Germline Analysis from Tumor-Germline Sequencing Dyads to Identify Clinically Actionable Secondary Findings

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ABSTRACT

In the past decade, tumor-germline next generation sequencing has become a routine part of personalized oncology care. Via this method, germline mutations are typically subtracted from those in the tumor to identify somatic mutations, thus negating the possibility of discovering germline variants. Previously, it has been proposed that the identification of germline variants could have significant clinical implications for patients with hereditary cancers and their family members. In this exploratory research study, we sought to investigate the prevalence of germline variants identified through clinical tumor-germline sequencing among a cohort of patients across ten major cancer types. Germline sequencing data from 439 individuals undergoing tumor-germline sequencing through the LCCC1108/UNCseq™ (NCT01457196) study were analyzed for genetic variants in 36 hereditary cancer susceptibility genes. Variants indicative of hereditary cancer predisposition were identified in 19 (4.3%) patients. For about half (10/19), these findings represent new molecular diagnostic information with potentially important implications for the patient and their family. Genes with pathogenic variants included the hereditary cancer genes: *ATM*, *BRCA1*, *BRCA2*, *CDKN2A*, and *CHEK2*. Furthermore, a substantial proportion of patients (178, or 40.5%) had Variants of Uncertain Significance (VUS), 24 of which had VUS in genes pertinent to the presenting cancer. Overall, with approximately 4% of cases harboring pathogenic variants in known hereditary cancer susceptibility genes, diagnostic germline findings such as these could be beneficial for patients and their families.

INTRODUCTION

Cancer is a leading cause of death worldwide, with an estimated 1.68 million new cases in 2015[1]. Recently, major technological advances in massively parallel sequencing coupled with dramatic reductions in cost have positioned next-generation sequencing as an integral tool used in cancer care. The application of this technology has enabled clinicians and scientists to recognize the potential of personalized oncology, particularly with respect to diagnosing tumors and determining effective courses of action for cancer patients[2].

Cancer is primarily a genetic disease caused by mutations in a wide variety of genes, including proto-oncogenes and tumor suppressor genes. These genetic changes can either be acquired post-conception (somatic mutation) or be present constitutively in all cells of the body as a result of inheritance or early post-zygotic events (hereafter referred to as constitutional or germline mutations)[3]. In patients with hereditary cancer syndromes, the presence of germline mutations can complicate the use of next-generation sequencing in identifying somatic mutations in a patient's tumor. Typically, when tumor-germline sequencing is performed on entire genomes, exomes, or selected genes, germline variants are "subtracted out" from those found in the tumor in order to identify somatic mutations in the tumor (figure 1). Previous studies have shown that germline variant subtraction enhances the specificity of detecting somatic mutations[4]. As a result, pathogenic germline mutations that predispose a patient to increased cancer susceptibility may be overlooked when seeking out somatic variants via tumor-germline sequencing. Thus, ignoring the germline variants post "subtraction" will likely miss these critical variants, which can have profound implications for cancer patients with hereditary cancer

syndromes. For instance, knowledge of these variants can guide the preventive care of the patient and their family members. Moreover, in a fraction of cases, knowledge of these variants can also influence treatment targets, as is the case with the use of PARP inhibitors in patients with *BRCA1* and *BRCA2* mutation-associated cancers[4].

