Somatic *TP53* variants frequently confound germline testing results

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Abstract

Purpose—Blood/saliva DNA is thought to represent the germline in genetic cancer risk assessment. Cases with pathogenic *TP53* variants detected by multi-gene panel tests (MGPT) are often discordant with Li-Fraumeni Syndrome (LFS), raising concern about misinterpretation of acquired aberrant clonal expansions (ACE) with *TP53* variants as germline results.

Methods—Pathogenic T*P53* variants with abnormal next-generation sequencing (NGS) metrics (e.g., decreased ratio [<25%] of mutant to wild-type allele, >2 detected alleles) were selected from a CLIA laboratory testing cohort. Alternate tissues and/or close relatives were tested to discern between ACE and germline status. Clinical data and LFS testing criteria were examined.

Results—Among 114,630 MGPT and 1,454 *TP53* gene-specific analyses, abnormal NGS metrics were observed in 20% of 353 *TP53* positive results, and ACE was confirmed for 91% of cases with ancillary materials, most due to clonal hematopoiesis. Only four met Chompret criteria. ACE cases were older (50 years vs 33.7; *P* = 0.02) and were more frequent among MGPT (66/285; 23.2%) vs *TP53* gene-specific tests (6/68; 8.8%, *P* = 0.005).

Conclusion—ACE confounds germline diagnosis, may portend hematologic malignancy, and may result in unwarranted clinical interventions. Ancillary testing to confirm germline status should precede Li-Fraumeni syndrome management.

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INTRODUCTION

DNA isolated from peripheral blood or saliva is typically considered representative of the germline for diagnosis of hereditary cancer. Since the advent of next-generation sequencing (NGS), multi-gene panel tests (MGPT) for hereditary cancer have become increasingly utilized in genetic cancer risk assessment.[1] MGPT are a potential cost- and time-effective alternative to sequential gene testing, yet clinicians may find themselves dealing with unexpected findings, such as detection of a cancer gene variant that is not known to correlate with the cancers in the patient and/or family.[2,3] Li-Fraumeni syndrome (LFS) was initially described by Frederick Li and Joseph Fraumeni in 1969[4] and associated with *TP53* in 1990.[5] The most frequently occurring tumors recognized as core cancers in LFS are sarcomas, breast cancers, central nervous system (CNS) tumors, and adrenocortical carcinomas.[6–9]

Various groups have published criteria to define LFS and identify patients for *TP53* gene-specific testing.[10–15] Given the broad tumor spectrum described in LFS families, *TP53* is included in most cancer-focused MGPT, and has resulted in the identification of carriers with a phenotype that is discordant with what has been reported for LFS,[16] suggesting the possibility of an expanded phenotype associated with germline *TP53* variants. However, the observation of a decreased mutant to wild-type allele ratio for a substantial portion of *TP53* variants detected through NGS raises the question of whether clonal populations in the blood or saliva that do not represent the germline or early post-zygotic mosaicism, were confounding clinical diagnosis. In most cases, a type of post-zygotic variation described by Forsberg et al. as aberrant clonal expansions (ACE) is suspected.[17] We previously applied the term 'somatic interference' to describe a circumstance wherein the analytical results of the test are technically valid (a pathogenic *TP53* variant was detected) yet the intent was to identify a bona fide germline predisposition to cancer.[18] However, for the purposes of this manuscript, we will refer to this phenomenon as ACE.

The term is ACE is used in distinction from the relatively infrequently documented phenomenon of classic mosaicism involving *TP53*, wherein a variant is acquired during embryogenesis and variably present in one or multiple germ layers, conferring increased cancer risk in the individual's respective tissues and the possible transmission of risk to offspring.[19,20] True somatic mosaicism has been well-documented for *NF1* and *NF2* cancer associated-genes and there has been one documented case for *PTEN* and Cowden syndrome.[21–24] There is a growing literature documenting the detection of mosaic mutations in disease genes detected by NGS, albeit in the context of developmental disorders manifesting in childhood.[25] However, in this study our focus is on the potential that late post-zygotic ACE, limited to the hematologic compartment or to a tumor, may be detected in the blood or saliva in the context of NGS-based testing to detect germline cancer predisposition. The magnitude of this phenomenon is yet unknown, and its potential impact on clinical care must be considered, given that more than 50,000 MGPT are conducted every year across a growing

number of commercial vendors, and many clinicians have limited experience ordering and interpreting MGPT.[1,26] Further, there is increasing evidence for the effectiveness of surveillance regimens prescribed for individuals with a germline *TP53* variant.[27,28] Nonetheless, these surveillance measures are resource intensive and have the potential for adverse events, so applying them to the truly at-risk individuals is an important consideration.

This study evaluated the prevalence and possible causes of apparent ACE involving *TP53* in a large series of patients who had clinical MGPT or *TP53* gene-specific testing.

