

# Detection of Three Distinct Clonal Populations Using Circulating Cell-Free DNA: A Cautionary Note on the Use of Liquid Biopsy

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## CLINICAL PRESENTATION AND FAMILY HISTORY

A 64-year-old man presented to a dermatologist with a pigmented lesion on his left forearm. The lesion was biopsied to reveal a 1.5-mm-thick malignant melanoma with 2 mitoses/mm<sup>2</sup> extending to the radial margin without ulceration. The patient subsequently underwent wide local excision with sentinel lymph node dissection. Pathology of the sample showed skin with melanoma in situ and no residual invasive melanoma identified with margins free of tumor. Two lymph nodes were identified with no tumor seen, as confirmed by Melanoma Antigen Recognized by T Cells 1 (MART1) immunostain. Final pathologic staging was stage IB (pT2aNO) based on the seventh edition of the American Joint Committee on Cancer melanoma staging system. The patient received no additional therapy at that time for his early-stage melanoma.

The patient was in good health for approximately 15 months until he reported bright red blood from his rectum. Colonoscopy revealed a left colon mass. Positron emission tomography/computed tomography revealed an approximately 3.1-cm colonic mass with a standardized uptake value of 26.4; several foci of abnormal [<sup>18</sup>F]fluorodeoxyglucose uptake in bilateral hepatic lobes and in the retroperitoneum and mesentery; a middle lobe lung nodule measuring 2 × 1.8 cm with a standardized uptake value of 7.5; and a right parietal brain lesion measuring 1.7 × 1.7 cm. Fine-needle aspiration was performed on a left iliac lymph node, which showed malignant cells positive for cytokeratin 20 (CK20) and caudal type homeobox 2 (CDX2) and negative for cytokeratin 7 (CK7), consistent with colorectal carcinoma. Reflex immunostains for mismatch repair (MMR) proteins showed loss of PMS1 homolog 2, mismatch repair system component (PMS2) (Fig 1A). Craniotomy was performed, with pathology showing metastatic melanoma positive for MART1, SRY-related HMG-box 10 (SOX10), and 100% soluble in ammonium sulfate at neutral pH (S100) in support of the diagnosis (Fig 1B). The patient reported no family history of colon cancer, but he did state that

his daughter was diagnosed with ovarian cancer at age 18 years.