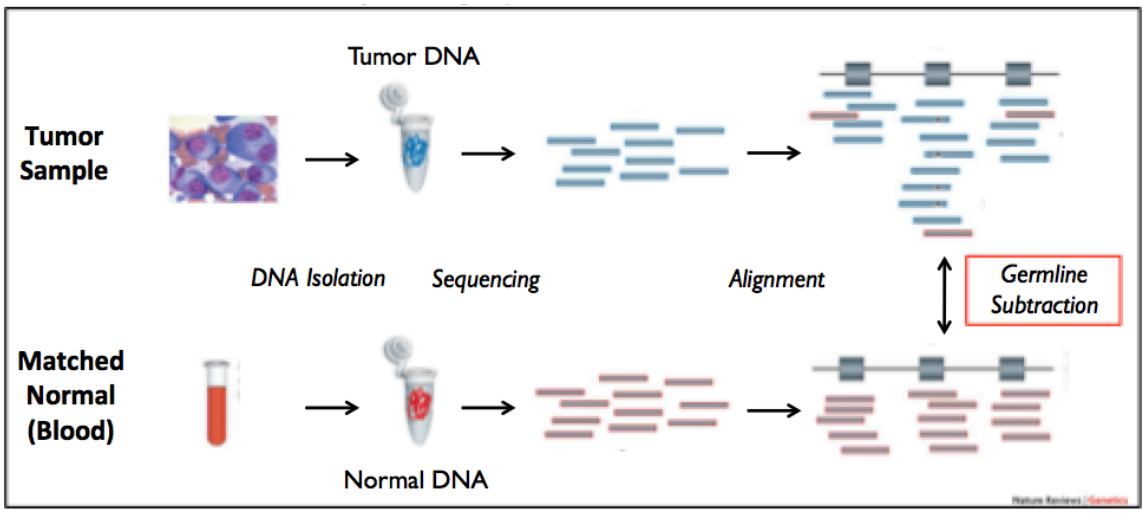

Figure 1. Tumor-germline sequencing workflow. Germline variants derived from normal tissue are typically subtracted out from tumor sequences in order to identify somatic mutations, thus negating the possibility of discovering germline mutations.

While the majority of cancer cases arise sporadically via acquired somatic mutations, inherited germline mutations are estimated to play a major role in roughly 5 to 10 percent of all cancers[5]. The burden of germline mutations in cancer patients varies by cancer type, with some tumor types having lesser-known germline etiology (e.g. lung cancer, kidney cancer, etc.) and others with well-known germline etiologies (e.g. breast cancer, ovarian cancer). Despite our knowledge of the relative frequencies of hereditary cancers, empirical data illustrating the burden of germline mutations identified through routine tumor-germline sequencing of patients with cancer remains insufficient[6].

Given the potential ramifications of missing critical germline variants in patients with cancer, we conducted an exploratory study within patients undergoing tumor-germline sequencing to explore the frequency of opportunistically identified pathogenic germline variants within cancer predisposing genes.

METHODS

Summary

The experimental design for this study can be divided into three main steps: (1) Tumor-germline sequencing, (2) Variant calling, and (3) Variant Classification. Germline sequencing data from 439 individuals undergoing tumor-germline dyad sequencing were analyzed for genetic variants in 36 hereditary cancer susceptibility genes. Patients across 10 major cancer types were included in the study. In order to realistically assess the burden of germline variants among individuals who receive tumor-germline sequencing, patients were included in the study irrespective of having prior clinical indicators of hereditary cancer predisposition. The germline variants found in the patients were then evaluated for pathogenicity using a variant classification framework previously published by the American College of Medical Genetics.

Steps (1) and (2) were performed by other members of the Berg Lab, while step (3) was conducted by Krunal Amin, Bryce Seifert, and Julianne O'Daniel.

(1) Tumor-germline sequencing

Participants were enrolled in the LCCC1108 study (UNC clinical sequencing study, referred to hereafter as UNCseq™). Informed consent and whole blood DNA (or

buccal as appropriate) were obtained from all patients through an institutional review board (IRB)-approved protocol at the Lineberger Comprehensive Cancer Center and the University of North Carolina, Chapel Hill (NCT01457196). The UNCseq™ study aims to associate known molecular alterations with clinical outcomes in oncology and uses this information to support treatment decisions through reporting of genetic profiling to clinicians. The overarching study consent describes the collection and analysis of both tumor and germline tissue including the explicit possibility for identification of an underlying hereditary cancer predisposition. Participants consent to the reporting of all results deemed clinically significant. Consent was obtained by UNCseq™ study staff for the primary study at enrollment. Patients were referred into the UNCseq™ study team by their clinic physician and enrolled according to their treated cancer (Table 1) and thus the tumor tissue to be analyzed. All patients enrolled between 11/2011 and 06/2014 for the cancer types listed in Table 1 were included in our data capture for exploratory germline analysis.

