False positive plasma genotyping due to clonal hematopoiesis

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Statement of Translational Relevance (106 words, 150 max)

Despite increasing use of plasma cell-free DNA (cfDNA) genotyping for cancer care, this biospecimen remains poorly understood. Here, we report recurrent mutations in \textit{JAK2}, \textit{TP53}, and \textit{KRAS} detected in cfDNA on validated plasma genotyping assays which are in fact derived white blood cells (i.e. clonal hematopoiesis, CH) rather than from tumor. Our approach highlights that clinicians must be aware that mutations detected in cfDNA, especially in CH-related genes, may not always reflect tumor genotype. Furthermore, future efforts to develop plasma cfDNA genotyping as a tool for cancer detection may require paired genotyping of peripheral blood cells so that CH-derived mutations are not misdiagnosed as occult malignancy.
Abstract (250 words, 250 max):

**Purpose:** Plasma cell-free DNA (cfDNA) genotyping is increasingly used in cancer care, but assay accuracy is debated. Because most cfDNA is derived from peripheral blood cells (PBC), we hypothesized that nonmalignant mutations harbored by hematopoietic cells (clonal hematopoiesis, CH) could be a cause of false positive plasma genotyping.

**Experimental Design:** We identified patients with advanced NSCLC with *KRAS*, *JAK2*, or *TP53* mutations identified in cfDNA. With consent, PBC DNA was tested using droplet digital PCR (ddPCR) or next-generation sequencing (NGS) to test for CH-derived mutations.

**Results:** We first studied plasma ddPCR results from 58 *EGFR*-mutant NSCLC patients. Two had *KRAS* G12X detected in cfDNA and both were present in PBC, including one where the *KRAS* mutation was detected serially for 20 months. We then studied 143 plasma NGS results from 122 NSCLC patients, and identified 5 *JAK2* V617F mutations derived from PBC. Additionally, 108 *TP53* mutations were detected in cfDNA; for 33 of the *TP53* mutations, PBC and tumor NGS were available for comparison, and 5 were present in PBC but absent in tumor, consistent with CH.

**Conclusions:** We find that most *JAK2* mutations, some *TP53* mutations, and rare *KRAS* mutations detected in cfDNA are derived from CH not tumor. Clinicians ordering plasma genotyping must be prepared for the possibility that mutations detected in plasma, particularly in genes mutated in CH, may not represent true tumor genotype. Efforts to use plasma genotyping for cancer detection may need paired PBC genotyping so that CH-derived mutations are not misdiagnosed as occult malignancy.
Introduction

Genomic analysis of plasma cell-free DNA (cfDNA) is increasingly clinically adopted as a method for noninvasive genotyping of advanced cancers. Such “liquid biopsy” approaches potentially allow cancer genotyping in the absence of adequate tumor tissue, resulting in the approval by the US Food and Drug Administration (FDA) of the Cobas EGFR mutation test v2 for plasma genotyping of non-small cell lung cancer (NSCLC) when tumor tissue is unavailable (1). More recent advances in the development of highly sensitive plasma next-generation sequencing (NGS) assays have motivated ambitious plans to expand the clinical use of plasma genotyping (2), to develop assays that can facilitate early detection of residual disease following curative therapy (3), and to eventually develop cancer screening tests.

Unfortunately, discordance between genotyping of tumor tissue and plasma cfDNA has hampered the use and interpretation of these new assays (4). One recognized cause of tumor/plasma discordance is variable shed of tumor DNA into the plasma, causing false negative plasma genotyping (5). A second well documented cause of tumor/plasma discordance is tumor heterogeneity, particularly in the setting of drug resistance (6). However, variable DNA shed and tumor heterogeneity fail to fully account for the recurring reports of inaccurate plasma genotyping reported in the literature (7,8).

Since a large proportion of cfDNA is derived from peripheral blood cells (PBC) (9), we hypothesized that somatic mutations within non-malignant hematopoietic cells, known clonal hematopoiesis (CH) (10), might be a recurring source of discordance between tumor genotyping and plasma cfDNA genotyping. Our specific aim was to identify false positives due to CH, i.e. instances where plasma genotyping, used with the intent of identifying NSCLC genotype, instead identified mutations derived from CH and not the tumor.