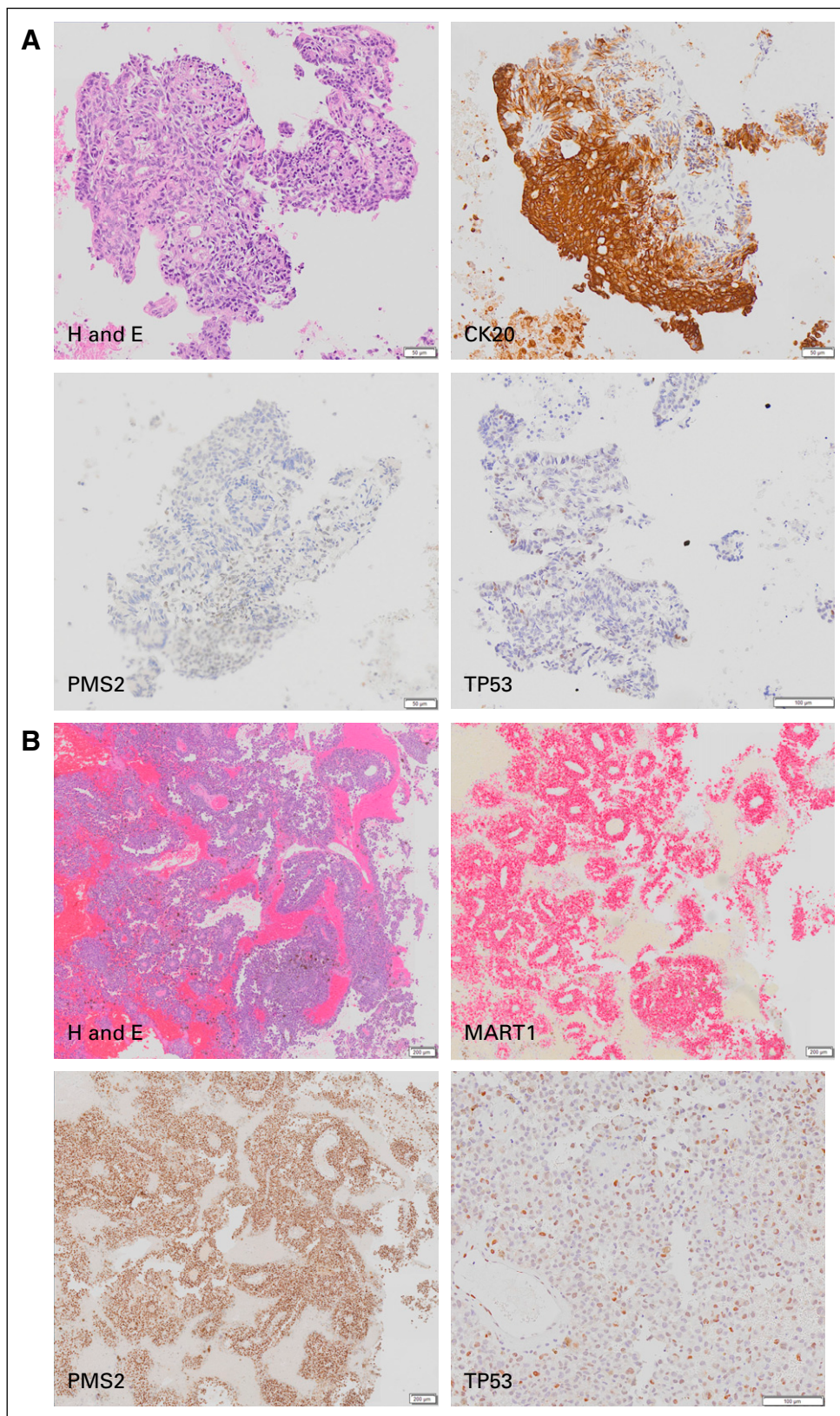
## GENOMIC ANALYSES

As part of reflex testing performed at our hospital for patients with advanced colon cancer and advanced melanoma, the patient's left iliac lymph node had *KRAS*, *NRAS*, and *BRAF* sequencing performed, whereas the craniotomy had *BRAF*, *NRAS*, and *KIT* sequencing. The colon cancer sample was found to have a *KRAS* c.183A>T, p.Gln61His mutation, whereas the melanoma harbored a *KIT* c.1669T>G, p.Trp557Gly mutation.

The patient subsequently had his blood analyzed for circulating tumor DNA (ctDNA) using the Foundation One Liquid platform (Foundation Medicine, Cambridge, MA), which consisted of hybrid capture sequencing of 70 genes for single nucleotide variants, indels, and copy number alterations with rearrangement detection performed for seven of these genes. This test is also able to identify microsatellite instability. Testing detected the *KRAS* c.183A>T, p.Gln61His mutation identified previously in the colon cancer specimen at 17.41% allele frequency and the *KIT* c.1669T>G, p.Trp557Gly mutation identified previously in the melanoma sample at 0.23%. The specimen was identified as having high microsatellite instability, consistent with the loss of PMS2 by immunohistochemistry (IHC) in the colon cancer specimen. Intriguingly, when all of the alterations identified using the ctDNA assay were viewed, there seemed to be two main allelic fractions, one with alterations at an allele frequency of less than 1% and another with allele frequencies from 13.43% to 35.9% (Table 1). The higher allele fraction seemed to correlate with known drivers of colon cancer tumorigenesis, which included the *KRAS* c.183A>T, p.Gln61His mutation; three *APC* mutations (c.4666\_4667insA, p.T1556fs\*3; c.1178C>A, p.S393\*; and c.904C>T, p.R302\*), and a *PTEEN* c.800delA, p.K267fs\*9 frameshift mutation. On the basis of clonal analysis of these variants and assuming ctDNA content of approximately 35% colon cancer cells,<sup>1</sup> the *KRAS* and *APC* variants had allele frequencies compatible with being heterozygous in the

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**FIG 1.** (A) Histology of left iliac lymph node involved with colorectal cancer. (B) Histology of craniotomy specimen with metastatic melanoma. H and E, hematoxylin and eosin.

