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Clonal hematopoiesis and mosaicism revealed by a multi-tissue analysis of constitutional *TP53* Status

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Abstract

Background: Though germline *TP53* pathogenic/likely pathogenic variants (PV) are associated with Li-Fraumeni syndrome, many detected by multigene panels represent aberrant clonal expansion (ACE), most due to clonal hematopoiesis (CH). Discerning ACE/CH from germline variants and post-zygotic mosaicism (PZM) is critically needed for risk assessment and management.

Methods: Participants in the Li Fraumeni & *TP53* Understanding & Progress (LiFT UP) study with a *TP53* PV were eligible. Demographics, personal/family cancer history and clinical laboratory test reports were obtained. DNA from multiple tissues was analyzed using a custom QIAseq[®] assay (ACE panel) that included *TP53* and other CH-associated genes; the ACE panel and eyebrow follicles were assessed in a workflow to discern *TP53* PV clinical categories.

Results: Among 134 participants there was a significant difference for the age at diagnosis ($p < 0.001$), component cancers ($p = 0.007$), and clinical testing criteria ($p < 0.001$), comparing germline vs PZM or ACE. ACE panel analysis of DNA from 55 sets of eyebrow follicles (mean 1.4 ug) and 36 formalin-fixed paraffin imbedded tissues demonstrated low variance (standard error

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Prior presentations

Portions of the work were presented as a poster at the 2020 annual meeting of the American Society of Clinical Oncology.

Conflicts of Interest

The other authors declare no conflict of interest.

3%; $p = 0.993$) for *TP53* variant allele fraction, with no significant difference ($p=0.965$) between tissue types, and detected CH gene PVs. Of 55 multi-tissue cases, germline status was confirmed for 20, PZM in 7, ACE for 25 and 3 were indeterminate. Additional CH variants were detected in 6 ACE and 2 germline cases.

Conclusions: We demonstrated an effective approach and tools for discerning germline *TP53* status.

Impact: Discernment of PZM and *TP53*-driven CH increases diagnostic accuracy and enables risk-appropriate care.

Keywords

Li-Fraumeni Syndrome; aberrant clonal expansion; clonal hematopoiesis; clonal hematopoiesis of indeterminate potential; germline; pathogenic variant; variant allele fraction

Introduction

Germline *TP53* pathogenic/likely pathogenic variants (PV) underlie Li-Fraumeni Syndrome (LFS), a rare inherited cancer susceptibility syndrome characterized by a predisposition to a broad spectrum of cancers and risk for multiple primary cancers at early ages (1–3). Core cancers include sarcoma, brain, breast, and adrenocortical cancers (4–6).

Putative germline *TP53* variants identified through hereditary cancer multi-gene panel tests (MGPT) are often found in individuals and families who share limited features with classic LFS (7), raising questions about the validity of established penetrance estimates. In addition, low (<30%) variant allele fraction (VAF) *TP53* PVs identified by next generation sequencing (NGS)-based MGPT are frequently due to aberrant clonal expansion (ACE) of marrow precursors with an acquired *TP53* PV (8–12), and must be distinguished from germline (constitutional) variants and true post-zygotic mosaicism (PZM), given the different clinical implications.

Clonal hematopoiesis (CH) is increasingly recognized as a somatic finding that has clinical implications regarding therapy-related hematologic malignancies and all-cause mortality (13). Skin biopsy with fibroblast culture may be used when a non-hematological source of germline DNA is needed, but is invasive, costly and time consuming, and not all diagnostic laboratories can process the samples. We developed tools for detecting CH and discerning constitutional *TP53* status, including an ACE panel to confirm the prevalence and clinical impact of *TP53*-associated ACE, determine co-occurrence of PVs in myeloid genes, and validate eyebrow follicles as a component of multiplex tissue analyses, including skin biopsies and formalin fixed paraffin-embedded (FFPE) normal and tumor tissues.

Finally, we include a schema and clinical criteria to validate the constitutional status of *TP53* PVs across tissues, and systematically characterize *TP53*-driven CH and PZM cases.

Materials and Methods

Participants

Eligibility criteria included all individuals (n=134) with commercial germline test results reporting *TP53* PV(s), including those with: (a) putative heterozygous germline, (b) VAF of less than 30%, (c) clinical history or molecular results suspicious for ACE (e.g., active hematologic neoplasia), or (d) an atypical clinical phenotype (i.e., not meeting *TP53* clinical testing guidelines). Affected cases with a *TP53* PV who met Li-Fraumeni syndrome (LFS) diagnostic criteria (supplemental table 1), and/or had informative cascade testing were considered germline (n=69), and additional tissues were requested for the remaining cases (n=65); tissues were obtained for 55 of the 65 cases (85%). All participants were accrued to the Li-Fraumeni & *TP53* Understanding & Progress (LiFT UP) study (<https://liftupstudy.org>) and provided written informed consent for City of Hope IRB-approved protocol number 96144 (NIH clinical trial ID [NCT04185935](https://clinicaltrials.gov/ct2/show/study/NCT04185935)) June 2002 through October 2020. Participants were referred to study from the Li-Fraumeni Exploration Consortium (LiFE) (14,15), the Li-Fraumeni Syndrome Association (<https://www.lfsassociation.org/>), Living LFS (<https://livinglfs.org/>), the Clinical Cancer Genomics Community Research Network (16,17), genetic testing laboratories, and self-referral via a RedCap survey on the LiFT UP website.