Materials and Methods

Six large publications studying CH were reviewed to identify genes of interest (11-16). Per patient prevalence estimate of mutations in relevant CH genes were calculated by review of publicly available data (Figure 1). The genes most commonly mutated (DNMT3A, ASXL1, and TET2) (11,12) are not covered by most clinically available plasma genotyping assays. However, several other genes with potential importance to cancer biology have been reported as occasionally mutated in CH: JAK2, TP53, GNAS, IDH2, and KRAS. For
this analysis, we focused on mutations in three cancer-associated genes which could be genotyped using available highly sensitive assays: JAK2, TP53, and KRAS.

**Patient cohort:** Patients with advanced NSCLC who had undergone plasma cfDNA genotyping as part of ongoing research or routine clinical care were identified. Plasma cfDNA genotyping was performed either using a validated ddPCR assay (performed in a research lab and described previously (5)), or using a commercially available plasma NGS assay (GuardantHealth, Redwood City). Under an IRB-approved protocol, patients were consented for blood collection so that PBC, which are usually discarded after spinning the plasma, could be saved for further genomic analysis.

**Droplet digital PCR (ddPCR):** DNA was extracted from PBCs using the DNeasy blood and tissue kit (Qiagen) per manufacturer’s recommendation. DNA was tested for recurring point mutations (e.g. KRAS G12X, JAK2 V617F) using ddPCR. Primer/probes for the JAK2 V617F assay were custom-made by Life Technologies.
- Forward primer sequence: 5’-AAGCTTTCTCACAAGCATTCTTTTGGTTT-3’.
- Reverse primer sequence: 5’-GAAAGCCTGTAGTTTTACTTACTCTCGT-3’.
- Probe sequences: 5’-VIC-CTCCACAGACACATACT-MGB-NFQ-3’, 5’-FAM-CTCCACAGAAACATACT-MGB-NFQ-3’.
- Cycling conditions: 10 min at 95 °C followed by 40 cycles of a two-step thermal profile of 15 s at 94 °C denaturation and 60 s at 58°C.

**Highly sensitive NGS:** NGS of PBC DNA was performed using a custom QIAseq v.3 amplicon-based panel containing PCR amplicons spanning the entire exome of TP53 and KRAS, and sequenced on an Illumina NextSeq at an average mean read depth of 1200. DNA libraries were prepared using a custom QIAseq v3 targeted DNA Kit principally as per manufacturer’s instructions. 15 ng of isolated DNA was enzymatically fragmented and end repaired in a 25ul reaction. The reaction conditions were: 4 °C for 1 min, 32 °C for 14 min and 65 °C for 30 min. Immediately after, adapter ligation reagents were directly added to the 25uL of DNA: 10uL 5X ligation buffer, 5uL DNA ligase, 2.8uL IL-N7## barcoded adapters and DNase free water were added to a total 50ul. The reaction continued at 20 °C for an additional 15 min after which the reaction was purified using 90uL of QIAseq beads and eluted into 12 uL of nuclease-free water. 9.4 uL of DNA was 5uL
of the Qiaseq targeted panel, 4uL of 5X TEPCR buffer, 0.8uL of IL-forward primer, and 0.8 uL of 0.8ul HotStarTaq DNA polymerase. The PCR enrichment condition was: 95 °C for 13 min, 98 °C for 2 min; six cycles of 98 °C for 15 s and 65 °C for 15 min; 72 °C for 5 min. Each reaction was cleaned once using QiAseq beads to remove unused primers. Enriched DNA was combined with 400nM IL-Universal primer, 400nM IL-Index primer, 1X UPCR buffer and 1ul HotStarTaq DNA polymerase in a volume of 20 ul. The universal PCR condition was: 95 °C for 13 min, 98 °C for 2 min; 20 cycles of 98 °C for 15 s and 60 °C for 2 min; 72 °C for 5 min. The DNA library was purified and sequenced on Illumina NextSeq (pair-end, 2x151 bp) following manufacturer’s user manual (Illumina, CA).

Tumor genotyping: Tumor NGS was performed at the Center for Advanced Molecular Diagnostics of Brigham and Women’s Hospital using a hybrid-capture based NGS platform spanning 275-447 genes. An institutional database of NSCLC tumor NGS results was used for comparisons (17).