**TABLE 1.** Variants Detected in Circulating Tumor DNA

Gene	Amino Acid Variant	Variant Allele Frequency, %	Total Depth	Chromosome and Position	Variant (in brackets) and Sequence Context	MTB Discussion
<i>ABL1</i>	M318T	0.22	3,594	chr9:133748292	GTTCA[T>C]GACCT	Putative melanoma
<i>KIT</i>	W557G	0.23	3,069	chr4:55593603	TACAG[T>G]GGAAG	Pathogenic/confirmed in melanoma
<i>CCND1</i>	P220T	0.24	3,321	chr11:69462845	GGAGC[C>A]CCAAC	Putative melanoma
<i>SMO</i>	T336A	0.26	4,632	chr7:128846076	TCCTC[A>G]CCTAT	Putative melanoma
<i>BRCA2</i>	H910R	0.27	3,640	chr13:32911221	ACTTC[A>G]TGAAA	Putative melanoma
<i>RAF1</i>	L546R	0.27	3,673	chr3:12626652	AAGGA[A>C]GCTCC	Putative melanoma
<i>MTOR</i>	N2219fs*60	0.29	3,400	chr1:11184560	CTGAG[T>–]TTTTTC	Indel in homopolymer region
<i>ATM</i>	S214P	0.32	2,820	chr11:108114823	TTTTT[T>C]CCAAG	Substitution in homopolymer region
<i>ALK</i>	N1095fs*14	0.36	4,448	chr2:29446284	TAGTT[G>–]GGGTT	Indel in homopolymer region
<i>MDM2</i>	Y287H	0.38	3,169	chr12:69230470	CTGTG[T>C]ATCAG	Pathogenic/putative melanoma
<i>NF1</i>	A1224V	0.41	3,163	chr17:29560194	TATAG[C>T]GATGG	Putative melanoma
<i>CDK12</i>	R298*	0.48	3,513	chr17:37619216	GTAGG[C>T]GACAG	Pathogenic/putative melanoma
<i>STK11</i>	F25L	0.48	3,127	chr19:1206985	ACACG[T>C]TCATC	Putative melanoma
<i>ERRF1</i>	F115fs*6	0.5	2,999	chr1:8074313	TTCTT[A>–]JAAACC	Pathogenic/indel in homopolymer region
<i>TP53</i>	G244C	0.54	2,795	chr17:7577551	GCCGC[G>T]CATGC	Pathogenic/putative CHIP
<i>MTOR</i>	L1460P	0.58	3,097	chr1:11217299	CCACA[A>C]GGGCA	Pathogenic/putative melanoma
<i>PTPN11</i>	V243A	0.7	3,122	chr12:112893839	TAAAG[T>C]CAAAC	Putative melanoma
<i>BRCA1</i>	S1387P	0.7	3,131	chr17:41242987	CTGAG[A>G]GGATA	Putative melanoma
<i>PDCD1LG2</i>	P186fs*14	1.71	4,155	chr9:5549529	CACCC[C>–]CTGGC	Indel in homopolymer region
<i>EGFR</i>	S1104A	1.9	3,527	chr7:55272987	CTGGC[T>G]CTGTG	Putative melanoma
<i>BRCA2</i>	L929fs*31	13.43	3,261	chr13:32911277	TGGTT[T>–]TATAT	Pathogenic/indel in homopolymer region/putative colon cancer
<i>KRAS</i>	Q61H	17.41	2,837	chr12:25380275	TCCTC[T>A]TGACC	Pathogenic/confirmed in colon cancer
<i>APC</i>	R302*	17.46	3,952	chr5:112151261	CACCT[C>T]GAAGG	Pathogenic/putative colon cancer
<i>APC</i>	S393*	18.21	4,294	chr5:112154907	TCACT[C>A]ACAGC	Pathogenic/putative colon cancer
<i>ATM</i>	K2465E	18.49	3,662	chr11:108201026	TATGT[A>G]AAGCA	Putative colon cancer
<i>GNAS</i>	R199H	20.22	3,036	chr20:57484415	GCTTC[G>A]CTGCC	Putative colon cancer
<i>APC</i>	T1556fs*3	22	4,169	chr5:112175957	AAAAA[–>A]CTATT	Pathogenic/indel in homopolymer region/putative colon cancer
<i>PTEN</i>	K267fs*9	35.9	3,309	chr10:89717774	TAAAA[A>–]JAGGTT	Pathogenic/indel in homopolymer region/putative colon cancer