Clinical Data

We obtained demographic information, personal/family cancer history, risk factor data via questionnaire, complete blood count and genetic testing reports (for the individual [including fibroblast testing from skin biopsies if available], as well as for cascade/transmission testing for family members) (17). We determined whether an individual met *TP53* testing criteria (supplemental table 1) (5,18). Quantitative VAF data was obtained from genetic testing laboratories when possible; Agilent hybrid capture sequencing library preparation methodology was used for most of the respective CLIA-certified Laboratory Designed Tests.

Biospecimens

Whole blood and/or a saliva sample, eyebrow plucks, and an archival normal and/or tumor FFPE tissue sample were collected. Blood (serial samples over time for a subset) and saliva were collected and processed as reported previously (16). Eyebrows were cleaned with an isopropanol wipe and 15–20 undyed eyebrow follicles were self-collected with a sterile tweezer into a falcon tube, dry, or in 1x PBS, or in alcohol-free mouthwash. Eyebrows were plucked close to the base of the shaft and in the direction of the growth (19). Both normal tissue (non-lymphatic) and tumor tissues were micro dissected from FFPE tissue blocks on 10-micron unstained slides for DNA extraction.

DNA Extraction

DNA was extracted from biospecimens using QIAGEN extraction kits (QIAGEN, Inc., Germantown, MD, USA); the Flexigene[®] DNA kit was used for whole blood; the Gentra Puregene[®] Cell Kit was used for mouthwash; the QIAamp[®] DNA Mini Kit was used for the eyebrow follicles (20) and for FFPE tissue extraction (COH Molecular Pathology Core).

Genomic DNA quality was determined by optical density (OD) 260/280 value, using a NanoDrop 2000c spectrophotometer (Thermo Fisher, Chino, CA).

Genotyping

We designed a custom QIAseq[®] (QIAGEN, Inc., Germantown, MD, USA) amplicon-based 81-gene panel (ACE panel) for detecting cancer predisposition variants, including *TP53*, and genes known or thought to be involved in CH (11,13,21,22). Content includes common ACE/CH-associated variants in genes such as *ASXL1*, *ATM*, *CBL*, *CHEK2*, *EZH2*, *ETV6*, *DNMT3A*, *GNB1*, *GNAS*, *IDH1*, *IDH2*, *JAK2*, *KIT*, *NF1*, *MPL*, *NPM1*, *NRAS*, *MYD88*, *PHF6*, *PPM1D*, *RUNX1*, *SETBP1*, *SF3B1*, *SRSF2*, *STAT3*, *TET2*, *TP53*, *U2AF1*, *ZRSR2*, and *FLT3*, and the design includes the 5' and 3' untranslated regions, full exonal gene coverage, and extends 10 base-pairs into introns, and results in a consistent coverage of over 300–500x. 150 base-pair, paired-end sequencing was performed on the HiSEQ 2500 Genetic Analyzer (Illumina Inc., San Diego, CA).

To assess test/retest reliability we created ACE panel libraries in triplicate for 14 samples. Sequencing libraries were also created with a custom 760 cancer gene panel (23,24) based on Agilent hybrid capture technology. *TP53* PV-specific Sanger resequencing was also performed for 6 samples.

Genotyping Curation and Quality Control

Variant call format (VCF) files were evaluated using Ingenuity Variant Analysis (IVA) version 4 (QIAGEN, Inc., Germantown, MD, USA) as per previous studies (21). We removed variants with a call quality less than 20, read depth less than 10x or alleles with a frequency greater than 3% in the 1000 Genomes project, National Heart, Lung and Blood Institute, Exome Sequencing Project exome or ExAC databases. According to contemporary definitions of CH, all identified PVs with >1% VAF and at least 50x locus coverage were considered (21).

Data Analysis

All cases were included in analyses of clinical characteristics, and cases with additional tissue(s) were analyzed by the ACE panel and categorized according to the schema in Figure 1. Statistical analyses were performed by SAS[®] (v9.4TS1M3, Cary, NC, USA) and RStudio (version 1.4.1103). χ^2 test of independence or Fisher's exact test was carried out for descriptive analysis. The two-sided significance level was set at 0.05. Performance characteristics of the ACE panel and eyebrow pluck DNAs were also analyzed. Within-participant analysis of variance (ANOVA) test was used to determine variability of VAFs for triplicate samples on the ACE panel. VAF measured by the ACE panel, the 760 gene Agilent capture panel, and original commercial laboratory testing (if available) were compared, noting sampling timeframe. Pearson correlation coefficient (*r*) that measures a linear association was performed between number of eyebrow follicles and extracted DNA quantity. Between-participant ANOVA test was conducted to compare VAFs among normal/tumor tissues and eyebrows.

Data Availability

All observed *TP53* PVs have been reported previously in ClinVar by the respective commercial laboratories. All clonal hematopoiesis gene variants were included in Figure 3, and are also reported in ClinVar. All data necessary to replicate our findings are included in the manuscript and supplemental tables.

Ethics Declaration

Protocol number 96144 (NIH clinical trial ID [NCT04185935](#)) was approved by the City of Hope IRB. All participants provided written informed consent.

Results

In total, 134 cases had commercial germline test results reporting *TP53* PV(s), along with 125 blood samples, 31 saliva samples, 57 eyebrow samples, 17 tumor blocks, and 19 normal tissue samples that were analyzed as part of the study. Each case was assigned to a clinical category by way of pre-existing ancillary data. After an initial review, 69 cases were determined to be germline. Of the remaining 65 cases, additional tissues were obtained for 55 (85%) cases. After applying our analyses, we were able to assign clinical categories for 122/134 cases (12 cases remained indeterminate).

