAs were identified but no cancer diagnosis was made. For 9 of them, the aberrant cfDNA profile originated from peripheral blood cells. For 15 others the origin of aberrations in cfDNA remains undetermined.

Conclusion(s): Genomewide profiling of cfDNA in apparently healthy individuals enables the detection of incipient hematologic malignancies as well as clonal mosaicism with unknown clinical significance. CNA screening of cellular DNA of peripheral blood in elderly has established that clonal mosaicism for these chromosomal anomalies predicts a 5- to 10-fold enhanced risk of a subsequent cancer. We demonstrate that cfDNA screening detects CNAs, which are not only derived from peripheral blood, but even more from other tissues. Since the clinical relevance of clonal mosaics in other tissues remains unknown, long-term follow-up is warranted. Taken together, this study demonstrates that genomewide cfDNA analysis has potential as an unbiased screening approach for hematological malignancies and premalignant conditions.

Key words: circulating plasma DNA, liquid biopsies, copy number alterations, early cancer detection

Introduction

With the increasing ageing population and concurrent expanding cancer incidence rates, a heavier focus on early cancer detection is required to complement new and improved treatments. As circulating tumor DNA (ctDNA) carries cancer-specific modifications, including DNA mutations and copy number alterations (CNAs), interest in its use as a noninvasive biomarker for detection of incipient tumors has been growing vastly. ctDNA represents a subset of the plasma cell-free DNA (cfDNA), the latter being normally present at low levels in the blood of healthy individuals and suggested to be primarily derived from normal cells of the hematopoietic lineage [1]. Most reports on ctDNA as a cancer marker focused on the detection of specific point mutations. Owing to its targeted nature, this approach only provides a partial glimpse of the tumor genome in plasma and must be customized for each patient based on a known tumor mutation profile. This is not feasible for screening purposes. CNAs, such as point mutations, are common for a large proportion of cancer types. About 90% of solid tumors and 50% of blood-related cancers are aneuploid and have somatic CNAs [2]. Furthermore, genomewide sequencing of plasma-derived DNA from cancer patients enabled the detection of tumor-associated copy number profiles in selected tumors prone to CNAs [3, 4].

We recently established a genomewide cfDNA analysis pipeline, coined Genomic Imbalance Profiling from cfDNA SEQuencing (GIPseq) for noninvasive prenatal testing, allowing genomewide detection of fetal and maternal chromosomal imbalances. Using this pipeline in ∼50 000 asymptomatic pregnant women we incidentally identified 8 malignancies upon the detection of cancer-like CNAs in cfDNA [4, 5] (unpublished data). Furthermore, we demonstrated that this approach allows detecting CNAs in cfDNA of patients with solid and hematological tumors [4, 6]. Building on these observations, we aimed to test this noninvasive unbiased genomewide GIPseq as a tool for cancer screening in people with an elevated risk of developing cancer. Plasma cfDNA of a large cohort of cancer-free, elderly individuals was screened for the presence of chromosomal aberrations that might reveal the presence of a malignancy. Participants were subjected to a 6-month clinical follow-up when aberrancies were detected. Furthermore, CNAs, present in cfDNA in otherwise healthy elderly, were cataloged, creating a unique map of anomalies in cfDNA in this ageing population.

Patients and methods

Participants

Aged people, 64 years and older, were recruited via senior societies (76% of participants) or via University Hospitals Leuven (Departments of Internal Medicine or Anaesthesiology, 24% of participants). Participants with a cancer history or reporting an early diagnosis were excluded. Peripheral blood (8–9 ml) was sampled in Streck tubes (Streck, Omaha). When a deviating GIPseq profile was detected, a second blood sample was taken for independent confirmation. Samples were collected between October 2015 and November 2017. The study was approved by the ethics committee of University Hospitals Leuven (S/55513). Written informed consent was obtained from all participants.

Cell-free plasma and genomic DNA extraction

Plasma was isolated through a standard, two-step centrifugation procedure. cfDNA was extracted from 2 to 4 ml plasma, using, respectively, the QIAamp circulating nucleic acid Kit (Qiagen, Hilden, Germany; manual extraction) or the Maxwell HT ccfDNA kit (Promega, Madison; automated procedure). Median cfDNA concentration in plasma was 5.5 ± 0.5 ng/ml (median $\pm$ SEM). Genomic DNA from blood cells or formalin-fixed paraffin-embedded (FFPE) tumor biopsies was extracted after macrodissection using the Qiagen Blood and Tissue kit. Following sonication (Covaris M220), samples were electrophoretically run on the Agilent 2100 Bioanalyzer system to verify fragmentation. The target DNA fragment size for library preparation was 150–200 bp.

Genomewide imbalance profile sequencing

DNA sequencing libraries were prepared using the TruSeq DNA Nano kit or Illumina ChipSeq kit (Illumina, San Diego). Whole-genome sequencing was carried out on a HiSeq2500 or 4000 (Illumina) using a V4 flowcell generating single-end 36 or 50 bp reads. Mean netto read count per sample was 9.6×10^6 reads. For Genomewide Imbalance Profiling (GIPseq), our previously described bioinformatics pipeline was applied, using genomewide parameters (quality score, QS), chromosomal parameters (*z*- and *zz*-score) and subchromosomal parameters [7]. QS was calculated as the standard deviation of the *z*-scores of all autosomes excluding the chromosomes with highest and lowest *z*-scores. A GIPseq profile was scored 'normal' when $QS < 2$ and no significant gains or losses were present across one of the chromosomes (i.e. $|z\text{-score}| < 3.0$ and $|zz\text{-score}| < 3.0$). The GIPseq profile was called 'aberrant' when $QS<2$, or when $QS < 2$ and one or more individual chromosomes were having $|z\text{-score}| \geq 3.0$ and $|zz\text{-score}| \geq 3.0$. Constitutional copy number variations were filtered out and GIPseq profiles were assessed by two independent researchers for their relevance as cancer-related CNAs, consulting online databases of chromosome aberrations in oncology [8, 9].