METHODS

Study population

A system-wide search of Ambry Genetics (Aliso Viejo, CA) Laboratory Information Management System (LIMS) for clinical cases tested with MGPT that included *TP53* or NGS-based *TP53* gene-specific tests between March 2013 and February 2016 was conducted. Cases were selected with test results reporting *TP53* pathogenic and likely pathogenic variants (*TP53* variants) with abnormal NGS metrics, including (1) a minor allele frequency (MAF) of less than 25% or (2) a MAF between 25–30% if the clinical history or molecular results were suspicious for ACE (i.e. LFS criteria not met, active hematologic malignancy, or multiple mutations detected, etc.) and Sanger results were consistent with the MAF. Multiple or atypical abnormalities on microarray looking at large rearrangements were also included.

All *TP53* variants detected by NGS were confirmed on Sanger sequencing; gross deletions/duplications were evaluated with multiplex ligation-dependent probe amplification (MLPA) and/or microarray.

Data collection

Demographic, personal and family history information was collected from test requisition forms (patient gender, age at testing, cancer type, age at cancer diagnosis, ER/PR/HER2 receptor status for breast cancer, and a family cancer history table), clinic notes, pedigrees, letters of medical necessity, and medical records submitted to Ambry Genetics. Additional information such as personal history of hematologic neoplasia, extended cancer family history (first- to third-degree relatives) and other genetic test results were collected through direct communication with respective healthcare providers. The study was approved by Solutions IRB and the City of Hope Institutional Review Board. Case specific details have been amended to obscure potentially identifiable characteristics.

Laboratory methods

Multigene panel tests and *TP53* gene-specific testing—Both MGPT and *TP53* gene-specific tests were performed from DNA isolated from whole blood or saliva samples. NGS analysis (Illumina, San Diego, CA) was performed in all coding domains plus at least five bases into the 5′ and 3′ ends of the introns and untranslated regions (5′UTR and 3′UTR) for all cancer susceptibility genes. *EPCAM* and *GREM1* were only analyzed for

gross deletions and duplications, if included on the panel. Depending on the panel ordered by the clinician, 5–49 genes, including *TP53*, were analyzed. Sanger sequencing was performed for any region with insufficient depth of coverage (<10X), for verification of all variants (other than known benign variants), and for those with decreased mutant to wild-type allele ratios. A targeted chromosomal microarray and/or MLPA was used for the detection of gross deletions and duplications.

A five-tier classification schema—pathogenic; variant, likely pathogenic; variant of unknown significance; variant, likely benign; and benign—was used to classify variants.[29]

Single-site analyses of ancillary tissues for known *TP53* variant(s)—Single-site analysis for previously identified *TP53* variants was performed on available tissue samples. DNA was extracted from formalin-fixed, paraffin-embedded tissue, fibroblasts cultured from skin, and eyebrow plucks.[30] Single-site analysis was conducted using Sanger sequencing for all cases, and in certain cases was also performed using NGS.

Single-site analysis in family members—Testing for transmission of the known *TP53* variant was offered to first-degree relatives. When two *TP53* variants were detected, Sanger sequencing for both variants was performed.

Data Analyses

Using descriptive statistics, exact binomial confidence limits were calculated at 95%. Tests of difference between >2 groups for binary variables use the Fisher exact test to generate two-tailed *P* values. The Student *t*-test was used to compare the mean of ages and years. The clinical features of each case were assessed against the published criteria for *TP53* testing (NCCN, Chompret, Classic) (Supplemental Table 1).[9,10,31,32]

Data Sharing

Ambry genetic testing data is deposited in ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), and access can also be obtained via application to the AmbryShare program (https://share.ambrygen.com/about-ambry-share).

RESULTS

Among 114,630 MGPT that included *TP53* and 1,454 gene-specific *TP53* analyses, 353 cases were identified with a *TP53* variant (representing only pathogenic or likely pathogenic variants, as noted in the methods). Seventy-two cases (20.4%) were selected with test results reporting *TP53* pathogenic and likely pathogenic variants (*TP53* variants) with abnormal NGS metrics, and these cases were selected for further study (study cohort)Sixty-six cases had a MAF of less than 25%, three had a MAF between 25%–30% with a clinical history and molecular results suspicious for ACE and Sanger results consistent with the MAF, and three had multiple or atypical abnormalities on microarray looking at large rearrangements. All cases were reviewed by a laboratory director before issuing a clinical report (Fig. 1, Table 1). The suspected ACE cases represented a significantly higher proportion (66/285; 23.2%) of MGPT cases compared to those undergoing *TP53* gene-specific testing (6/68; 8.8%, *P* = 0.005). The mean age at testing was significantly older (58.4 vs 44.3 years; *P* <

0.0001) for suspected ACE cases compared to the cases with unambiguous NGS metrics among the MGPT cases; a similar pattern was observed among the six *TP53* gene-specific cases (39.5 vs 30.1 years).