Participant characteristics and clinical categories are summarized in Table 1. Most participants were female (89%), white (57%) or Hispanic (26%), under the age of 50 years (70%) at accrual (average 43 years; range 1 – 82 years), had a personal history of cancer (89%) with an average age at diagnosis of 37 years (range 1 – 80 years). The most common cancer types included breast (n=100), sarcoma (n=28), lung (n=10), colorectal (n=9), thyroid (n=8), leukemia (n=7), brain (n=3), and adrenocortical (n=3). There was a significant difference for the age at entry ($p=0.001$), age at diagnosis ($p<0.001$), component cancers ($p=0.007$), and presence/absence of clinical testing criteria ($p<0.001$), among those discerned to be germline vs PZM or ACE (Table 1).

Clinical categories were discerned according to a derived flowchart (Figure 1) for the 55 participants with multiple tissues analyzed with the ACE panel (Table 2 and supplemental figure 1), and additional CH-driver PVs were detected (Table 3).

High quality genomic DNA was obtained from 55 of 57 eyebrow samples. Two failed due to insufficient quantity of follicles. DNA yield from eyebrows ranged from 0.5 μg to 4.7 μg ; (average 1.4 μg) (Supplemental Table 2); OD 260/280 value ranged from 1.77 to 2.96 (average 2.10), and there was a linear correlation between number of follicles and DNA quantity (Supplemental Figure 2). Eyebrow DNA yield (range 0.5 μg to 18.4 μg ; average 3.61 μg ; average OD ratio 2.19).

We compared *TP53* VAFs in eyebrows to normal and tumor tissues (Supplemental Table 3). Normal and tumor tissues were micro-dissected from archival tumor blocks and showed comparable *TP53* VAF. *TP53* VAFs detected in eyebrows were highly correlated with VAFs in normal and tumor tissues, with a between-participant ANOVA test of $F \sim F_{(2,35)} = 0.0357$ and $p = 0.965$.

There was no significant variability ($p = 0.993$) of VAF for within-participant ACE panel analysis (standard error 3%) with 3 separate libraries created from the same DNA sample (Figure 2). In addition, we compared the result from our custom capture, and the VAF obtained from commercial laboratory MGPT. There was no significant difference for VAF between ACE panel and Agilent capture/commercial testing ($p=0.895$). Semi-quantitative Sanger sequencing was done for 6 cases, but not included in the statistical analysis.

Beyond quantification and validation of the reported *TP53* VAF, all 55 cases in the multi-tissue component cohort were also analyzed by the ACE panel for evidence of known CH driver PVs. Additional CH-associated PVs (*ATM*, *DNMT3A* [n=3], *TET2* [n=2] and *TP53* PVs [n=2]) were identified in addition to the known *TP53* PV in 6 of 25 ACE cases and in 2 germline cases (Table 3). Note that detection of additional CH-driver PVs, while of great interest and more prevalent in the ACE cases, were not used to categorize a given case.

The VAF for these CH-PV also demonstrated consistent results on test-retest for the 4/7 cases included in the validation experiment (marked with * in Figure 2; respective VAFs noted in Table 3) and approximated that for the known *TP53* PV for 2 cases; the others were at a lower VAF (range 1–3.4%). Four of seven cases with an additional CH-PV received prior cytotoxic therapy for cancer, and one is in long-term clinical remission from aplastic anemia. One case with two *TET2* PVs was a 79-year-old male with a family history of pancreatic cancer and was unaffected at the time of commercial testing but was diagnosed with locally advanced prostate cancer one year later.

There were 69 affected cases with a *TP53* PV who met Li-Fraumeni syndrome (LFS) diagnostic criteria, and/or had informative cascade testing and were considered germline. Additional tissues were obtained for 55 of the remaining 65 cases (85%). Multi tissue discernment according to the schema in Figure 1 was successful for 52 of 55 cases. Figure 3 depicts the proportions in context for the total cohort of 134: 89 (66%) were germline, 26 (19%) were ACE, and 7 (5.2%) were PZM; each of the PZM cases demonstrated the presence of the *TP53* PV in tissues representing at least 2 germ layers. Of the 12 (9%) cases that remained indeterminate, tissues were not available for 8 cases, 3 cases the secondary biospecimen failed and 1 case (case 54) where potential infiltrating inflammatory cells confounded our categorization. Quality check indicated that the eyebrow samples for these cases failed due to collection of < 5 follicles. There is a direct relationship between the number of follicles and DNA quantity and quality, with a yield of at least 0.5 ug from 15 follicles (Supplemental Table 2 and Supplemental Figure 2).

Discussion

Prior to the advent of NGS based-MGPT, commercial germline testing of *TP53* has been limited to individuals and families who met specific LFS testing criteria, such as classic, Chompret, Birch, Eeles, or NCCN (5,18,25). Our multi-tissue testing schema enabled discernment of patients with *TP53* PVs identified on MGPT that did not have clinical characteristics (or meet *TP53* testing criteria) of LFS, revealing a high proportion with ACE. These participants rarely met *TP53* testing criteria (4%), had no core LFS cancers beyond breast cancer (5), and if affected were diagnosed with cancer at ages that were more typical

of sporadic disease. Two recent studies noted a similar discordance between testing criteria and clinical category in the context of ACE/CH cases (12,26).