Array comparative genomic hybridization and fluorescent *in situ* hybridization

Array comparative genomic hybridization (aCGH) analysis was done as before [10], using the 4×180K CytoSure Syndrome Plus Leuven Design microarray or the 8 ×60K CytoSure ISCA v2 microarray and analyzed with the CytoSure Interpret Software (OGT, Oxford, UK). Fluorescent *in situ* hybridization (FISH) was carried out according to standard procedures. DNA probes are described in supplementary Table S1, available at *Annals of Oncology* online.

Peripheral blood analyses

Analysis of hematological parameters (including cell counts and cytology), clinical biochemistry and protein electrophoresis of peripheral blood was done at the Laboratory Medicine of University Hospitals Leuven following standard procedures.

Whole-body MRI screening

When two sequential plasma samples independently confirmed the presence of an aberrant GIPseq profile, subjects were sent for 3 Tesla whole-body diffusion Weighted MRI (WB-DWI MRI) imaging to be screened for the presence of cancer-like lesions, as described before [11]. We choose WB-DWI MRI due to its absent ionizing radiation, *in-house* experience, and similar diagnostic performance compared with PET/CT for detecting primary and metastatic malignancies [5, 12]. As WB-DWI MRI was designed for diagnostic purposes rather than for screening purpose and from ethical considerations, we applied a low threshold for detection of possible tumor and incidental findings favoring high sensitivity for lesion detection over specificity. Detected lesions were examined via dedicated investigations in a second stage. When no lesions, indicative of

malignancy, were observed, the participant was referred for two additional WB-DWI MRIs with a 3-month time interval.

Statistical analysis

Two-tailed *t*-testing, assuming equal variances in unpaired samples was used to compare plasma cfDNA concentrations among study subgroups. Fisher's exact test was applied to determine the association between the frequency of chromosomal aberrations in plasma cfDNA and the age of the index.

Results

A subset of elderly people has chromosomal imbalances in plasma cfDNA

Plasma cfDNA of 386 men and 616 women, with a median age of 72 years (range 64–96 years), was subjected to GIPseq analyses. Ninety-five percent of participants had a normal GIPseq profile. For 5% of cases, an aberrant profile was detected and a second blood sample was analyzed (Figure 1A). One participant refused secondary sampling. Eighteen other samples, for which aberrations could not be confirmed upon second sampling, were reclassified as normal. In the remaining 30 cases (20 men and 10 women), representing 3% of the study population, the GIPseq profile of the second blood sample reproduced the CNAs found in the first sample. There was no association between the detection of chromosomal aberrations in plasma cfDNA and the age of the participants (frequency of chromosomal aberrations: 2.7% in people aged 64–69 years; 3.2% in people aged 70–79; and 3.3% in the 80+ group; $P > 0.5$). Cell-free DNA concentrations did not significantly differ between participants with a reproducible aberrant GIPseq profile and those with a normal score (mean ± SEM: 9.6 ± 0.5 ng/ml plasma for 'normal' cases; 6.3 ± 0.6 ng/ml for 'aberrant' cases; $P = 0.66$).

Two types of chromosomal anomalies were observed upon cfDNA profiling: (i) genomewide aberrations with gains and/or losses on multiple chromosomes, affecting whole chromosomes and chromosome arms, characterized by an elevated GIPseq QS-score ($n = 12$ cases; Figure 2A and Table 1) and (ii) isolated aberrations in which only one chromosome or chromosomal segment was affected, with an overall QS < 2 ($n = 18$ cases; Figure 2B and Table 2). Isolated anomalies encompassed: a segmental del(3p), del(5q), del(9q), del(20q) or del(22q); segmental gains on 9p and 9q, or 14q, or a trisomy 12 or 15.

For the 30 participants with aberrant GIPseq profiles, aCGH on peripheral blood cell DNA was carried out to investigate the possibility of an underlying hematological malignancy. Furthermore, general peripheral blood parameters (including cell count and cytology) were examined and subjects were screened via WB-DWI MRI for the presence of potentially malignant lesions. The results are summarized in Tables 1 and 2 and Figure 1B.

Genomewide chromosomal imbalances in plasma cfDNA may point to an underlying (pre)malignancy

Five cases, with genomewide chromosomal imbalances in cfDNA, were diagnosed with cancer or a premalignant condition upon clinical follow-up (Table 1): one non-Hodgkin lymphoma (NHL)-type chronic lymphocytic leukemia (CLL) (Rai I–Binet A); one classical Hodgkin lymphoma (HL) (stage II); one myelodysplastic syndrome (MDS) with excess blasts (type 1, IPSS 2); one NHL-type mucosa-associated lymphoid tissue (MALT; stage I) and one high-count monoclonal B-cell lymphocytosis (MBL), a premalignant condition at risk for progression to B-cell malignancy. Molecular investigations in biopsies from the indices, confirmed that the CNAs detected in cfDNA were, at least partially, derived from tumor DNA. One additional individual, having an isolated trisomy 12 in cfDNA, was eventually identified with NHL-type small lymphocytic lymphoma (SLL; stage III), with the trisomy 12 present as a low-grade mosaicism in peripheral blood cells. Supplementary document S1, available at *Annals of Oncology* online, comprehensively describes follow-up investigations in these six cases. Figure 3 depicts the results of the molecular and clinical examinations for the NHL, type CLL. Analogous results for the HL, MDS, MALT, SLL and MBL cases are shown in supplementary Figures S1–S5, available at *Annals of Oncology* online.

In the remaining 24 individuals, of whom 7 presenting with multiple and 17 having isolated chromosomal anomalies in cfDNA, no definitive cancer diagnosis was made (Tables 1 and 2 and Figure 1B). For eight of them, no complete clinical follow-up was carried out, either due to the participant's voluntary withdrawal, and hence preventing a final diagnosis, or because the abnormalities in cfDNA were believed not to be cancer-related (indicated with an asterisk, Tables 1 and 2). For one such case (case 22022016-03), again having genomewide CNAs in cfDNA but not in peripheral blood DNA, WB-DWI MRI revealed supraclavicular adenopathies, being suggestive of a lymphoma. No confirmatory diagnosis was made since this woman refused subsequent clinical investigations because of her age. Additionally, one male (case 30032017-02) having genomewide aberrations in cfDNA, was independently of our investigations, diagnosed with a lip cancer, but no correlation was found between the imbalances in cfDNA and the copy number profile in tumor DNA (data not shown).