The study cohort was predominately female (95.8%), with a personal history of cancer in 68 of 72 individuals (94.4%). Figure 2a depicts the types and prevalence of cancers observed in the study cohort. Eighteen cases had multiple primary cancers. The average age of onset for first cancer was 48.2 years (2–80 years). The average age at the time of genetic testing was 57 years (15–86 years) (Table 1).

Ninety-two cancers were reported among the 68 affected individuals (Fig. 2a). Breast cancer was the most common diagnosis (53%), with an average age of diagnosis at 46 years (19–72 years). Seven cases had a breast cancer diagnosis <31 years (NCCN criterion).[32] The status of the hormone receptors (estrogen and progesterone) and HER2 amplification was available for 25 breast cancer cases; of which, 18 were estrogen receptor positive (72%), two were triple positive (8%), and six were triple negative (24%).

Ovarian cancer ($n = 19$; 27.9%) was the second most common diagnosis, with an average age of diagnosis at 60.1 years (43–74 years) (Fig. 2a). The average time between ovarian cancer diagnosis and genetic testing was four years (0–12 years). Seven (37%) of these cases had at least one additional primary cancer.

Fifty-seven individuals reported a family history of cancer among first- and/or second-degree relatives (Fig. 2b). Two reported a relative with childhood cancer (diagnosis <18 years). Breast, colorectal, prostate, and uterine cancers were the most frequently reported among relatives. Two reported a relative with sarcoma (soft tissue or bone), five with a CNS tumor, and one with an adrenocortical carcinoma. Four of 72 (5.6%) cases met Chompret or classic diagnostic LFS criteria.

The majority (91.6%) of cases suspicious for ACE were identified through MGPT. Of these, four (6.1%) had two distinct *TP53* variants, both with a low MAF. Eight cases (12.1%) also had a pathogenic variant in another cancer predisposition gene (Table 2), including one in *PMS2* with a low MAF. The other seven (*ATM, BRCA2, BRIP1, MLH1, MUTYH, PALB2, RAD50*) appeared to be germline findings, among which four had a clinical phenotype compatible with the respective gene.

Five of seven cases with a history of hematological neoplasia were determined to have evidence of active disease at the time of testing—three with myelodysplastic syndrome (MDS), one with chronic lymphocytic leukemia (CLL), and one with acute myeloid leukemia (AML).

Ancillary testing, consisting of additional tissue analyses and/or single-site analysis of relatives, was performed on 35 of 72 cases (48.6%) (Fig. 1). Twenty two cases had a total of 62 relatives undergo single-site analysis. Germline status was confirmed in three of the 35 (8.6%) cases with additional testing: The respective *TP53* variant was detected in family member(s) for two of the *TP53* gene-specific testing cases. One was a 34 year-old female with breast cancer whose twin sister (zygosity uncertain) was also found to carry the *TP53*

variant, and the other was a 15 year-old male with acute leukemia and history of rhabdomyosarcoma and MDS in early childhood, who had two *TP53* variants (one missense variant and one large deletion). Only the missense *TP53* variant was detected in his mother, who had a neuroendocrine tumor, and in the offspring of his maternal aunt who had osteosarcoma at age 16 and a brain tumor at age 25. Single-site analyses of family members for all the other cases (*n* = 20) were negative for transmission of the respective *TP53* variant. The third case, a 19 year-old woman with triple negative breast cancer, was one of the MGPT cases and the respective *TP53* variant was detected in cultured skin fibroblasts.

Additional tissue was collected from 19 cases, of which seven had more than one unique tissue type available for analysis. The respective *TP53* variant was not detected in 27 non-lymphoid tissue samples, supporting a conclusion of ACE for the original sample submitted for clinical germline testing. Among the cases where the tumor tissue was tested, none appeared to be the origin of the *TP53* variant detected on germline testing. In one illustrative case, a 79-year old unaffected man had MGPT because his brother died of pancreatic cancer wherein a *TP53* variant (MAF = 12.7%) was detected. Subsequent testing of eyebrow plucks and cultured skin fibroblasts did not reveal the respective *TP53* variant. One year after MGPT, his PSA had risen to 16 and a biopsy confirmed Gleason grade 7–8 prostate cancer. Sequencing of the tumor tissue was reported as negative for the variant. However, review of the aligned sequence data noted that the variant was present in 1% of greater than 10,000 reads, below the threshold for validation, but consistent with reported inflammatory cells in the biopsy specimen. These findings support a conclusion of ACE.

Analysis of benign tissue of lymphoid origin in two cases identified the respective *TP53* variant. One was a 38 year-old female with a diagnosis of splenic angiosarcoma at age 38. Benign splenic tissue adjacent to the angiosarcoma detected the *TP5*3 variant at a low level (MAF = 17%), commensurate with the MAF seen in the blood (18%). The other case was a 66 year-old female with a history of breast cancer at age 45, and rectal carcinoid and lung cancer at age 56. The variant was not detected in benign duodenal and stomach tissues. It was detected in a benign lymph node and in benign colon tissue with prominent lymphocytic infiltrate; the MAF for the variant was less in the colon tissue than in the blood (9% vs 22%). Germline testing was negative for two daughters. These findings support a conclusion of ACE for both cases.