Our approach also illuminated participants with mosaicism. We observed that 59% of the cases selected for multi-tissue analysis were either ACE (46%) or PZM (13%), presumably reflecting selection bias from focus on cases that raised concerns about the phenotype. The overall categorical assignments across our clinical genetic testing cohort reflect a 19% rate of ACE plus 5% PZM (total is 24%), which is similar to the reported rates of ACE from three commercial laboratory series wherein 22–40% MGPT-detected *TP53* PVs represent ACE (8,9,12,26,27). Clearly one cannot discern PZM cases, where there is an undefined but likely increased cancer risk depending on tissue distribution, without a multi-tissue approach representing the respective germ layers (9,28,29). Based on our study, we believe there is likely a similar proportion of PZM cases among the ACE cases in the other reported studies. Similar to a report from one genetic testing vendor (12), we observed apparent PZM among 13% of the 55 cases selected for multi-tissue analysis; this represents 5% of our overall genetic testing cohort (Figure 3). Importantly, *TP53*-associated PZM is still potentially transmissible to offspring (30) and is likely associated with increased cancer risk depending on tissue distribution of the PV. In addition, beyond potential misapplication of LFS-related care, failure to exclude ACE and PZM cases would bias *TP53* penetrance estimates. VAF for a heterozygous germline PV is typically around 50% but can range from 30 – 60% (31). PZM is suspected when multiple tissues demonstrate a comparable low VAF (<30%). Our data for the PZM cases showed that the VAF varied, and was as high as 47% which overlaps the germline range. Further *TP53*-associated ACE may also have a VAF overlapping with the range seen in PZM. Thus, *TP53* testing of ancillary tissues and transmission testing is paramount to distinguish between these two states.

In many cases, ACE reflects CH of indeterminate potential (CHIP) (32), which is observed in healthy populations at increasing frequency with increasing age (33,34). We created an amplicon-based MGPT that includes ACE/CH-specific genes for this study (LiFT UP). We demonstrated the validity of the ACE panel through test/re-test ($p=0.993$; 3% within-participant standard error) as well as across other NGS-based MGPT platforms ($p = 0.895$). The ACE panel performed equally well on all biospecimens, thus allowing comparison across tissues, and performed as designed to detect additional CH-driver PVs. *TP53*-associated CHIP is the 5th most common finding in previous studies, and along with other acquired myeloid neoplasia-related gene PVs, has been linked with increased risk of hematological neoplasia, cardiovascular diseases, and all-cause mortality (11,13,33).

The prevalence of additional CH variants in ACE cases is unknown, since hematopoietic genes are not included in most available clinical MGPT. We observed additional PVs in 24% of ACE cases and 10% of the germline cases that were analyzed with the ACE panel. Previous reports on CH suggest that there is an increased risk for progression to hematologic neoplasia when more than 1 driver PV is identified (13,32,35,36). Data from a large institutional tumor sequencing project provided evidence for both induction of CH-PVs in hematologic stem cell precursors by cytotoxic cancer therapies, as well as selection for CH (36). Understanding the clinical implications of *TP53*-driven CH is important, given the evidence suggesting that CH initiating variants often precede exposures associated with

therapy-related myeloid neoplasia (tMN) (36–38), rather than the traditional thought that tMN develops from cancer therapy per se. Further, there is evidence from studies of the genomic landscape of MDS that highlight the critical effect of acquired *TP53* allelic state in particular, demonstrating poorer outcome for multi-hit cases, and affirm the relevance of other CH PVs (e.g., *TET2*, *ASXL1*) in the setting of monoallelic *TP53* PVs (22). Our observation of additional CH-PVs documented in 2 of 20 (10%) germline cases poses a related question about whether a patient with a germline *TP53* PV is at risk for developing CH and/or myeloid neoplasms. Though leukemia was part of the original LFS phenotype (39), it is no longer considered a core cancer in the syndrome. Further studies of the prevalence of CH among germline *TP53* carriers is warranted. There may be clinical utility for serial CBCs and ACE panel analyses (ideally with a CLIA certified assay) in ACE/CH cases to measure VAF change over time, subsequent acquisition of other CH-driver PVs, and monitoring for the potential development of hematological neoplasia. Given limited access to germline hematologic panels and lack of consensus in guidelines regarding workup of apparent CH/CHIP, clinicians may consider use of the ACE panel developed for this study, exploratory though we believe such a tool should be made clinically available.

Clinically, a skin biopsy with fibroblast culture is used when a non-hematological source of germline DNA is needed. Our data shows reliable and reproducible extraction of high-quality genomic DNA from eyebrows, correlation with results from skin biopsies and FFPE normal and tumor tissues. This supports the integration of eyebrows as a biospecimen type by commercial laboratories, as suggested by a recent statement of the American College of Medical Genetics and Genomics (40). Plucked hair follicles are an easily collected and less invasive alternative to skin biopsy, particularly if a small quantity of high-quality DNA will suffice for clinical diagnostics. We recently used eyebrow follicles to help characterize the distribution of a *DNMT3A* PV in a patient with PZM for that gene (28). Our procedures and schema (Figure 1) may be applicable to other testing scenarios where there is the possibility of ACE confounding diagnosis, such as *ATM* or *CHEK2* PVs with low VAF (8).

Selection of cases for application of the multi-tissue strategy was biased toward inclusion of those lacking classic LFS features or evidence of transmission among family members, so it was not surprising that half of the germline cases did not meet testing criteria for LFS, and that ACE was prevalent (46%; supplemental figure 2). Cascade or transmission testing was subsequently able to independently discern germline status in 3 cases. When considering our cohort as a whole (Figure 3), the proportion of cases with ACE and PZM was 24%, supporting that the respective population was comparable to published series from commercial laboratories, where the rate of ACE ranges from 22%–40% (8,9,12,27).

For all ACE and PZM cases, more than one additional tissue was needed for discernment, and demonstrated the absence of the *TP53* PV in additional tissues associated with ACE/CH or the presence of the *TP53* PV in low levels (2–30%) across other tissues in the case of PZM. Somewhat surprising was the prevalence of PZM (5% overall; 13% among cases selected for analysis), which could not have been discerned without the multi-tissue approach. Transmission testing is indicated as a part of clinical care due to the possibility of transmission to offspring and should be evaluated in all suspected ACE/PZM cases, though it may only help in discernment of the clinical category if it is positive (30).