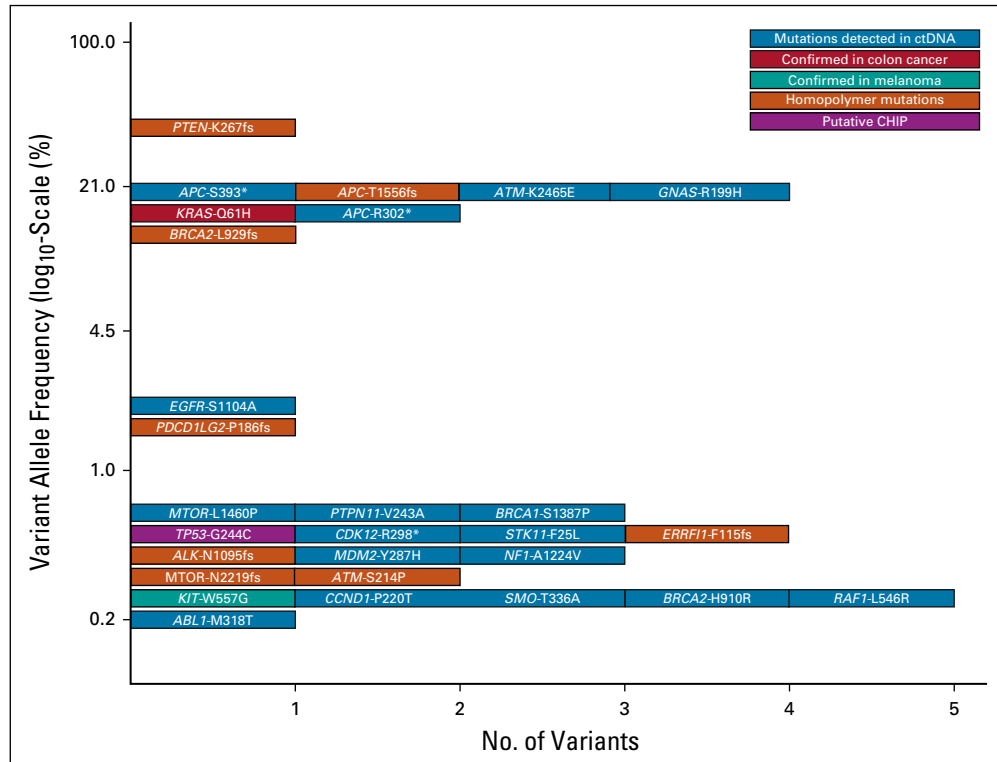
NOTE. Variants described as pathogenic were reported on the front page of the Foundation One Liquid assay, Foundation Medicine (Cambridge, MA) report in genomic findings. Other listed variants were reported by Foundation Medicine as variants of unknown significance.

Abbreviations: CHIP, clonal hematopoiesis of indeterminate potential; MTB, molecular tumor board; Total Depth, total depth of sequencing reads.