Twenty-nine of 30 MGPT cases with ancillary testing (96.7%) supported a conclusion of ACE (Table 1, Fig. 1). Comparison of these cases to the remaining suspected ACE MGPT cases with no ancillary testing (*n* = 36) showed no significant difference in age at diagnosis of breast cancer (mean = 43.7, [25–72] years vs mean = 49.1 [31–69] years, respectively) (*P* = 0.12), age at genetic testing (*P* = 0.2), and time between first cancer diagnosis and genetic testing (*P* = 0.31).

DISCUSSION

TP53 variants are increasingly detected on MGPT across diverse patient scenarios.[3,16,33–36] Although these findings may suggest a broader phenotype than is typically associated with LFS, we demonstrated that ACE in germline testing is a clinically important phenomenon,

involving nearly a quarter of MGPT wherein *TP53* variants were detected in blood or saliva. A recent short report from another commercial diagnostic laboratory reported 38.8% of MGPT detected *TP53* variants had abnormal NGS germline metrics, though ancillary testing was not performed.[37] We observed a similar proportion of suspect *TP53* results and evaluated ancillary tissues, providing evidence supporting the conclusion of ACE in most cases. Further, the criteria defining abnormal NGS metrics are not uniform among commercial genetic testing laboratories, so the true prevalence of the phenomenon and the inclusion of qualifications on the report to alert clinicians are uncertain. Nonetheless, ancillary studies performed on appropriate tissues can eliminate consideration of specific germ layers (e.g., epithelial by virtue of a negative result in skin biopsy or eyebrow pluck), but cannot ultimately prove that post-zygotic mosaicism does not exist.

In most cases, the ACE is likely due to clonal hematopoiesis of indeterminate potential (CHIP), which can be demonstrated in healthy populations at increasing frequency with increasing age.[33,34,38] Previous studies of CHIP have demonstrated increased risk (approximately 1%/year) for the development of overt hematologic neoplasia and increased overall mortality, especially if there is a variant in more than one gene involved in hematologic neoplasias (e.g., *ASXL1, DNMT3A*).[33,38] However, outside of *TP53* and *ATM*, genes that are frequently mutated in clonal hematopoiesis are not included on most hereditary cancer MGPT.

Just 7.2% (5/69) of cases in our series that were deemed unlikely to be germline had evidence of overt hematological neoplasia as a likely cause of ACE (Supplemental Table 2), only one of which was noted on test requisition form (the others were clarified by queries related to this study). Therefore, there should be clear instructions from genetic testing laboratories regarding the unsuitability of blood and/or saliva as a source of DNA for germline testing for cases with a history of hematologic abnormalities. Further, careful examination of the patient's complete blood count and peripheral smear may be warranted in all cases reporting the discovery of a *TP53* variant.

Thirty-five of the 72 (45.8%) cases in this study had ancillary materials and/or clinical data to interrogate germline status, and there were no significant differences in breast cancer age, age at testing, and age from diagnosis to testing for those with and without ancillary data. The latter were labeled 'indeterminate' in our analysis (Supplemental Table 2). However, assuming no bias in access to ancillary data or tissues, and given the apparent lack of differentiating clinical features between those with and without ancillary material, and the fact that none of the cases had a clinical phenotype of LFS, we speculate that ACE is probably the explanation for the majority of the remaining cases. Nonetheless, testing ancillary tissues would be necessary to confirm or exclude germline status.

Given that clinical information for a portion of the cases was limited to that available on the clinician completed test requisition form, supplemental efforts to obtain comprehensive personal medical and family history data were made for all cases and ancillary tissues were obtained when possible. Follow-up e-mails and phone calls to ordering providers with request for additional information were moderately successful, but standardized family history collection was not uniform across the series.

Historically, when a patient's blood or saliva are not the appropriate specimen for germline genetic testing due to history of hematologic neoplasia or allogenic bone marrow/stem cell transplant, skin fibroblasts have been utilized for testing. However, coordination of skin punch biopsy and cell culturing can be costly and challenging to facilitate. One innovative approach employed in this study was the use of eyebrow plucks as a surrogate for skin biopsies and the growth of fibroblasts. We documented a yield of 0.5–1.2 micrograms of high quality DNA from 10–15 eyebrow hair follicles. A technical manuscript outlining the specifications and standard operating procedure for processing eyebrow hair follicles to obtain genomic DNA is in process.[30]

Beyond cultured skin fibroblasts, alternatives to white blood cells from blood or saliva may be considered for interrogation of the germline. One can use normal solid tissues or solid tumor tissue derived from archival surgical specimens. The possibility that circulating tumor cells could explain ACE is suggested by detection of tumor related variants in cell-free DNA ('liquid biopsy').[39] However, none of the cases with ancillary testing of tumor tissue in our series showed the respective *TP53* variant. If the variant is found in the tumor, however, one cannot distinguish whether it represents ACE (as a result of circulating tumor DNA) or a germline finding. Discerning germline from somatic variants in tumor tissues is another emerging challenge for clinicians. Finally, orthogonal testing with different methods (e.g. NGS and Sanger sequencing on the same sample) provides evidence that the skewed MAF is not likely due to a technical problem with differential allelic amplification.