(2) Variant Calling

Library preparation and gene capture methods have been described previously[7]. Briefly, DNA was extracted from blood using a Puregene DNA Purification kit (Gentra Systems), DNeasy Blood and Tissue Kit (Qiagen), or a Maxwell MDx16TM (Promega, Inc.). In each methodology, DNA was extracted according to the manufacturer's protocol. DNA was fragmented to approximately 180-225 base pairs 7 using a Covaris E220 focused ultrasonicator instrument (Covaris, Inc.). Postfragmentation, the sample was enriched for appropriately sized fragments using an automated separation step

employing AMPure beads (Beckman Coulter). Fragment size enrichment and subsequent library preparation steps involving precise liquid handling steps were performed using the Agilent basic Bravo A and/or the Bravo B robot(s) (Agilent Technologies). Gene capture was performed using a SureSelectXT custom capture kit according to the manufacturer's protocol (Agilent Technologies). All exons of the 247 genes on the UNCseq™ panel were sufficiently captured with average coverage depth of 750X (see Supplementary Table 1-Capture V6 within Jeck et al. listing all 247 genes).

Library quality was assessed with a Bioanalyzer or Tapestation 2200 (Agilent Technologies) using either D1K Screentapes or High Sensitivity D1K Screentapes (Agilent Technologies). Completed libraries were normalized and pooled using Bravo robots guided by vWorks automation control software (Agilent Technologies), and sequenced at the UNC High Throughput Sequencing Facility (HTSF) using a HiSeq2500TM (Illumina). Alignment and variant calling of the sequencing reads have been described previously, with the addition of Isaac and FreeBayes for variant calling as well as ABRA for read realignment[7-10]. In brief, germline sequencing reads were mapped to the hg18 reference genome using the Burrows Wheeler Aligner and ABRA. ABRA is a bioinformatics platform designed to improve indel detection and accuracy for estimation of variant allele frequency[11]. The germline variants were then called using Varscan[12], FreeBayes haplotype-based variant detector[9], and Isaac to improve calling near indels by local realignment[13]. Lastly, variants were 8 annotated using ANNOVAR[14]. Generally, mean target coverage for all patients ranged from 100-2000X, with the average being approximately 750X. Germline variants and variant annotations were stored in a local PostgreSQL database.

While the NGS methods used here may detect copy number variation (CNV), we did not use it for this purpose. If we had, any CNV would have been verified through a Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory at UNC Chapel Hill. Validation of the assay including assessment of sensitivity and specificity to detect germline variants was not performed because this is an exploratory research study. Any variants deemed clinically significant, and thus warranting return to the patient, are confirmed on a new sample through an orthogonal method within the CLIA certified Molecular Genetics Laboratory at the University of North Carolina at Chapel Hill.

(3) Variant classification

Variants were first filtered through a list of 36 known hereditary cancer genes and then prioritized for analysis based on minor allele frequencies, protein effect, and existence in databases of previously reported pathogenic variants (see Table 1 for analyzed genes). Allele frequency data were obtained from The 1000 Genomes Browser (http://browser.1000genomes.org/index.html), National Heart, Lung, and Blood Institute Exome Variant Server ESP6500 Data Set (http://evs.gs.washington.edu/EVS/), and/or The Exome Aggregation Consortium (ExAC, http://exac.broadinstitute.org/). In order to focus our analysis on rare, highly penetrant variants, variants with a maximum allele frequency of 0.001 were filtered out (Figure 2).