Circulating tumor DNA (ctDNA) in an advanced case may confound blood/saliva-based testing and may require ancillary testing at different timepoints to distinguish from PZM or germline. For example, case 48, the single ctDNA case in our cohort, was categorized as such as the *TP53* variant was not initially detected in blood used as companion normal to his colorectal tumor testing, but was detected approximately a year later when he underwent MGPT pre-terminally with widespread metastatic disease. Had the initial blood sample not been reviewed, one may have categorized this case as germline based on our proposed schema.

In case 54, the absence of the *TP53* PV in eyebrow plucks and normal tissue are the strongest indicator that this is an ACE case with likely CH. The gradient of 23% VAF in blood compared to 5% in tumor suggested contamination of the tissue with hemorrhage/inflammatory cell infiltrate. Nonetheless, to be conservative we designated the case as indeterminate. As for case 48 above, this illustrates that nuanced judgement of the multi-tissue genomic data may be warranted in some cases beyond the suggested workflow in Figure 1. Cases cited in our previous publication demonstrated low level detection of the respective PV in the tumor and/or “normal” tissues commensurate with inflammatory cell infiltrate (9). Thus, reliably “normal” tissues such as skin fibroblasts or in our case eyebrows were the most valuable for discernment. Given that 8 cases remained indeterminate due to inability to obtain additional samples such as fibroblast cultures or eyebrow plucks, and 3 cases in which the secondary biospecimen failed, FFPE tissue analysis may be valuable for discernment if preferred ancillary normal tissues are not available and for more in depth interrogation of PZM cases.

To conclude, our work has direct translational impact, as discerning the appropriate *TP53* clinical category will increase the diagnostic accuracy of genetic testing and direct clinical care for individuals with *TP53* PVs (40), as well as allow identification of additional CH-associated PVs and the potential for progression to myeloid neoplasia. False positive or incidental findings on surveillance can lead to diagnostic procedures and surgical misadventure and can have enormous psychological and financial impacts on individuals and families. In addition, unrecognized *TP53*-associated ACE will confound studies of prevalence and penetrance for individuals with LFS (8). We are currently using the tools and approach in this study to validate *TP53* status and answer these questions in LiFT UP, a large prospective cohort study, that also includes a prospective natural history study of *TP53*-driven CH. More precise estimation of penetrance with specific *TP53* PV subtypes, coupled with new insights about clinical and molecular genetic risk modifiers, and reliably discerning *TP53* clinical category will enable risk-appropriate allocation of cancer surveillance and risk-reduction interventions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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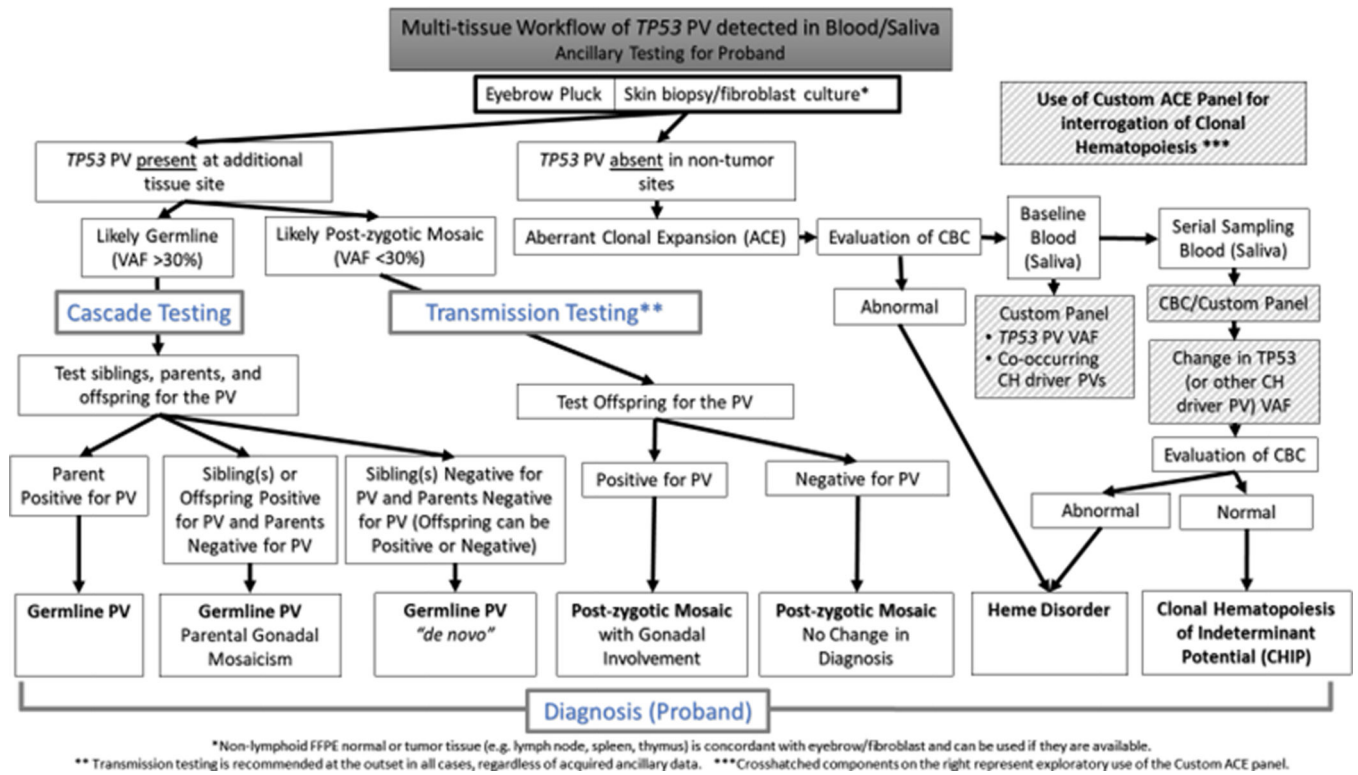


Figure 1.