Normal (non-cancer) tissues are preferred for confirmatory studies, and tissues in the lymphoid compartment or with inflammatory cell infiltrate should be avoided, as they may reflect the clonal hematopoietic findings from the blood. For example, two cases of ACE in our series demonstrated the variant in benign tissue with heavy representation of lymphocytes; lymph nodes, spleen, and colon tissue with predominant inflammatory cell infiltrate. The spleen and lymph nodes are arguably part of the hematopoietic compartment. The MAF of the *TP53* variant in the lymphoid-derived tissue in each of the cases in our series approximated that observed in the blood. Thus, we recommend the avoidance of ancillary tissues from the hematopoietic compartment such as bone marrow, lymph nodes, spleen, or tissues identified through histologic analysis to have significant lymphocytic infiltrates.

Apparent CHIP was especially prevalent among the ovarian cancers in our series. The *TP53* variant was not detected in any of the ancillary tissues analyzed in five ovarian cancer cases. Using NGS to analyze a set of cancer genes in the peripheral blood of women with ovarian cancer, Swisher et al. demonstrated that somatic mosaic variants in *PPM1D* were associated with chemotherapy exposure and older age at time of blood draw.[36] Ruark et al. speculated about mosaicism in lymphocytes and concluded that *PPM1D* mutations predisposed to breast and/or ovarian cancer, though they were not able to detect the *PPM1D* mutations in any of the respective tumors, thus we believe the more likely explanation is clonal hematopoiesis.[40] Swisher et al. further observed the emergence of pathogenic *TP53* variants in the blood after exposure to chemotherapy.[36] With approximately four years between ovarian cancer diagnosis and subsequent genetic testing in our series, interval chemotherapy exposure is a common feature of cases with apparent ACE. There are many potential causes

for ACE including circulating tumor DNA, however the only evidence present in this case series was CHIP or an evolving hematologic neoplasia.

Using a large clinical series we demonstrated that the phenomenon of ACE was common and most often due to clonal hematopoiesis. This finding has important clinical implications regarding potential application of unwarranted clinical interventions. Further, the finding of clonal hematopoiesis itself may portend adverse clinical outcomes, such as the development of hematologic neoplasia and increased non-hematologic mortality.

Currently, there are no standard guidelines for NGS quality control measures for detecting or reporting potential ACE. Laboratories need to be transparent about their policies regarding the detection, reporting, and follow-up of cases with potential ACE.