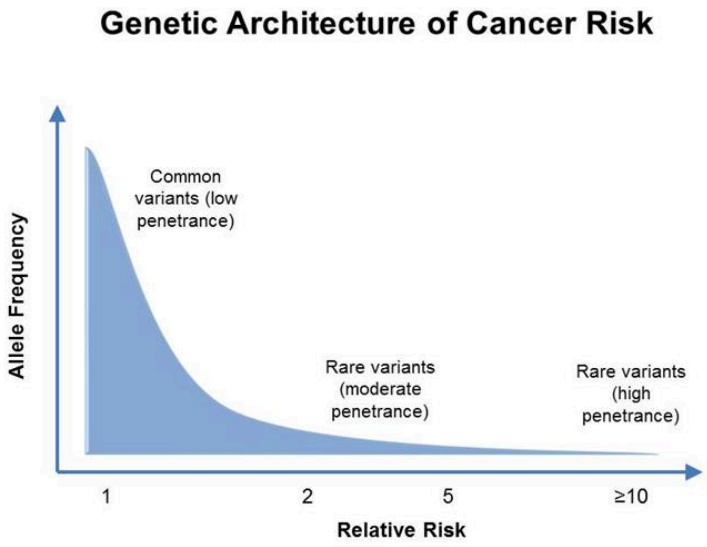

Figure 2. Analysis was focused on rare, highly penetrant variants. Given the low incidence of most hereditary cancer syndromes in the population, common variants are unlikely to contribute to hereditary cancer predisposition.

Online resources for variant classification included The National Center for Biotechnology Information ClinVar database (http://www.ncbi.nlm.nih.gov/clinvar/), the Leiden Open-Source Variation Database (LOVD, http://www.lovd.nl/2.0/index_list.php), and the Catalogue of Somatic Mutations in Cancer (COSMIC, http://cancer.sanger.ac.uk/cosmic). COSMIC was used to determine if a variant existed in tumors from similar tissues of origin. After a preliminary computational classification, variant counts were generated using an in-house python script and validated manually. Variants underwent tiered review by trained molecular analysts in conjunction with discussion in a multidisciplinary group. Evidence curation and variant classification was performed in a manner similar to the more recently published guidelines from the American College of Medical Genetics and Genomics and the Association for Molecular Pathology[10] (Figure 3). As the patients were not selected for clinical or family histories

suggestive of a hereditary cancer predisposition, this phenotype information was not available during the variant review process. Therefore, the molecular analysts utilized an incidental or secondary variant analysis approach such that a high threshold for pathogenicity must be met for variant result. The medical and family history presented in Table 2 was obtained from medical record review after variant analysis. Following stringent review, variants classified as Likely Pathogenic or Known Pathogenic were identified as eligible for return to patients. Prior to results return, these variants will be confirmed through analysis of a new sample via an orthogonal method (e.g. Sanger sequencing) and verified by an American Board of Medical Genetics and Genomics (ABMGG)-certified molecular pathologist. The confirmation step was ongoing at the time of submission. Once confirmed, the hereditary cancer predisposing variants will be returned to the patients through a board certified genetic counselor experienced in hereditary cancer. When medical record review documented a clinically known hereditary cancer predisposing variant, no additional steps for confirmation and results return were performed.

Cancer Type (Subtype)	Cases (% of Total)	Hereditary cancer susceptibility genes evaluated
Colorectal	53 (12.1%)	*MLH1,MSH2, MSH6, APC, PTEN, SMAD4, STK11*
Ovarian	29 (6.6%)	*BRCA1, BRCA2, MRE11A, TP53, MSH6, CHEK2*
Breast (Ductal, Lobular, Other)	114 (26.0%)	*BRCA1, BRCA2, ATM, CHEK2, CDH1, MRE11A, PTEN, STK11*
Musculoskeletal	41 (9.3%)	*TP53*
Lung (Non-small cell, Small cell, Other)	31 (7.1%)	*TP53*
Kidney	30 (6.8%)	*VHL, MET*
Brain/CNS (Astrocytoma, Glioma, Oligodendroglioma, Other)	54 (12.3%)	*NF1, NF2, TSC1, TSC2, TP53*
Skin (Melanoma, Non-melanoma, Other)	39 (8.9%)	*CDKN2A, PTCH1*[#]
Hematologic (ALL, AML, CLL, Other)*	29 (6.6%)	*RUNX1, CEBPA, TP53*
Pancreas	19 (4.3%)	*BRCA1, BRCA2, CDKN2A, ATM,TP53*
Total	439	
Other hereditary cancer genes	*AKT1, ATR, CBL, CDC73, CDKN1B, EGFR, MEN1 NTRK1, PIK3CA, RB1, RET, SMARCA4, SMARCB1,WT1*	

*Hematologic cancer abbreviations: Acute Lymphoblastic Leukemia (ALL), Acute Myeloid Leukemia (AML), Chronic Lymphocytic Leukemia (CLL).