About one-third of chromosomal imbalances in plasma cfDNA of asymptomatic individuals originates from peripheral blood cells

In 9 of the 24 (37.5%) individuals without a cancer diagnosis, the aberrant cfDNA species were shown, upon aCGH, FISH and/or low-pass sequencing of peripheral blood DNA, to originate from a low-grade mosaicism in peripheral blood cells, but without fulfilling the criteria for a hematological diagnosis. It concerned cases with an isolated anomaly on chromosome 3p, 5q, 15, 20q or 22q (Table 2). GIPseq profiles of each of these imbalances, and confirmatory analyses in peripheral blood cells, are shown in supplementary Figures S6–S8, available at *Annals of Oncology* online. For 15 out of the 24 undiagnosed cases (62.5%), the chromosomal imbalances observed in cfDNA were not detected in peripheral blood DNA.

Four incidental cancer diagnoses were made despite a normal GIPseq profile

Four out of the 953 cases, with a normal GIPseq profile, were incidentally diagnosed with one of the following cancers: prostate adenocarcinoma (stage pT3aN0M0; diagnosed 5 months after GIPseq analysis), bronchus adenocarcinoma (stage cT3N3M1b;

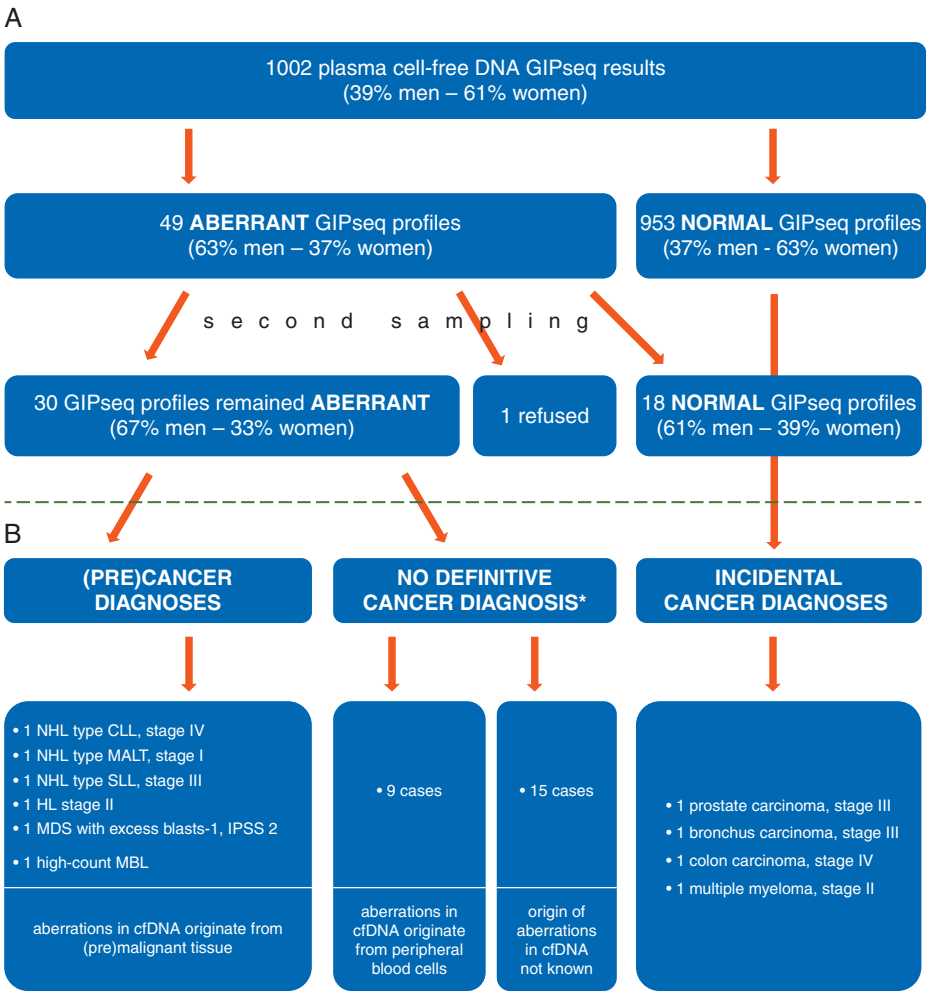

Figure 1. Flow chart of analyses and clinical outcome. (A) Flow chart of plasma sampling and subsequent GIPseq profiling. (B) Clinical follow-up results of the 30 cases with a reproducible GIPseq profile. *For 8 of the 24 cases, for whom no cancer diagnosis was made, clinical follow-up data for either whole-body diffusion weighted MRI examination(s) or general hematological analysis are missing, either due to withdrawal of the participant during the course of the study or because the observed abnormalities in cfDNA were believed not to be cancer-related aberrations. CLL, chronic lymphocytic leukemia; HL, Hodgkin lymphoma; MALT, mucosa-associated lymphoid tissue; MBL, monoclonal B-cell lymphocytosis; MDS, myelodysplastic syndrome; NHL, non-Hodgkin lymphoma; SLL, small lymphocytic lymphoma.

diagnosed 35 days after GIPseq analysis), liver-metastasized colon adenocarcinoma (stage pT4N1cM1; 1 month after GIPseq analysis) or multiple myeloma IgG kappa (stage 2; 27 days after GIPseq testing). For the prostate adenocarcinoma, additional plasma analysis at the time of diagnosis again showed a normal GIPseq profile (supplementary Figure S9A, available at *Annals of Oncology* online). Whole-genome shallow sequencing was carried out on FFPE biopsy DNA of the solid tumors, and FISH analysis was done on bone marrow plasma cells from the multiple myeloma patient. The genomes of the prostate, bronchus and colon adenocarcinomas and that of the plasma cell tumor displayed noticeable focal and/or broad CNAs (supplementary Figures S9–S11, available at *Annals of Oncology* online).