Confirming the validity of germline *TP53* test results may be necessary to evaluate the associated phenotype(s) and enable accurate identification and management of germline carriers. Ancillary tissues should be obtained and tested to determine whether a given variant is present in any tissue other than the blood. Beyond using NGS quality control measures, clinician recognition of test results inconsistent with a LFS phenotype should create an index of suspicion, and caution is urged in the medical management of patients in whom the only criterion for LFS is a *TP53* variant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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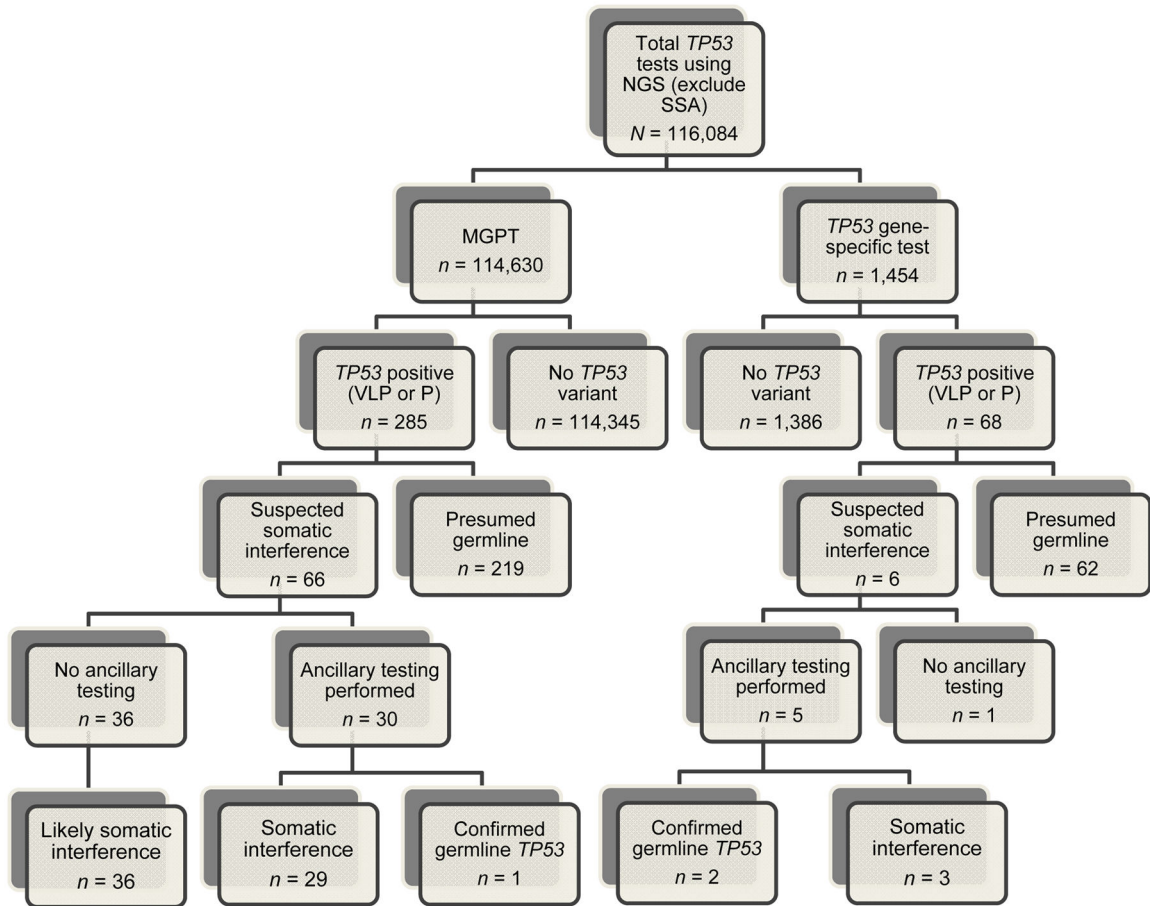
Figure 1.
Consort Diagram of the Clinical Study; The total sample of cases evaluated at the genetic testing laboratory is indicated followed by the respective subsets of MGPT and single-gene *TP53* tests and the subsets meeting eligibility criteria. Cases with and without ancillary testing are noted, as is the final assignment of aberrant clonal expansion status based on consideration of the data.