tumor, whereas the *PTEN* variant was at an allele frequency compatible with being homozygous in the tumor as a result of loss of heterozygosity or gene conversion to inactivate the second wild-type allele. These observations, in addition to the *KIT* mutation having an allele fraction of 0.23%, raised the possibility that the lower allele fraction alterations may be arising from the patient's melanoma (Fig 2). Several studies have shown that ctDNA correlates with tumor burden, and it may be that the only melanoma tissue mass in this patient was in his brain.<sup>2,3</sup> The reflex testing of the patient's colon cancer and melanoma was performed using

the Illumina Trusight Tumor 15 gene panel (Illumina, San Diego, CA), with results masked to only report out specific genes depending on the indication (ie, *KRAS*, *NRAS*, and *BRAF* for colon cancer and *BRAF*, *NRAS*, and *KIT* for melanoma). When the original sequencing results were examined for all 15 genes, the *KRAS* c.183A>T, p.Gln61His mutation was only seen in the patient's colon cancer at 43.4% allele frequency, and the *KIT* c.1669T>G, p.Trp557Gly mutation was only seen in the patient's melanoma at 38.8% allele frequency. Astonishingly, despite the high allele frequencies of the *KRAS* and *KIT*





**FIG 2.** Allele frequencies of variants detected in circulating tumor DNA (ctDNA). CHIP, clonal hematopoiesis of indeterminate potential.

mutations, the *TP53* c.730G>T, p.G244C mutation identified in ctDNA was not identified in either sample. Studies have shown that clonal mutations in the hematopoietic compartment may be detected on tumor tissue testing.<sup>4,5</sup> The results suggest that ctDNA analysis in this patient identified not only clonal DNA from two separate solid tumors, but also an additional third clonal mutation in the hematopoietic compartment, as has been recently described.<sup>6</sup> The patient was evaluated at Rutgers Cancer Institute of New Jersey and provided informed consent to participate in a prospective study trial for tumor genomic profiling (ClinicalTrials.gov identifier: [NCT02688517](https://clinicaltrials.gov/ct2/show/study/NCT02688517)). The results of genomic profiling, clinical course, and pathology were reviewed at the Rutgers Cancer Institute of New Jersey Molecular Tumor Board (MTB), which was approved by the Rutgers University New Brunswick Health Sciences Institutional Review Board (Pro2012002075). The patient is now deceased, but his informed consent to participate in the NCT02688517 trial covers approval for publication of these results.

As a result of the colon cancer being deficient for PMS2, the ctDNA showing high microsatellite instability, and the benefit in a subset of patients with melanoma, the primary MTB recommendation was to use immune checkpoint inhibitors to treat this patient.<sup>7</sup> Interestingly, when the genomic context of alterations identified in the ctDNA analysis was analyzed, three alterations in homopolymer

regions were identified at higher allele frequency, whereas five homopolymer alterations at low allele frequency were found, raising the possibility that the melanoma was also MMR deficient (Table 1 and Fig 2).

For the patient's melanoma, the MTB discussed data showing responses to imatinib in patients with activating *KIT* mutations.<sup>8</sup> These data suggested acquired *NRAS* mutations or *KIT* amplification as a resistance mechanism. A published report of a patient with rectal melanoma harboring an identical *KIT* c.1669T>G, p.Trp557Gly mutation with a 7-month response to sunitinib was presented. When this patient ultimately experienced relapse, a new *NRAS* p.Q61K mutation was detected.<sup>9</sup> The MTB noted that this patient had a previously described activating mutation in *MTOR* (c. 4379T>C, p. L1460P) at allele frequencies compatible with being present in the patient's melanoma.<sup>10</sup>

Other alterations that the MTB discussed as alternative targets of treatment included a *BRCA2* c.2786delT, p.L929fs\*31 truncating mutation identified in ctDNA at 13.43% allele frequency. It was likely that only one allele was inactivated in *BRCA2* in the colon cancer based on the allele frequency being compatible with it being heterozygous. Because the alteration occurred in a homopolymer region and *BRCA2* is a large gene, this may have been a passenger alteration as a result of the tumor being deficient in MMR. Nonetheless, because ctDNA could not

determine whether both alleles were inactivated, the MTB suggested that platinum drugs or poly (ADP-ribose) polymerase inhibitors could be effective. In addition, a *CDK12* c.892C>T, p. R298\* inactivating mutation was identified as possibly being in the patient's melanoma on the basis of its allele frequency. Recent work has shown that *CDK12* regulates DNA damage response genes, including *BRCA1*, and is sensitive to DNA-damaging agents and poly (ADP-ribose) polymerase inhibitors.<sup>11,12</sup> The MTB suggested that these agents may be an option with the potential to target both the *BRCA2* truncating mutation and the *CDK12* inactivating mutation. In addition, during the MTB meeting, accumulating evidence suggesting that loss of genes involved in DNA damage repair, such as *BRCA2* or *CDK12*, may have a role in sensitivity to immune checkpoint inhibitors was presented.<sup>13,14</sup>