Discussion

We here report unique data on the potential of genomewide CNA detection in plasma cfDNA for cancer screening in a large cohort of individuals without a cancer history. Five out of the 1002 screened participants were diagnosed with a hematological cancer (3 NHL, 1 HL and 1 MDS-EB1), with the CNAs in cfDNA originating, at least partially, from malignant cells. This frequency of cancer detection (0.5%) is three to four times lower than the expected prevalence of all cancers together, but is approximately four times higher than reported incidence rates for NHL, HL and MDS combined, in people aged 65 years and above [13–15]. Importantly, for two of our participants, the malignancy was not yet reflected in abnormal blood parameters, pointing to the potential of GIPseq for presymptomatic detection of these hematological cancers. Whereas current incidence numbers are based on the usual clinical presentations and conventional diagnostic criteria for hematologic malignancies, genetic screening approaches for clonal DNA mosaicisms in blood may result in a higher detection rate: especially in indolent disorders, the observation of clonality can lead to diagnosis of a malignancy before clinical signs or symptoms bring it to attention. The preponderance of hematological diagnoses might be plausible when

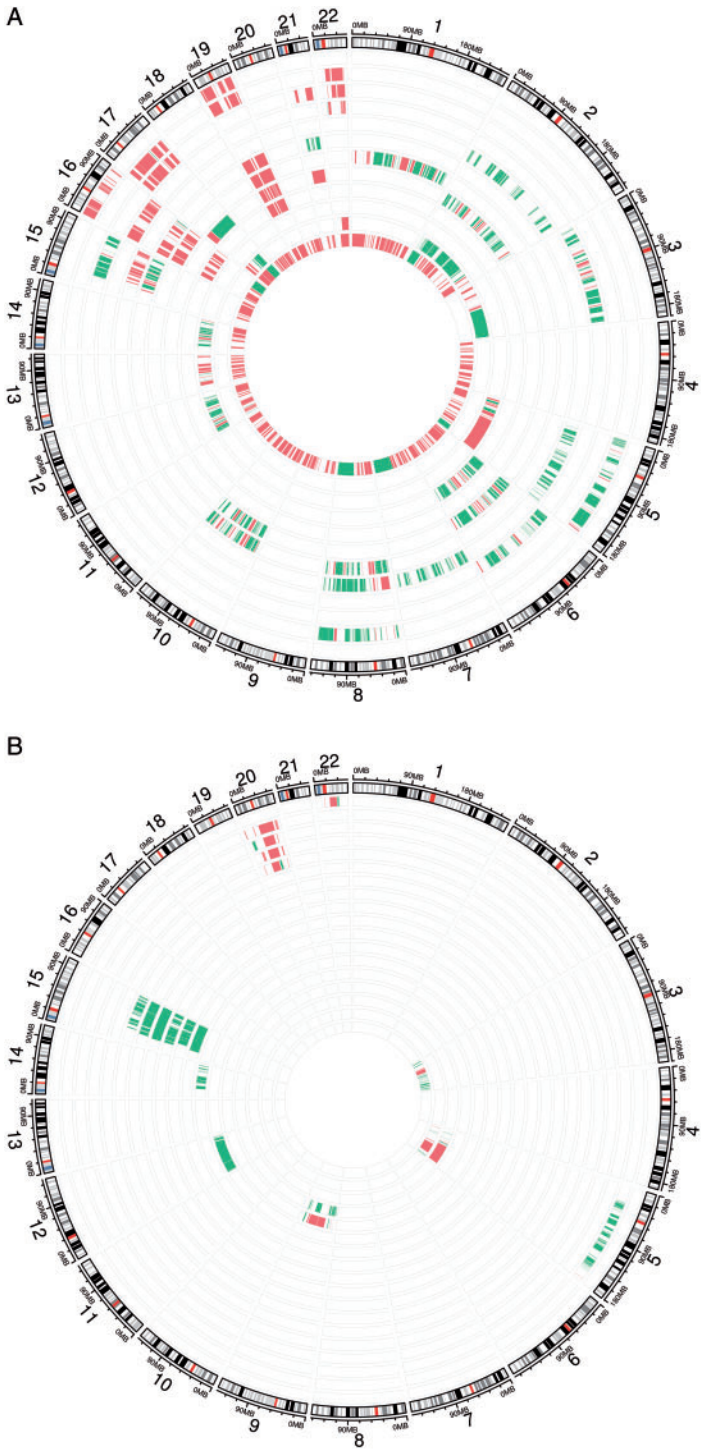

Figure 2. Chromosomal aberrations observed in cell-free plasma DNA. Representative circos plots showing reproducible chromosomal anomalies detectable in cfDNA. Plotting is based on the GIPseq results of the second plasma sample of each case, showing those chromosomal anomalies that are reproducible in the two independent plasma samples and with a chromosomal *z*-score ≥3.0 (suggesting gain; in green) or ≤3.0 (suggesting loss; in red). Chromosomal regions with clear reproducible amplifications and deletions, resulting in a neutral *z*-score are displayed as well. For every case, the genomic representation profile of the autosomal chromosomes is shown in clockwise order, aligned with chromosomal ideograms (outer circle). (A) Cases with genomewide chromosomal aberrations (*n*=12). For some of these cases, high *z*-scores for almost every chromosome were observed. This indicates that either all chromosomes are indeed affected, or the *z*-scores of particular individual chromosomes or chromosomal fragments might be skewed due to excessive presentation of other, highly amplified chromosomes or chromosome arms. (B) Cases with single chromosomal aberrations (*n*=18). For one del(20q) case focal gains on chromosome 5 emerged upon second cfDNA sampling (second last case; see also supplementary Figure S8, available at *Annals of Oncology* online). Cases are shown from the periphery to the center in the same order as in Tables 1 and 2.