Results

False positive plasma ddPCR due to CH

We first queried our multi-year institutional experience performing plasma genotyping of key EGFR and KRAS mutations in patients with advanced NSCLC using a recently validated ddPCR platform with a negligible false positive rate (5). Reviewing a cohort of 221 patients with advanced NSCLC undergoing plasma ddPCR for EGFR and KRAS mutations, 58 were known to harbor an EGFR mutation based on tumor genotyping (Figure 2A). Two of these cases (3%) had plasma ddPCR which was positive for a KRAS codon 12 mutation. PBC DNA was collected and tested and determined to be the source of the KRAS mutation in both cases.

The first case was a 78-year-old female with newly diagnosed advanced EGFR-mutant NSCLC whose initial plasma ddPCR revealed both an EGFR exon 19 deletion (2.2% allelic fraction, AF) and a KRAS G12D (2.0% AF) mutation. Treatment with erlotinib resulted in a durable response on imaging and clearance of the plasma EGFR mutation, however the KRAS mutation persisted in cfDNA over the course of 20 months on therapy (Figure 2B-C). Occult malignancy was suspected but imaging with abdominal ultrasound and CT colonography revealed no occult malignancy. Noting that KRAS mutations have been reported in rare patients with CH (12,14), the KRAS G12D mutation was retested in paired cfDNA and PBC, and detected in plasma at
1.2% AF and PBC at 0.5% AF, whereas the EGFR exon 19 deletion was undetectable in PBC (Supplemental Figure 1). To pinpoint the origin of this KRAS mutation, PBC were sorted via fluorescence-activated cell sorting (Figure 2D); ddPCR detected the KRAS mutation only in lymphocytes (1.7% AF), and across four lymphoid subpopulations. NGS confirmed the presence of the KRAS G12D mutation, as detected with ddPCR, but the absence of any other mutations in 8 cancer-associated genes (Supplemental Figure 1). These data suggest a common lymphoid progenitor acquired a KRAS G12D mutation and underwent clonal expansion, consistent with CH (5).

The second case was a 61-year-old female with advanced EGFR-mutant NSCLC being treated with erlotinib. Prior tumor NGS of a resection specimen had detected an EGFR L858R mutation but no KRAS mutation. Upon acquired resistance to erlotinib, commercial plasma NGS did not detect the EGFR L858R mutation but did detect a KRAS G12S mutation; the KRAS G12 mutation was then confirmed at our institution using ddPCR at 0.38% AF. We performed ddPCR of DNA from PBC which revealed a KRAS G12 mutation (0.41% AF) and no detectable EGFR L858R. NGS of a tumor biopsy performed following erlotinib resistance detected the EGFR L858R mutation and a new T790M mutation but no KRAS mutation.

False positive plasma NGS due to CH

We then studied 143 commercial plasma NGS results from 122 patients with advanced NSCLC treated at our center (Figure 3A). 14 mutations were detected in three genes associated with CH (JAK2, GNAS, IDH2), including 6 cases (4.9%) positive for a JAK2 V617F mutation, which is rarely seen on tumor genotyping of NSCLC (0.26%, p=0.0001). PBC were available for analysis in 5 cases and ddPCR detected the JAK2 V617F mutation in each (AF 0.13-4.66%, Table 1, Supplemental Figure 2); 3 had tumor NGS available and all were JAK2 wildtype.

Finally, we studied TP53 mutations found on plasma NGS as these are common in NSCLC but also can be seen in CH. Reviewing the 143 plasma NGS results, 108 TP53 mutations were detected. Interestingly, the number of TP53 mutations per specimen was higher than expected with 7 specimens having >2 different TP53 mutations, which is rare on tumor NGS, and suggests a polyclonal process (Figure 3B). For 33 of the TP53 mutations detected on plasma NGS, PBC and tumor NGS were available for comparison. 14 TP53 mutations were detected on tumor NGS but not in PBC, consistent with tumor-derived variants, while the
majority of the TP53 mutations were not detected on tumor NGS (Table 1). For 5 mutations, NGS of PBC detected the TP53 mutation but tumor NGS did not, consistent with CH; in some of these cases, TP53 mutations from the NSCLC and CH were both found concurrently in plasma (Figure 3C). 14 TP53 mutations were neither detected on tumor NGS nor PBC NGS such that their source could not be determined; 13 of these had an AF ≤0.5% plasma NGS, near the limit of detection (LOD) of most plasma genotyping assays and making validation using an orthogonal assay extremely difficult.