Abbreviations: NGS, Next-Generation Sequencing; SSA, Single Site Analyses; MGPT, Multigene Panel Test; VLP, Variant Likely Pathogenic; P, Pathogenic; Aberrant Clonal Expansions, ACE
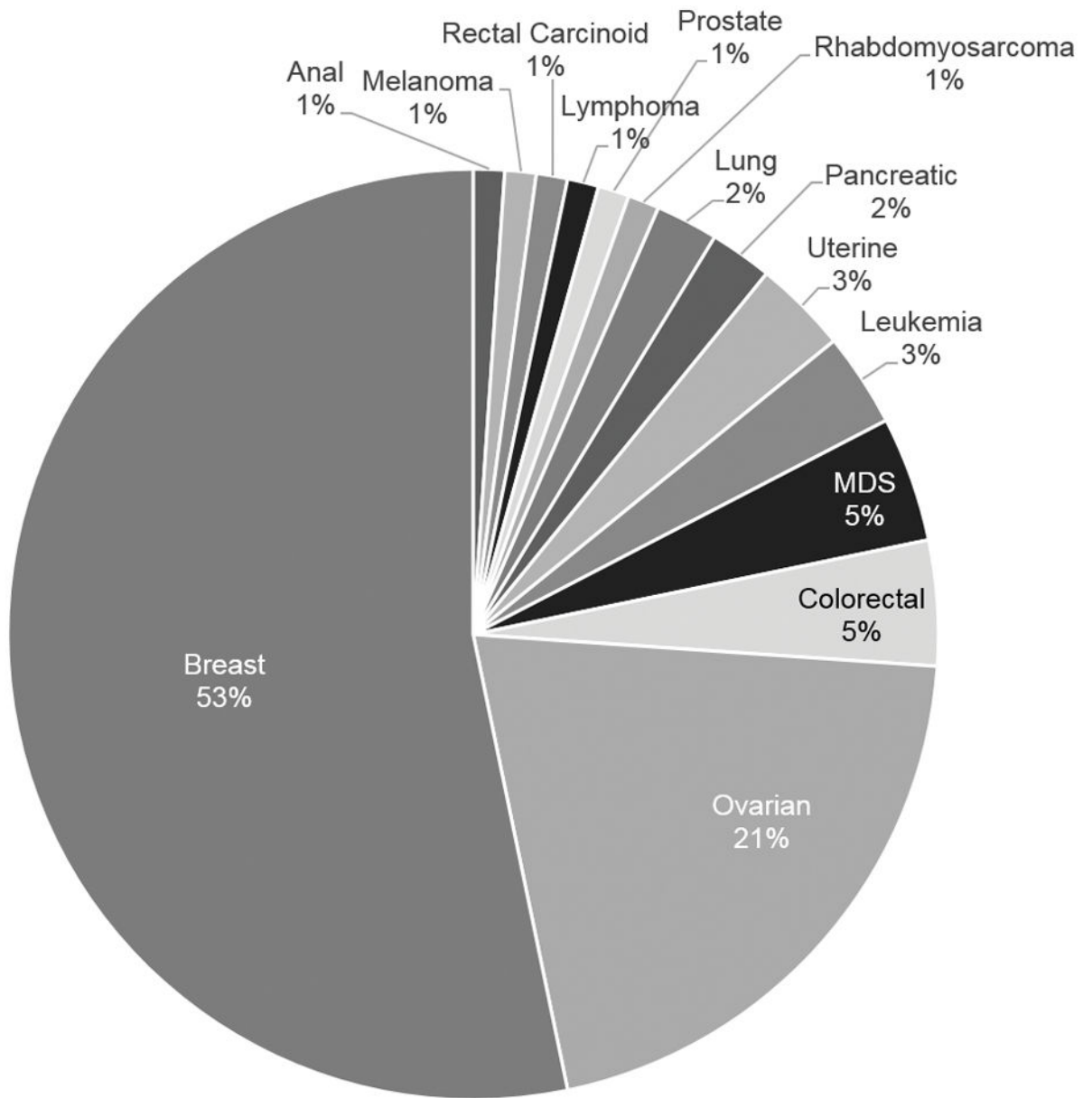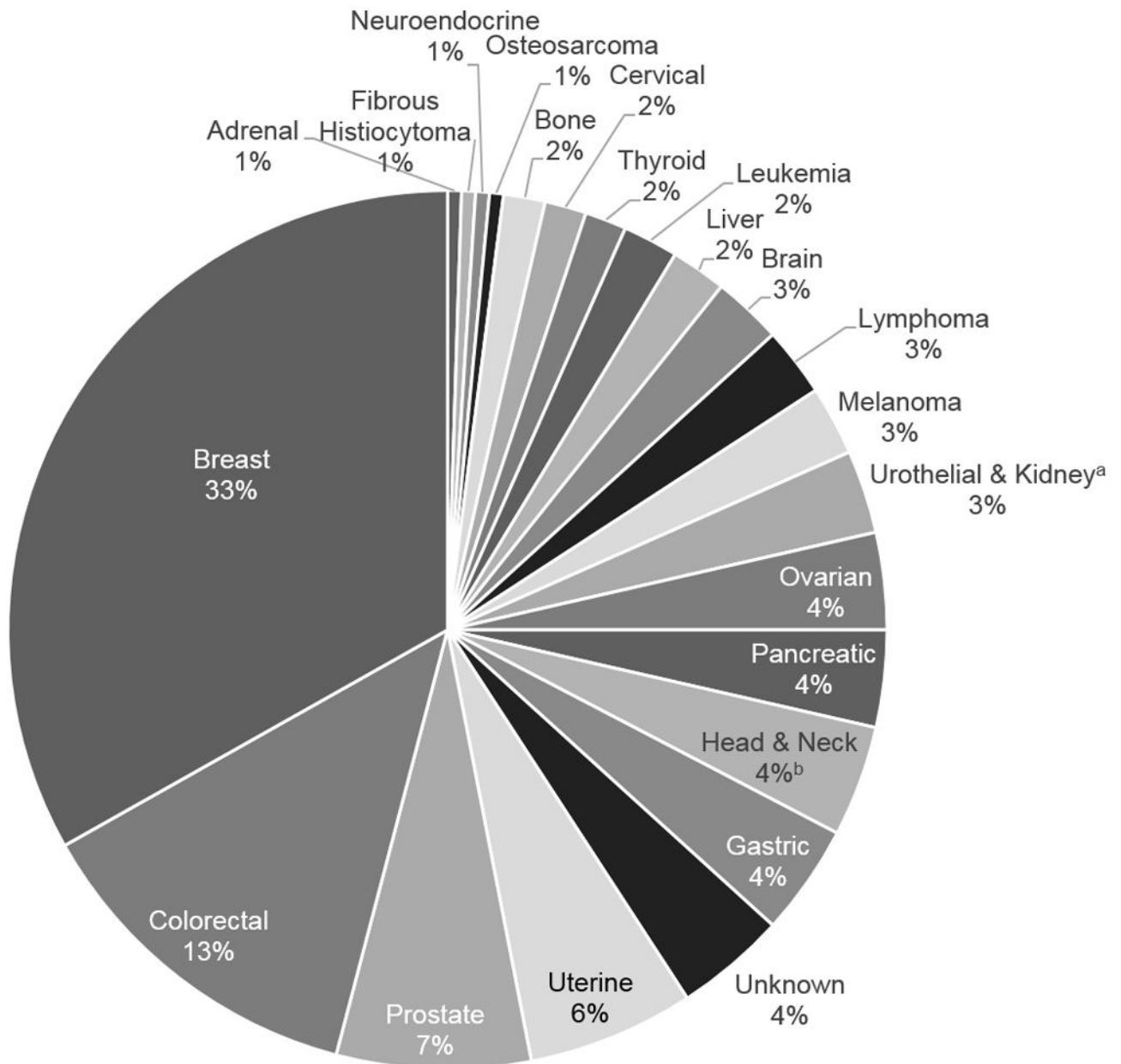
Figure 2.