It is unusual that three truncating *APC* mutations that likely arose from the patient's colon cancer at heterozygous levels were identified. *APC* mutations are typically correlated with microsatellite-stable colon cancer, whereas one mutation of each allele would be sufficient to inactivate the protein. It may be that the colon cancer initially developed along the microsatellite-stable pathway with biallelic inactivation of *APC* before acquiring an additional frameshift mutation as a result of loss of MMR function, with *APC* being particularly susceptible to being damaged by a loss of MMR because it is a large gene. One *PTEN* allele seems to have been inactivated as a result of the defect in MMR with loss of heterozygosity or gene conversion of the second allele, whereas *BRCA2* likely only had one allele inactivated by MMR deficiency in a similar manner to the third *APC* mutation.

Additional recommendations from the MTB included genetic counseling with the patient harboring a PMS2-deficient colon cancer and having a daughter with a questionable history of ovarian cancer at a young age, PMS2 IHC to be performed on the patient's craniotomy melanoma specimen, and consideration for sequencing washed peripheral-blood mononuclear cells to determine whether the *TP53* p.G244C mutation was present in the hematopoietic compartment.

### TREATMENT OUTCOMES

The patient was started on nivolumab. Shortly after, the patient became septic, developed endocarditis, and ultimately died during an operation to remove the aortic valve.

After the MTB meeting, PMS2 IHC was performed on the melanoma from the craniotomy, which showed intact

PMS2 (Fig 1B). Therefore, the five lower allele frequency variants showing alterations in homopolymer regions most likely represent subclonal alterations in the colon cancer as a result of the defect in MMR. Also after the MTB meeting, sequencing of a lymph node negative for tumor from the patient's original sentinel lymph node dissection on the Illumina Trusight Tumor 15 gene panel was performed. This was also found to be negative for *TP53* at c.730G>T, p.G244C. Although finding this mutation in the lymph node would have provided strong evidence that it arose in the hematopoietic compartment, not finding it does not disprove this hypothesis. The *TP53* p.G244C mutation was present at an allele fraction of 0.54% in the blood, and although it would be expected to be detected in the melanoma or colon cancer tissue samples if it was present, it could be present at this relatively low fraction in the blood and not be present in a lymph node. In addition, because the lymph node was removed 18 months before the patient's blood was sequenced, the *TP53* mutation may not have even been present at that time or present at even lower levels. As ancillary confirmation, TP53 IHC was performed on both the craniotomy with melanoma and the iliac lymph node fine-needle aspiration with colon cancer. In both cases, TP53 was negative in tumor cells, although melanin and occasional TP53-positive cells were observed in the melanoma (Figs 1A and 1B). *TP53* somatic missense mutations in cancer are generally reported to be positive by IHC, and p.G244C was reported to be positive in 17 of 18 samples.<sup>15</sup> In total, the negative sequencing and IHC results strongly suggest the possibility of the *TP53* p.G244C mutation having arisen in the hematopoietic compartment.

In summary, ctDNA profiling identified alterations at different allele frequencies that seemed to correspond with mutations present in his colon cancer and melanoma. Remarkably, there was a *TP53* mutation identified in the liquid biopsy, with subsequent sequencing and IHC results indicating it was unlikely to be present in either of the solid tumors and suggesting a third clonal mutation in the hematopoietic compartment. When ctDNA analysis is performed, knowledge of genomic alterations in the primary cancer may not be available, and occasionally, there may be a second unknown primary cancer. This patient's case demonstrates potential confounding factors in genomic profiling of ctDNA and the value in presentation at MTB, where members with different expertise can help in properly interpreting the results.

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