Multi-tissue workup of TP53 pathogenic variants (PV) identified in germline (blood/saliva) commercial testing. This diagram depicts a diagnostic flowchart for establishing the presence/absence and variant allele fraction (VAF) of TP53 PVs in multiple tissues, often representing different embryonic germ layers, to discern and subclassify germline status including parental gonadal mosaicism, “de novo” germline status, post-zygotic mosaicism (PZM), or clonal hematopoiesis (CH) as ACE in a single tissue compartment. CH is further differentiated into clonal hematopoiesis of indeterminant potential (CHIP) if the complete blood count (CBC) is normal or extant hematologic neoplasia if not, and the approach for prospective evaluation of clonal evolution and genomic drivers of CH, and clinical state change is outlined.

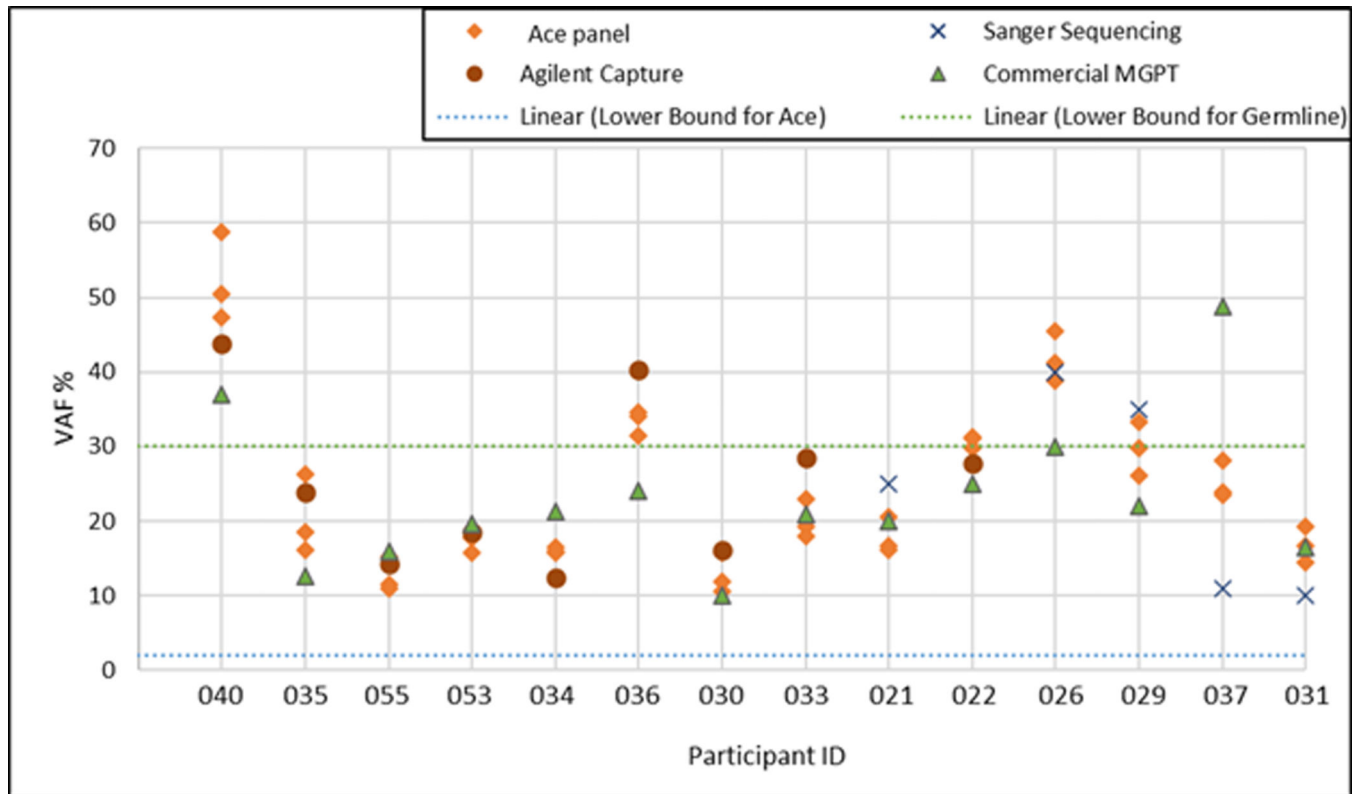


Figure 2.

Variant allele fraction (VAF) of a TP53 pathogenic variant tested on different platforms. The custom The QIAseq[®] amplicon-based 81-gene panel (ACE Panel) was used for assessment of VAF across blood samples from 14 participants. Test-retest variability (reproducibility) was assessed in triplicate. TP53 VAFs detected on the ACE panel were highly reproducible, as the within-participant standard error was 3%, with no statistically significant variability by ANOVA ($p = 0.993$). There was consistency/correlation of VAF across samples as well as across platforms (commercial laboratory multigene panel testing (MGPT); custom Agilent Hybrid capture panel) ($p=0.895$). Sanger sequencing values noted but not included in analysis as a semi-quantitative method. Note that Table 3 lists additional CH driver variants detected by the ACE panel, wherein, the test/retest VAF consistency is also shown for these secondary CH variants.