Discussion

The enormous enthusiasm behind the potential clinical applications of plasma cfDNA analysis has begun to outpace our biologic understanding of this new biospecimen. A range of data indicate that plasma cfDNA is a complex mixture of DNA from many sources including germline, fetal, infectious, and malignant (2,18). For example, it is clear that mutations detected on plasma genotyping can be of somatic or germline origin (18), and can be truncal tumor mutations or heterogeneous resistance mutations (6). Here we demonstrate an added element of complexity – that mutations in the cfDNA can be of hematopoietic origin, yet risk being mistaken as tumor-derived mutations. These CH-derived mutations can be seen at a range of AF in cfDNA, with a median AF of 0.9% across the 10 CH-derived mutations we detected.

Our findings impact the use of plasma genotyping for tumor profiling in search of targeted therapy options. We and others have advocated for the use of plasma genotyping in patients with advanced NSCLC where tumor tissue is unavailable for genomic analysis, with the caveat that a positive result can trusted while a negative result should reflex to tumor genotyping. The finding that KRAS and TP53 mutations detected in plasma may not represent tumor genotype add complexity to this approach. Importantly, targetable mutations in genes like EGFR, BRAF, and MET have not been described in CH – if these are detected in plasma with a rigorously validated assay, they can likely be trusted and used for initiation of targeted therapy. However, the same may not be true for KRAS or TP53 mutation – a clinician might mistakenly assume these mutations in cfDNA are tumor-derived and indicate the absence of a targetable genotyping. Our finding of CH-derived mutations in KRAS and TP53 supports the established paradigm that treatment can be initiated based upon a
targetable mutation detected in cfDNA, while absence of a targetable mutation in cfDNA should be followed by genotyping of a tumor biopsy specimen (6).

Second, our findings impact the development of plasma genomics as a tool for detecting minimal residual disease (MRD) or as a cancer screening assay. For example, in a patient who has completed curative therapy for cancer, detection in plasma of mutations in cancer-associated genes like TP53, KRAS or JAK2 could be interpreted as indicating the presence of residual cancer (3), while these mutations might in fact be derived from CH. It has been reported that CH is seen in 25% of patients with non-hematologic cancers (16); indeed, TP53 mutations may be even more common in PBC of patients previously treated with chemotherapy as part of curative therapy (19), increasing the chance of finding such mutations at a timepoint when MRD is being evaluated. The clearest way to overcome this challenge would be paired genotyping of both plasma cfDNA and PBC DNA, which could allow an assay to screen out hematopoietic-derived mutations. Such a step would certainly increase the complexity and cost of cfDNA analysis, and would require the saving of PBC which are routinely discarded when spinning plasma. Mutations detected in cfDNA and not in the PBC could then be considered potentially tumor-derived and indicative of risk. Of course, it is then possible that the incidental detection of JAK2 mutations in plasma or in PBC could lead to the overdiagnosis of hematological malignancies which are clinically nonsignificant. This is merely one symptom of a broader challenge, which is that there remains no established management strategy for patients incidentally diagnosed with CH (10).

Further prospective study is needed to better capture the prevalence of CH-related false positives, and their potential impact on the PPV of these assays. In our analysis the prevalence of CH-derived KRAS mutations was 3%, which, given KRAS mutations are common in many cancer types, is unlikely to impact the PPV of KRAS plasma genotyping. In contrast, JAK2 mutations are rare in solid tumors so the PPV of JAK2 plasma genotyping may be low – most of these mutations in cfDNA are likely to be CH-derived. We note that others have found rare JAK2 mutations in plasma NGS from a large cohort of patients with colorectal cancer (20); however this study did not perform PBC and tumor sequencing to confirm the hematopoietic etiology of these mutations. In another case report, an IDH2 mutation found in the cfDNA of a patient with metastatic colon cancer was determined to be CH-derived though sequencing of a bone marrow biopsy specimen (21); our data highlights that bone marrow biopsy is not needed for detection of CH-derived mutations, as these can
be easily detected in PBC. Our most surprising result is the finding that many TP53 mutations in cfDNA may be CH-derived - only 14 of 33 TP53 mutations could be confirmed to be tumor-derived, though many low AF TP53 mutations could not be found in PBC as well. Thus, the PPV of TP53 plasma genotyping remains unclear but could be lower than previously believed. Some might disagree with whether mutations in cfDNA due to CH should in fact be considered false positives as these are not technical errors but rather real variants derived from non-tumor tissue. However, since plasma cfDNA genotyping is intended to offer a “liquid biopsy” of the tumor, the clinical impact of such CH-derived mutations is a false positive result that potentially misdiagnoses a cancer patient with an incorrect genotype. Because CH-derived mutations cause a misleading diagnostic result, they should be acknowledged as false positives, much like a false positive d-dimer in a patient with cancer undergoing work-up for thromboembolism (22), or a false positive CT scan in a patient undergoing lung cancer screening who is found to have benign nodules (23).