Table 1. Molecular and clinical outcome in cases with aberrant copy number profiles in two independent plasma cfDNA samples: genomewide chromosomal aberrations

Case	Sex	Age (years)	QS GIPseq-1	QS GIPseq-2	Time GIPseq-1 to -2 (days)	Affected chromosomes in cfDNA[a]	Array CGH and/or FISH	WB-DWI MRI[b]	Hematological analyses	Diagnosis
12012016-28	M	74	9.42	10.66	93	1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22	arr[hg19] 2p25.3p22.3 (189743_35576176)×3 [0.4], 5p15.33p15.2 (22149_11762145)×1[−0.42], 5q33.3q35.3 (159255273_180696832)×3 0.3], 7q21.11q36.3 (85783873_159128555)×3[0.4], 8q21.13q24.3 (82560146_146274890)×3[0.4], 17q22q25.3 (51084223_81050817)×3[0.2][c]	• #1: infra- and supra-diaphragmatic adenopathies	Leukocytosis; atypical lymphoid cells and Gumprechts shadows	Non-Hodgkin lymphoma (NHL)-type CLL, Rai I – Binet A
25022016-07	M	65	1.56	4.04	98	2, 3, 17, 22	nuc ish 3q27 (BCL6×7), 8p11.1-8q11.1 (D8Z2×5 [18/20], 11q22 (ATM×2), 12p11.1-q11 (D12Z3×3–4)[4/20], 17p13 (TP53×1–2), 17p11.1-q11 (D17Z1×2–3)[5/20][d]	• incidental post-operative MRI with cancer diagnosis	Normal parameters	Classical Hodgkin lymphoma, stage II
02102015-03	F	69	2.23	3.35	117	5, 12, 13, 14	arr(1–22, X)×2[c]	• #1: lesion in endometrium: benign pathology • #2: lesion in breast: benign mammographic findings	Anemia	Myelodysplastic syndrome, with excess blasts-1, IPSS 2
09022017-08	M	70	1.50	2.18	123	6, 16, 19	arr(1–22)×2, (X, Y)×1[c]	• incidental CT-scans for leiomyoma and MALT	Normal	NHL-type MALT, stage I
16032017-13	F	76	2.20	2.26	119	2, 17, 19, 21	ish del(17)(TP53-, D17Z1+, RARA+), del(21)(RUNX1-).nuc ish(RUNX1T1×2, RUNX1×1)[114/200], (PML×2, RARA×3) [13/100], (TP53×1, D17Z1×2)[44/100][c,e,f]	• #1–3: watch and wait policy for very small kidney lesion	Leukocytosis	High-count monoclonal B-cell lymphocytosis
05102015-02	M	66	2.42	2.42	113	1, 6, 10, 16, 19	arr(1–22)×2, (X, Y)×1[c]	• #1–3: normal	Mild lymphocytosis, monocytosis	None
14122015-02[g]	M	69	2.50	2.58	140	8, 10, 15, 16, 19, 21	arr(1–22)×2, (X, Y)×1[c]	ND, claustrofobia	Mild anemia	None
22022016-03[g]	F	91	2.16	2.35	80	2, 5, 7, 8, 15	arr(1–22, X)×2[c]	• #1: supraclavicular adenopathies	Monoclonal gammopathy of undetermined significance	None, voluntary withdrawal

Continued

Table 1. *Continued*

Case	Sex	Age (years)	QS GIPseq-1	QS GIPseq-2	Time GIPseq-1 to -2 (days)	Affected chromosomes in cfDNA[a]	Array CGH and/or FISH	WB-DWI MRI[b]	Hematological analyses	Diagnosis
19012017-04[g]	M	66	2.19	2.16	133	3, 6, 16, 22	arr(1–22)×2, (X, Y)×1[c]	• #1–2: normal • #3: voluntary withdrawal	Mild leukocytosis	None
02022017-08[g]	M	77	2.66	2.60	106	15, 17, 21, 22	arr(1–22)×2, (X, Y)×1[c]	• Voluntary withdrawal	ND	None
06022017-06	M	67	2.10	2.77	155	5, 8, 17, 19, 22	arr(1–22)×2, (X, Y)×1[c]	• # 1–3: normal	Anemia, macrocytosis	None
16032017-14	M	67	2.13	1.96	175	16, 19	arr(1–22)×2, (X, Y)×1[c]	• #1–3: normal	Normal	None

[a]Chromosomes for which |z-score|≥3 in the GIPseq profiles of the two independent plasma samples.
[b]WB-DWI MRI #2 and #3 were carried out, respectively, 3 and 6 months after WB-DWI MRI #1.
[c]In peripheral blood cells.
[d]In rare Reed–Sternberg cells.
[e]Array not interpretable.
[f]Conventional cytogenetic analysis was not carried out but the aberrations described here were identified by ish.
[g]Cases for whom partial data on WB-DWI MRI examinations or general hematological analyses are available, either due to withdrawal of the participant during the course of the study or because the observed abnormalities in cfDNA were believed not to be cancer-related aberrations.
GIPseq-1, -2, GIPseq analysis of sample 1 and 2, respectively; F, female; M, male; ND, not done; QS, quality scores.

assuming that cfDNA is largely derived from hematopoietic cells [1]. Three of the four incidental cancer diagnoses, following a normal GIPseq profile, were solid tumors and the genomes of these cancers were characterized by CNAs. Either these tumors did not shed DNA into the circulation or the load of ctDNA species was too low to be detected via GIPseq. Also, plasma cfDNA concentrations did not differ between participants with and without CNAs in cfDNA, suggesting that, even with a higher turnover of malignant cells, the ctDNA fraction remains limited.

Besides detecting hematological malignancies, GIPseq profiling might allow detecting premalignant conditions. Firstly, one high-count MBL diagnosis was made, being recognized as a precursor lesion of CLL [16]. Secondly, in 9 of the 24 cases (38%) without a cancer diagnosis, the isolated anomalies in cfDNA were originating from a low-grade mosaicism in peripheral blood cells, some of these aberrations being typically associated with MDS and myeloproliferative neoplasms [17, 18]. Two of our cases had, respectively, a mild anemia and monocytosis, potentially pointing to an underlying, developing myeloid neoplasm. Previous studies on cellular DNA from peripheral blood found that clonal mosaicisms for these chromosomal anomalies in blood cell DNA are present in ∼2.5% of aged people and, importantly, that they predict a 5- to 10-fold increased risk of a subsequent cancer [19, 20].

For about two-third of study participants (15 of 24 cases), with anomalies in cfDNA but no cancer diagnosis, the chromosomal aberrations were not detectable in peripheral blood DNA. This does not necessarily preclude that the observed abnormalities were from hematopoietic origin. It might be possible that aberrant cfDNA species originated from neoplastic clones in bone marrow without resulting in aberrant peripheral blood cell descendants. Such an observation was previously reported by Yeh et al. in the context of MDS [21]. Similarly, the cases identified with MDS and MALT in our study had a normal molecular karyotype in peripheral blood cells. Molecular testing of bone marrow biopsy DNA in cases with detectable anomalies in cfDNA but not in peripheral blood could help identifying the origin of aberrant cfDNA species. However, this is not ethically justifiable in otherwise healthy individuals. Nevertheless, there remains the possibility that the cfDNA clones might be derived from other than hematopoietic cells and reflect other non-neoplastic conditions [22]. Future analyses, exploiting supramolecular information contained in cfDNA, may help tracing the tissue from which the detected aberrant cfDNA species originated [23].