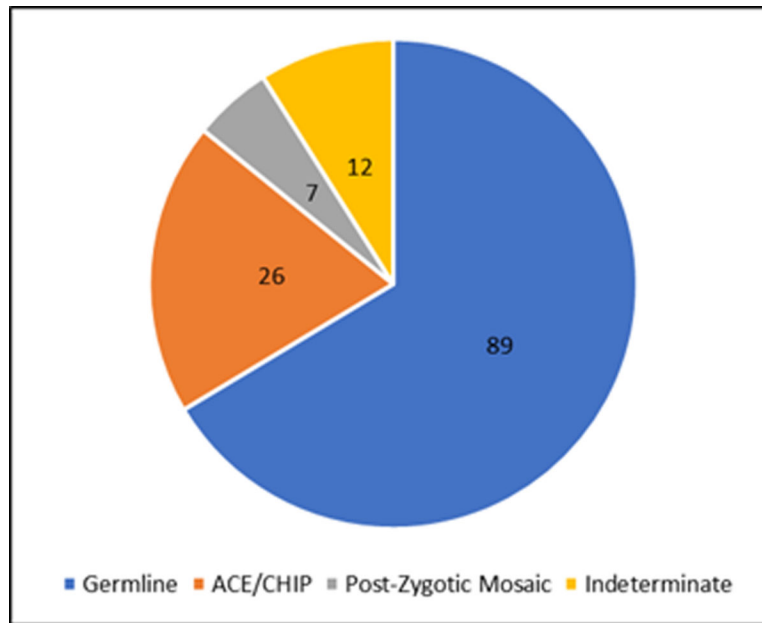


Figure 3. Clinical category assignment for individuals with a *TP53* pathogenic variants. The figure depicts the overall proportion of the clinical categories (germline, ACE/CHIP, PZM) found in this clinic based genetic testing cohort. Based on cascade/transmission testing, clinical criteria, and/or multi tissue analyses, 89/134 (66%) cases had evidence for germline, 26/134 (19%) were categorized as ACE, and 7/134 (5.2%) had evidence for PZM. Additional tissue or transmission data would be needed to discern PZM from broader ACE category for the 12/134 (9%) that were indeterminate.

Table 1.

Participant characteristics.

Characteristic	Range/Groups	N (%) ¹	Clinical Categories ¹			p-value ⁷
			Germline	PZM	ACE	
Sex	Male	15 (11)	10	0	4	0.61
	Female	119 (89)	79	7	22	
Age at entry	0–50	89 (70)	68	6	10	0.001
	51–71+	45 (30)	21	1	16	
Race	White	76 (57)	47	5	20	0.559
	Hispanic	35 (26)	28	2	3	
	Asian American	13 (10)	9	0	2	
	American Indian	4 (3)	1	0	1	
	African American	1 (0.8)	1	0	0	
	Pacific Islander	1 (0.8)	1	0	0	
	Unknown	4 (3)	2	0	0	
Cancer status	No	15 (11)	7	1	3	0.502
	Yes	119 (89)	82	6	23	
Age at cancer diagnosis ²	0–50	92 (77)	71	6	11	<0.001
	51–71+	27 (23)	11	0	12	
Cancer type ³	Breast ⁴	100	63	5	26	0.007
	Sarcoma	28	28	0	0	
	Adrenocortical carcinoma	3	3	0	0	
	Brain	3	3	0	0	
	Lung	10	9	1	0	
	Colorectal	9	5	1	1	
	Thyroid	8	4	1	3	
	Leukemia	7	3	0	4	
	Other ⁵	31	18	2	9	
TP53 Testing	Meets	71 (53)	74	1	0	<0.001
Criteria ⁶	Does Not Meet	63 (47)	15	6	26	

¹Total includes 12 indeterminate cases; these are not included in the statistical analyses of the clinical categories (n=122).

²Age at first cancer diagnosis if multiple primaries

³Percentage not provided due to some participants developing multiple primary cancers.

⁴Including invasive breast cancer (n=90) and ductal carcinoma in situ (n=10).

⁵ Other cancers include: ovarian (n=5), melanoma (n=4), uterine (n=4), renal (n=4), gastric (n=3), pancreatic (n=2), aplastic anemia (n=1), bladder (n=1), cervical (n=1), non-Hodgkin's lymphoma (n=1), parathyroid (n=1), phyllodes (n=1), prostate (n=1), tongue (n=1), and unknown (n=1).

⁶ *TP53* Testing Criteria found in Supplemental table 3

⁷ χ^2 test of independence or Fisher's exact test on the three clinical categories.

PZM = post-zygotic mosaic; ACE = aberrant clonal expansion

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Table 2.*TP53* pathogenic variant (PV) subtypes and variant allele fractions (VAFs) in different tissues