Our approach was technically limited in its leveraging of ongoing commercial plasma NGS efforts, which increases the clinical relevance of our findings but means we were unable to perform plasma and PBC sequencing with identical assays. Prospective efforts that include paired NGS of cfDNA and PBC DNA using identical assays will be needed. It should be acknowledged that available estimates of CH prevalence (5-13% of persons over 70 years old (11,13), and 25% of cancer patients (16)) are based upon moderate-depth sequencing efforts with a limit of detection around ~2% AF. With the highly sensitive NGS approaches being offered by commercial plasma NGS laboratories (LOD ~0.5% AF or lower), CH may in fact be much more common, and thus a condition we will need to be ready to recognize as a part of our routine cancer care and a potentially common finding in plasma genotyping results.
Author Contributions:

Study concept and design: Y. Hu, B.C. Ulrich, C.P. Paweletz, G.R. Oxnard


Writing, review, and/or revision of the manuscript: Y. Hu, B.C. Ulrich, J. Supplee, Y. Kuang, P.H. Lizotte, N.B. Feeney, N.M. Guibert, M.M. Awad, K.K. Wong, P.A. Jänne, C.P. Paweletz, G.R. Oxnard

Administrative, technical, or material support: Y. Hu, B.C. Ulrich, J. Supplee, N.B. Feeney, N.M. Guibert, C.P. Paweletz

Study supervision: C.P. Paweletz and G.R. Oxnard

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Table 1: Genotyping of peripheral blood cells identifies clonal hematopoiesis in patients with plasma genotyping positive for JAK2 mutations (top) or TP53 mutations (bottom).

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<th>Tumor NGS result</th>
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AF: allelic fraction; CH: clonal hematopoiesis; NGS: next-generation sequencing; PBC: peripheral blood cells; ddPCR: droplet digital PCR
Figure Legends:

**Figure 1:** Genes commonly mutated in clonal hematopoiesis (CH). Review of six publications studying CH reveals three commonly mutated genes: *DNMT3A*, *ASXL1*, and *TET2* (left). Additionally, several cancer-related genes (right) are less commonly mutated in CH but are covered by many commercial plasma genotyping assays. Note, genes not reported in a given publication are marked with an asterisk.

**Figure 2:** Testing for clonal hematopoiesis (CH) using droplet digital PCR (ddPCR) of peripheral blood cells (PBC) from patients with advanced non-small cell lung cancer. (A) 221 patients completed plasma ddPCR for *EGFR* and *KRAS* driver mutations. Two patients with *EGFR* mutations on tumor next-generation sequencing (NGS) had *KRAS* G12X mutations detected in plasma. PBC for both were positive for the KRAS mutation. (B) One of these patients had advanced *EGFR*-mutant lung cancer with a durable response to erlotinib therapy (arrow). (C) Serial ddPCR of plasma cell-free DNA reveals clearance of the *EGFR* exon 19 deletion, but sustained low levels of a *KRAS* G12D mutation over 20 months on therapy. (D) Genotyping of DNA from the PBC identifies the presence of the *KRAS* G12D mutation, and further fluorescence-activated cell sorting (FACS) determined the mutation to be present in lymphocyte subpopulations but not present in monocytes or granulocytes.