To allow exploiting the full potential of GIPseq for cancer screening, a longer follow-up of our study population should be carried out to determine the clinical and biological significance of chromosomal imbalances in cfDNA of apparently healthy individuals. Either these chromosomal events represent malignant precursor clones or they reflect the normal clonal variation in aged people. Both scenarios impact future cancer screening programs searching for cancer-specific CNAs in liquid biopsies. Furthermore, long-term follow-up will allow determining the false negative rate in our population. Finally, further investigations are necessary to understand why, in about one-third of cases, the initial deviating GIPseq profile could not be reproduced upon subsequent plasma sampling, and whether this irregularity has a technical and/or a biological nature.

Table 2. Molecular and clinical outcome in cases with aberrant copy number profiles in two independent plasma cfDNA samples: aberrations affecting one chromosome

Case	Sex	Age (years)	QS GIPseq-1	QS GIPseq-2	Time GIPseq-1 to -2 (days)	Type of anomaly in cfDNA	Estimated size (Mb)	Array CGH and/or FISH	WB-DWI MRI[a]	Hematological analyses	Diagnosis
Chromosome 3											
08122015-04	M	75	1.04	1.00	196	3p−	22.9	nuc ish (3p26.3, RP11-88H12)×2[100][b].arr[hg19] 3p14.2p12.3 (60240594_79219598)×1[0.4][c]	• #1–3: normal	Normal	None
Chromosome 5											
29042016-27	F	67	1.21	0.79	132	5q−	64.2	nuc ish5p15.2 (D5S23×2), 5q31 (EGR1×2)[100].arr(1–22, X)×2[c]	• #1: lesions in the stomach: benign pathology • #3: lesions in rectum: benign pathology	Normal	None
16062016-33	F	72	0.95	0.92	159	5q−	94.6	nuc ish5p15.2 (D5S23×2), 5q31 (EGR1×1)[10/100].arr(1–22, X)×2[c]	• #1: lesions in breast: benign pathology • #3: normal	Normal	None
Chromosome 9											
17112016-02[d]	M	75	1.33	1.27	140	9p+/9q+	23.5 (9p); 22.6 (9q)	arr[hg19] 8q13.2 (69469335_70279670)×1[0.9][c]	ND	Normal	None
20042017-10[d]	F	78	0.77	1.20	140	9q−	39.8	arr(1–22, X)×2[c]	• #1: normal • #2–3: voluntary withdrawal	Mild anemia	None
Chromosome 12											
15092016-04	M	66	1.01	1.23	112	+ 12	126.4	nuc ish11q22 (ATM×2), 12p11.1q11 (D12Z3×3)[12/200].arr(1–22)×2, (X, Y)×1[c]	• #1: infra- and supra-diaphragmatic adenopathies	Lymphocytosis	NHL-type SLL, stage III
Chromosome 14											
21042016-06	M	71	1.07	1.11	140	14q+	41.2	arr(1–22)×2, (X, Y)×1[c]	• #1: normal • #3: lesion in colon: benign pathology	Normal	None
Chromosome 15											
21062016-02	M	83	1.06	1.11	156	+15	82.6	nuc ish15q24 (PML×3), 17q21 (RARA×2)[32/200].arr(1–22)×2, (X, Y)×1[c]	• #1: lesion in prostate: benign on ultrasonography • #2–3: stable findings	Elevated mean cell volume; mild anemia	None
18022016-01	M	69	1.39	1.02	95	+15	75.4	nuc ish15q24 (PML×2), 17q21 (RARA×2)[183/200].arr(1–22)×2, (X, Y)×1[c,e]	• #1–2: lesion in the skull base; • fMRI: benign	Mild monocytosis	None
14032016-08	M	74	1.39	1.18	214	+15	82.5	arr(1–22)×2, (X, Y)×1[c]	• #1–3: normal	Normal	None
06122016-12	M	88	1.18	1.38	119	+15	82.7	nuc ish15q24 (PML×3), 17q21 (RARA×2)[41/200].arr(1–22)×2, (X, Y)×1[c]	• #1–3: normal	Normal	None
16022017-08[d]	F	71	0.94	0.96	147	+15	35.0	arr(1–22, X)×2[c]	ND	Normal	None

Continued

Table 2. *Continued*

Case	Sex	Age (years)	QS GIPseq-1	QS GIPseq-2	Time GIPseq-1 to -2 (days)	Type of anomaly in cfDNA	Estimated size (Mb)	Array CGH and/or FISH	WB-DWI MRI[a]	Hematological analyses	Diagnosis
30032017-02	M	81	1.33	1.39	168	+15	80.4	Nuc ish15q24 (PML×2), 17q21 (RARA×2)[244/253].arr(1–22)×2, (X, Y)×1[c,e]	• #1–3: normal	Normal	None
Chromosome 20											
18042017-05	F	68	1.08	0.85	86	20q−	16.6	arr[hg19] 20q11.21q13.12 (30946217_43063241)×1[0.21][c]	ND[e]	Normal	None
04122015-02	M	79	1.25	1.45	186	20q−	23.5	arr[hg19] 20q11.21q13.13 (31974422_46786648)×1[0.3][c]	ND[e]	Mild monocytosis	None
11122015-01	F	73	1.01	0.92	130	20q−	20.8	arr[hg19]20q11.21q13.2 (31021315_46569323)×1[0.3][c]	ND[e]	Normal	None
29042016-02	M	73	1.04	1.15	157	20q−	24.5	arr[hg19] 20q11.21q13.2 (30658819_50505718)×1[0.2][c]	ND[e]	Normal	None
Chromosome 22											
23012017-28[d]	F	72	0.98	0.93	246	22q−	16.0	arr[hg19] 22q12.1q12.3 (27016168_34544054)×1[0.2][c]	ND	Normal	None

[a]WB-DWI MRI #2 and #3 were carried out, respectively, 3 and 6 months after WB-DWI MRI #1.
[b]In buccal swab cells.
[c]In peripheral blood cells.
[d]Cases for whom partial data on WB-DWI MRI examinations or general hematological analyses are available, either due to withdrawal of the participant during the course of the study or because the observed abnormalities in cfDNA were believed not to be cancer-related aberrations.
[e]One out of the 17 cells (case 18022016-01) and 2 out of the 9 cells (case 30032017-02) with an abnormal genetic constellation showed a gain of 15q24.
GIPseq-1, -2, GIPseq analysis of sample 1 and 2, respectively; F, female; M, male; ND, not done; QS, quality scores.