<i>TP53</i> PV SUBTYPES	Sample ID	Cancer Status (Y/N)	Cancer Diagnosis ¹	Age at Diagnosis (year) ²	Blood VAF (%)	Eyebrow VAF (%)	Normal Tissue VAF (%)	Tumor Tissue VAF (%)	Skin Fibroblast VAF (%)	<i>TP53</i> Testing Criteria	Transmission Testing
GERMLINE	001	Y	Br	54	53	47	43	46	-	N	U
	002	Y	Gastric	47	50	50	-	-	-	Y	-
	003	Y	Br	47	52	50	-	-	-	N	U
	004	Y	Br	28	50	47	36	-	-	Y	U
	005	Y	Br	35	50	48	-	-	-	Y	-
	006	Y	Br	28	48	50	-	-	-	Y	-
	007	Y	Br	51	53	50	-	-	-	N	-
	008	Y	CML	33	50	51	-	-	-	Y	Y
	009	Y	Mel	43	46	48	-	-	-	N	Y
	010	Y	Ov	31	52	56	-	-	-	Y	-
	011	Y	Pan	46	44	52	-	-	-	N	-
	012	N	-	35	53	53	-	-	-	N	U
	013	N	-	24	54	47	-	-	-	N	-
	014	Y	Ut	42	49	51	-	-	-	N	-
	015	Y	Br	33	48	52	-	-	-	Y	-
	016	Y	Ut	26	50	50	-	-	-	Y	-
	017	Y	Lung	69	44 ³	47	-	-	-	Y	-
	018	Y	Br	43	35	15	41	47	>30	N	U
	019	Y	Br	33	46 ³	50	51	-	-	Y	-
PZM	020	Y	Thyroid	26	43	45	-	-	-	N	Y
	021	Y	CRC	27	18 ³	47	7	6	-	N	-
	022	Y	Br	28	28	18	20	-	20	Y	U
	023	N	-	30	28	14	-	-	29	N	U
	024	Y	Br	36	15	36	12	9	-	N	-
	025	Y	DCIS	42	14	8	8	33	-	N	-
	026	Y	Br	45	40	-	20	0	20	N	-
	027	Y	Br	43	38 ³	17	30	-	-	N	-
ACE/CH	028	Y	Br	37	20	0	-	-	-	N	-
	029	Y	Br	52	33	0	-	-	-	N	-
	030	Y	Br	42	13	0	-	-	-	N	-
	031	Y	Br	57	17	0	0	0	-	N	-
	032	N	-	42	9.4	0	-	-	-	N	-
	033	N	-	38	20 ³	0	-	-	-	N	-
	034	Y	Br	61	15 ³	0	0	-	-	N	-
	035	Y	Pr	80	20 ³	0	0	-	0	N	U

<i>TP53</i> PV SUBTYPES	Sample ID	Cancer Status (Y/N)	Cancer Diagnosis ¹	Age at Diagnosis (year) ²	Blood VAF (%)	Eyebrow VAF (%)	Normal Tissue VAF (%)	Tumor Tissue VAF (%)	Skin Fibroblast VAF (%)	<i>TP53</i> Testing Criteria	Transmission Testing
	036	Y	Br	28	33	0	-	-	-	Y	-
	037	Y	Br	56	20	0	0	-	0	N	-
	038	Y	Ov	65	23	0	0	-	-	N	-
	039	Y	Br	43	36	0	-	-	-	N	-
	040	Y	Br	63	52	0	0	-	0	N	-
	041	Y	Br	41	19	0	-	-	-	N	-
	042	Y	Br	37	39	-	-	-	0	N	-
	043	Y	Br	32	22	-	-	-	0	N	-
	044	Y	Br	72	33	-	-	-	0	N	-
	045	Y	DCIS	46	50	-	-	-	0	N	U
	046	Y	Thyroid	50	17	-	-	-	0	N	-
	047	N	-	68	22	0	-	-	-	N	-
	048	Y	CRC	20	0, ^{<} 30 ⁴	-	-	>30 ⁵	-	N	-
	049	Y	Br	67	<30 ⁶	0 ⁶	0	0	-	N	-
	050	N	-	26	36	0	-	-	-	N	-
	051	Y	Br	61	16	0	-	-	-	N	-
	052	Y	Renal	71	30	0	-	-	-	N	-
INDETERMINATE	053	Y	Br	31	20	-	-	-	-	N	-
	054	Y	Br	56	23	0	5	0	-	N	U
	055	Y	Ov	63	16	-	-	-	-	N	-

ACE/CH=ABERRANT CLONAL EXPANSION/CLONAL HEMATOPOIESIS; PZM=POST-ZYGOTIC MOSAICISM; U=PERFORMED; UNINFORMATIVE; Y=PERFORMED; INFORMATIVE

¹FIRST CANCER, IF MULTIPLE. ABBREVIATIONS: BR=BREAST CANCER; CML=CHRONIC MYELOGENOUS LEUKEMIA; MEL=MELANOMA; OV=OVARIAN CANCER; PAN=PANCREATIC ADENOCARCINOMA; UT=UTERINE CANCER; CRC=COLORECTAL CANCER; DCIS=DUCTAL CARCINOMA IN SITU;

²AGE AT ENTRY IF UNAFFECTED.

³ADDITIONAL CH DRIVER VARIANTS FOUND IN BLOOD.

⁴CASE TESTED TWICE.

⁵DOCUMENTED TO BE CIRCULATING TUMOR DNA.

⁶WHOLE GENE DELETION; SEMI-QUANTITATIVE FROM COMMERCIAL LABORATORY; NOT DETECTED BY ACE PANEL, THOUGH CNV ANALYSES NOT YET VALIDATED.

Table 3.

Additional CH variants found on the ACE panel.

Gene	Variant	VAF
ACE		
<i>ATM</i>	c.8096C>T	3.8, 2.7, 3.8 ^I
<i>DNMT3A</i>	c.*3595del	2.2
<i>TET2</i>	c.1360A>T	23.4, 18.7, 21.3 ^I
<i>TET2</i>	c.3467del	1.1, 1.4, 1.1 ^I
<i>TP53</i>	c.350G>T	16.5, 13.2, 15.0 ^I
<i>TP53</i>	c.669del	1.0, 1.0, 1.0 ^I
Germline		
<i>DNMT3A</i>	c.1308C>G	2.0
<i>DNMT3A</i>	c.894dup	2.8

^ITest/retest VAF consistency