**Figure 3:** Testing for clonal hematopoiesis (CH) through next-generation sequencing (NGS) of peripheral blood cells (PBC) from patients with advanced non-small cell lung cancer. (A) 122 patients had plasma NGS, revealing 14 mutations in CH-related genes and 108 mutations in *TP53*. PBC were available for analysis for 5 *JAK2* mutations and 33 *TP53* mutations (see Table 1). (B) Comparing the number of coding *TP53* mutations across 80 plasma specimens and 1245 tumor specimens with a *TP53* mutation detected using NGS, more *TP53* variants were detected per sample in plasma as compared to tumor (p<0.0001, chi-squared test), with 0.5% of tumor samples showing >2 mutations compared to 8.8% plasma samples. (C) Three cases had multiple *TP53* mutations found on plasma NGS, shown in relationship to the known oncogenic driver mutations (black), where some *TP53* mutations were derived from tumor (green) and some were derived from CH (orange).
Supplementary Figure 1: Droplet digital PCR (ddPCR) detected the *KRAS* mutation in peripheral blood cells (PBC, A) but did not detect the *EGFR* driver mutation (B). Highly sensitive next-generation sequencing (NGS, C) confirmed the presence of the *KRAS* G12D mutation in PBC and cellular subpopulations (D) at a similar allelic fraction (AF) as found with ddPCR.

Supplementary Figure 2: Droplet digital PCR (ddPCR) of the peripheral blood cell DNA from 4 cases with *JAK2* V617F found on plasma next-generation sequencing (NGS). In each, the JAK2 mutation was detected (top), consistent with clonal hematopoiesis, while not detected in a control sample.
References


Figure 1

- DNMT3A
- TET2
- ASXL1
- JAK2
- TP53
- GNAS
- IDH2
- KRAS

Prevalence (%) among CH-patients

Genovese (n=308)
Jaiswal (n=746)
Xie (n = 58)
McKerrell (n=105)
Zink (n = 299)
Coombs (n=1353)

* * * * * * * **

Research.
Figure 2

A Plasma ddPCR cohort

- **221** subjects with NSCLC who had plasma ddPCR for *EGFR* & *KRAS*

- **58** subjects with tumor genotyping positive for an *EGFR* mutation

- **2** of these with *KRAS* G12X mutations on plasma ddPCR

- **2** with PBC available for *KRAS* ddPCR

- **2** detected in PBC, consistent with CH

B

![Baseline 6.5 months 20 months](image)

C

![EGFR exon 19 del KRAS G12D](image)

D

- **Plasma cfDNA** 1.2% AF

  - Mixed Cell Fraction 0.5% AF

  - Monocytes: N/D
  - Lymphocytes: 1.7% AF
  - Granulocytes: N/D

  - CD45+/CD19+ -- B-Cells: 2.4% AF
  - CD45+/CD3+/CD8+ -- Cytotoxic T cells: 0.9% AF
  - CD45+/CD3+/CD4+ -- Helper T cells: 1.6% AF
  - CD45+/CD8-/CD4- -- γδ T Cells: 4.4% AF
143 plasma NGS specimens from 122 subjects with non-squamous NSCLC

129 specimens with mutations detected on plasma NGS, totaling 486 coding mutations detected

108 mutations detected in TP53

14 mutations in 3 CH-related genes (JAK2, GNAS, IDH2)

33 with PBC and tumor NGS available for TP53 NGS

5 with PBC available for JAK2 ddPCR

5 detected in PBC, consistent with CH

5 detected in PBC, consistent with CH

A Plasma NGS cohort

B

Number of TP53 variants

Percent of samples

Plasma (N = 80)

Tumor (N = 1245)

p < 0.0001

C

Allelic Fraction (%)

Patient 426

Patient 4188

Patient 3557

EGFR 19 del

EGFR K754E

PIK3CA E545K

TP53 Y220C (CH)

TP53 S127F (tumor)

TP53 V157L (unknown)

EGFR T790M

KIF5-RET fusion

TP53 Y220C (tumor)

CDKN2A R80*

TP53 M237I (CH)

TP53 C277Y (unknown)

MET exon 14 skipping

TP53 F134L (tumor)

KIT G961S

TP53 H193P (CH)

MET D1246N

TP53 C238Y (unknown)
False positive plasma genotyping due to clonal hematopoiesis


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