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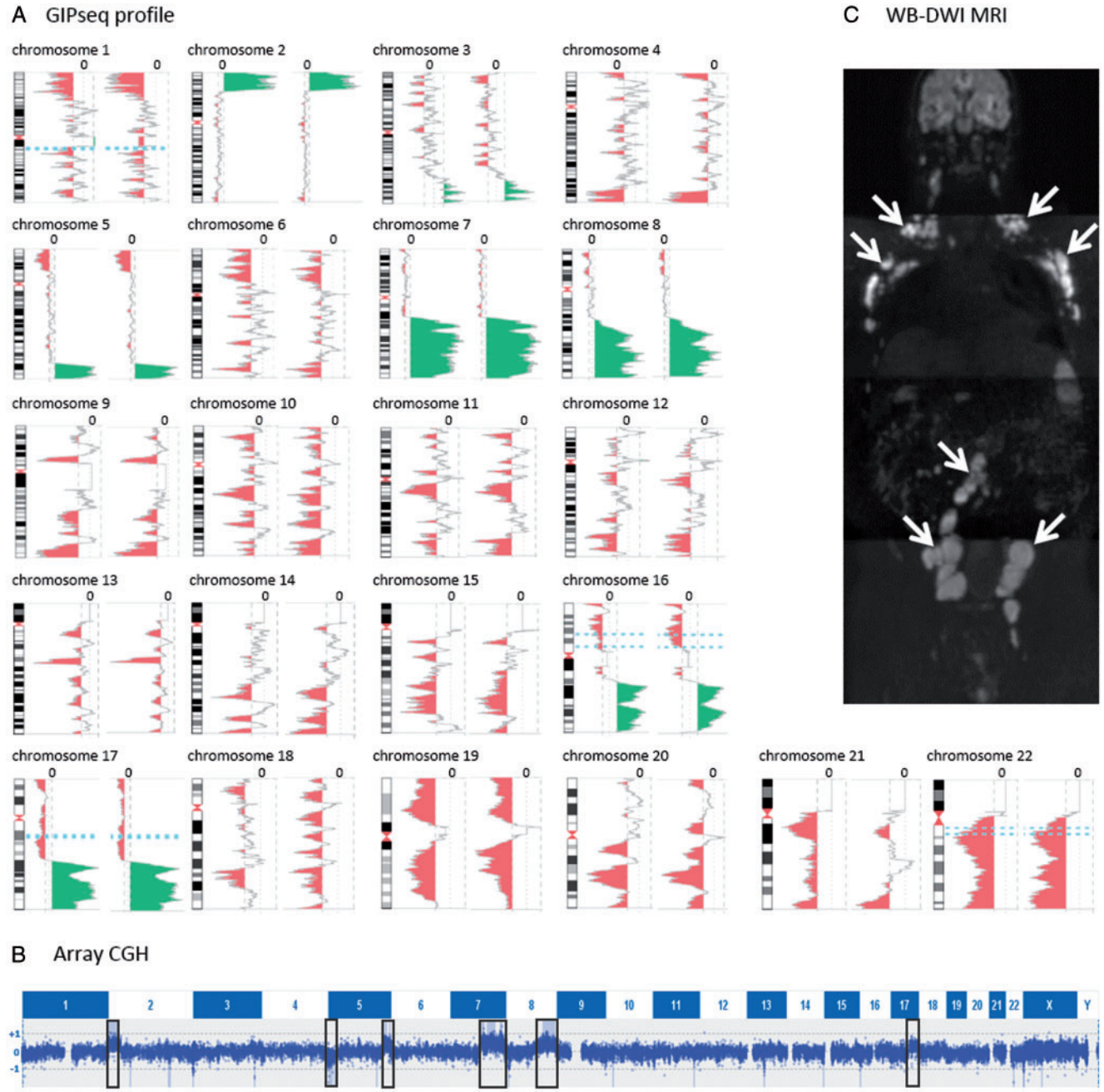

Figure 3. GIPseq profiles, array CGH and WB-DWI MRI analyses for the case with non-Hodgkin lymphoma-type CLL. (A) GIPseq-profiles of the two consecutive blood samples of case 12012016-28. For every chromosome, the ideogram is shown at the left, together with the genomic representation profile of sample one (immediately at the right) and sample two (far right, taken three months after sample one). The vertical graph represents the actual genome representation profile: dotted lines on either side of the axis, plus or minus ×1.5; red areas, likely deleted regions; green areas, likely duplicated or amplified regions. (B) Array CGH on peripheral blood DNA. The column labels represent the numbered chromosomes plus X and Y; the *y*-axis, represents the log 2 of the intensity ratios; each graphed point represents an array probe. (C) WB-DWI MRI. Arrows indicate the presence of multiple infra- and supradiaphragmatic adenopathies, indicative of a lymphoproliferative process.

Together, these data underscore the potential of genomewide cfDNA profiling, as an unbiased screening approach for chronic hematological malignancies and premalignant conditions, such as HL, NHL, MDS or MBL. Such a secondary prevention strategy could be particularly valuable for patient groups at risk, e.g. after genotoxic cancer therapy. As shown above, plasma cfDNA analysis might allow detecting a broader range of chromosomal abnormalities than do examinations restricted to peripheral blood cells. Identifying individuals with an indolent hematological malignancy or a clonal mosaicism at risk of subsequent malignant evolution would allow a close follow-up and the introduction of specific measures for the management of the

early-stage disease [24]. Finally, potential consequences for the patient's mental health need to be taken into account: the discovery of clonal mosaicism in cfDNA and/or an indolent hematological malignancy together with uncertainty about its future evolution may cause anxiety, akin to that described in patients diagnosed with CLL that were approached with a strategy of active surveillance and 'watchful waiting' [25, 26]. Lastly, this study also provides a baseline of CNAs expected to be present in plasma cfDNA.