Figure 2a. Spectrum of cancers among *TP53* cases suspected to be due to ACE; The cancer subtypes and their respective proportions are indicated in the pie chart. Note that in some cases (n=18) some individuals had multiple tumor types.

Abbreviations: MDS, Myelodysplastic syndrome

Figure 2b. Spectrum of cancers among relatives of cases suspected to be due to ACE; The cancer subtypes (excluding non-melanoma skin cancers) reported among first and second degree relatives and their respective proportions are indicated in the pie chart.

[a]Urothelial & Kidney: Bladder, Kidney, Renal Pelvis

[b]Head & Neck: Esophageal, Laryngeal, Nose, Throat

Table 1

Clinical characteristics and ancillary testing summary

	Totals *n* (%)	MGPT	*TP53* gene-specific	*P* value
Total testing inclusive of *TP53*	116084	114630	1454	
Total *TP53* positive cases	353	285	68	
Evidence for aberrant clonal expansions (ACE)	**72 (20.4%)**	**66 (23.2%)**	**6 (8.8%)**	**P = 0.005**
Gender				
Female	69 (95.8%)	64 (97%)	5 (83.3%)	
Male	3 (4.2%)	2 (3%)	1 (16.7%)	
Average age at testing (years)	57	58.5	39.5	P = 0.009
Two *TP53* mutations	5 (6.9%)	4 (6.1%)	1 (16.7%)	ns
Other pathogenic variant	8 (11.1%)	8 (12.1%)	N/A	
Personal history of any cancer[a]	68 (94.4%)	62 (94%)	6 (100%)	ns
Age at diagnosis 1st primary cancer (years)	48.5	50	33.7	P = 0.02
Meets criteria for *TP53* testing				
Breast cancer diagnosis < 31 years	7 (9.7%)	6 (9.1%)	1 (16.7%)	ns
Chompret criteria	4 (5.6%)	2 (3%)	2 (33.3%)	P = 0.002
Any relatives[b] with cancer[a]	59 (81.9%)	54 (81.8%)	5 (83.3%)	ns
Any relatives[b] with childhood cancer[a]	2 (2.8%)	1 (1.5%)	1 (16.7%)	P = 0.031
Cases with ancillary testing	35 (48.6%)	30 (45.5%)	5 (83.3%)	
Had relatives undergo testing	22 (30.6%)	18 (27.3%)	4 (66.7%)	P = 0.046
Had relatives test positive	2 (9.1%)	0 (0%)	2 (50%)	P = 0.002
Tissue testing performed	19 (26.4%)	17 (25.8%)	2 (33.3%)	ns
Non-lymphoid tissue positive	2 (11.8%)	1 (5.9%)	1 (50%)	P = 0.062
Results of ancillary testing				
Evidence confirming ACE	32 (91.4%)	29 (96.7%)	3 (60%)	P = 0.007
Evidence supporting germline	3 (8.6%)	1 (3.3%)	2 (40%)	ns

Abbreviations: ns, non-significant;

[a] Excluding non-melanoma skin cancer

[b] First or second degree relatives

Table 2

Cases with multiple pathogenic variants

Case	Variant 1 & Allele Frequency (%)	Variant 2 & Allele Frequency (%)[a]	Variant 3 & Allele Frequency (%)[a]
10	*TP53* (11.3)	*TP53* (17.5)	
15	*TP53* (22.2)	*TP53* (14.3)	
27	*TP53* (27.2)	*TP53* (10.7)	
53	*TP53* (24.9)	*TP53* (19.2)	*MLH1*[b]
34	*TP53* (16.6)	*ATM*[b]	
39	*TP53* (20.2)	*BRCA2*[b]	
40	*TP53* (22.1)	*BRIP1*[b]	
51	*TP53* (26.4)	*RAD50*	
54	*TP53* (14.8)	*PMS2*[c,d]	
61	*TP53* (23.1)	*PALB2*	
70	*TP53*[c]	*MUTYH*	
72	*TP53*[c]	*TP53*	

[a]The allele frequency is listed for those that fall below the NGS quality threshold; all others were reported by the laboratory as heterozygous based on available data.

[b]This reported germline mutation is concordant with the clinical presentation of the patient.

[c]Large deletion

[d]MLPA data shows shifts below threshold for heterozygous call for all probes in the gene.