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Disclosure

The authors have declared no conflicts of interest.

References

1. Aucamp J, Bronkhorst AJ, Badenhorst CPS, Pretorius PJ. The diverse origins of circulating cell-free DNA in the human body: a critical re-evaluation of the literature. Biol Rev Camb Philos Soc 2018; 93(3): 1649–1683.
2. Heitzer E, Ulz P, Geigl JB, Speicher MR. Non-invasive detection of genome-wide somatic copy number alterations by liquid biopsies. Mol Oncol 2016; 10: 494–502.
3. Heitzer E, Ulz P, Belic J et al. Tumor-associated copy number changes in the circulation of patients with prostate cancer identified through whole-genome sequencing. Genome Med 2013; 5: 30.
4. Vandenberghe P, Wlodarska I, Tousseyn T et al. Non-invasive detection of genomic imbalances in Hodgkin/Reed-Sternberg cells in early and advanced stage Hodgkin's lymphoma by sequencing of circulating cell-free DNA: a technical proof-of-principle study. Lancet Haematol 2015; 2: e55–e65.
5. Amant F, Verheecke M, Wlodarska I et al. Presymptomatic identification of cancers in pregnant women during noninvasive prenatal testing. JAMA Oncol 2015; 1: 814–819.
6. Przybyl J, Chabon JJ, Spans L et al. Combination approach for detecting different types of alterations in circulating tumor DNA in leiomyosarcoma. Clin Cancer Res 2018; 24: 2688–2699.
7. Bayindir B, Dehaspe L, Brison N et al. Noninvasive prenatal testing using a novel analysis pipeline to screen for all autosomal fetal aneuploidies improves pregnancy management. Eur J Hum Genet 2015; 23: 1286–1293.
8. Mitelman F, Johansson B, Mertens FE (eds). Mitelman Database of Chromosome Aberrations and Gene Fusions in Cancer 2018. http://cgap.nci.nih.gov/Chromosomes/Mitelman.
9. Huret JL, Ahmad M, Arsaban M et al. Atlas of genetics and cytogenetics in oncology and haematology in 2013. Nucleic Acids Res 2013; 41: D920–D924.
10. Brady PD, Delle Chiaie B, Christenhusz G et al. A prospective study of the clinical utility of prenatal chromosomal microarray analysis in fetuses with ultrasound abnormalities and an exploration of a framework for reporting unclassified variants and risk factors. Genet Med 2014; 16: 469–476.
11. Michielsen K, Dresen R, Vanslembrouck R et al. Diagnostic value of whole body diffusion-weighted MRI compared to computed tomography for pre-operative assessment of patients suspected for ovarian cancer. Eur J Cancer 2017; 83: 88–98.
12. Li B, Li Q, Nie W, Liu S. Diagnostic value of whole-body diffusion-weighted magnetic resonance imaging for detection of primary and metastatic malignancies: a meta-analysis. Eur J Radiol 2014; 83: 338–344.
13. Krok-Schoen JL, Fisher JL, Stephens JA et al. Incidence and survival of hematological cancers among adults ages >/=75 years. Cancer Med 2018; 7(7): 3425–3433.
14. Noone AH, Howlader N, Krapcho M et al. (eds). SEER Cancer Statistics Review, 1975–2015. Bethesda, MD: National Cancer Institute. https://seer.cancer.gov/csr/1975_2015/, based on November 2017 SEER data submission, posted to the SEER web site, April 2018.
15. Cancer Research UK Cancer Statistics. All Cancers Excluding Non-Melanoma Skin Cancer, European Age-Standardised Incidence Rates, By Age, UK, 1993-2015. https://www.cancerresearchuk.org/health-professional/cancer-statistics/incidence/age#heading-Three (accessed July 2018).
16. Quintanilla-Martinez L. The 2016 updated WHO classification of lymphoid neoplasias. Hematol Oncol 2017; 35(Suppl 1): 37–45.
17. Hosono N, Makishima H, Mahfouz R et al. Recurrent genetic defects on chromosome 5q in myeloid neoplasms. Oncotarget 2017; 8: 6483–6495.
18. Bacher U, Haferlach T, Schnittger S et al. Investigation of 305 patients with myelodysplastic syndromes and 20q deletion for associated cytogenetic and molecular genetic lesions and their prognostic impact. Br J Haematol 2014; 164: 822–833.
19. Jacobs KB, Yeager M, Zhou W et al. Detectable clonal mosaicism and its relationship to aging and cancer. Nat Genet 2012; 44(6): 651–658.
20. Laurie CC, Laurie CA, Rice K et al. Detectable clonal mosaicism from birth to old age and its relationship to cancer. Nat Genet 2012; 44(6): 642–650.
21. Yeh P, Dickinson M, Ftouni S et al. Molecular disease monitoring using circulating tumor DNA in myelodysplastic syndromes. Blood 2017; 129(12): 1685–1690.
22. Chan RW, Jiang P, Peng X et al. Plasma DNA aberrations in systemic lupus erythematosus revealed by genomic and methylomic sequencing. Proc Natl Acad Sci USA 2014; 111(49): E5302–E5311.
23. Lo YM, Lam WK. Tracing the tissue of origin of plasma DNA-feasibility and implications. Ann N Y Acad Sci 2016; 1376(1): 14–17.
24. Strati P, Shanafelt TD. Monoclonal B-cell lymphocytosis and early-stage chronic lymphocytic leukemia: diagnosis, natural history, and risk stratification. Blood 2015; 126(4): 454–462.
25. Steensma DP. Clinical Implications of Clonal Hematopoiesis. Mayo Clin Proc 2018; 93(8): 1122–1130.
26. Shanafelt TD, Bowen DA, Venkat C et al. The physician-patient relationship and quality of life: lessons from chronic lymphocytic leukemia. Leuk Res 2009; 33(2): 263–270.