[#]*PTCH1* variants were considered relevant only in skin cancer cases that were of the non-melanoma type.

Table 1. UNCseq™ cancer cases and hereditary susceptibility genes analyzed

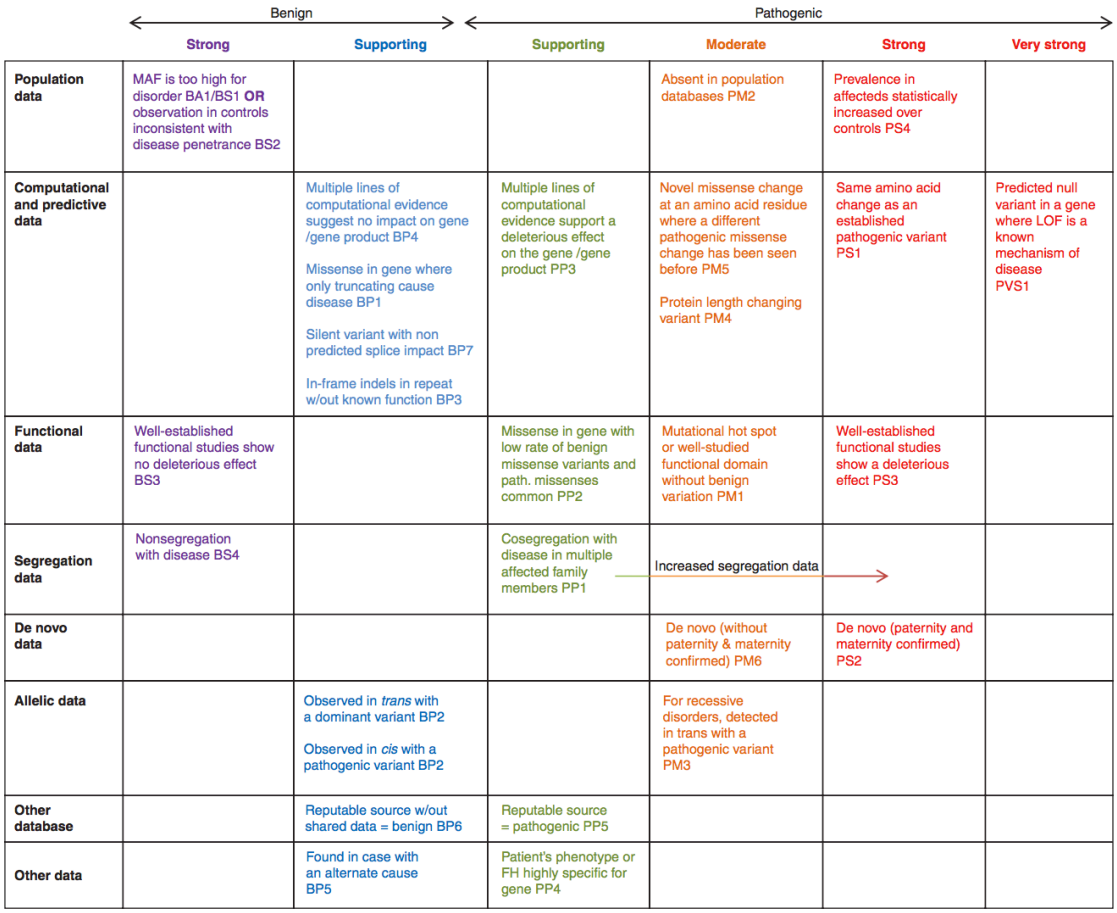

Figure 3. Evidence framework. This chart published by the American College of Medical Genetics organizes each of the criteria by the type of evidence as well as the strength of the criteria for a benign (left) or pathogenic (right) assertion.

RESULTS

To assess the frequency of pathogenic variants in a group of unselected cancer patients undergoing tumor sequencing, we analyzed germline variants from 439 patients ascertained through the UNCseq™ study. Although all 247 genes of the UNCseq™ panel were sequenced, we specifically investigated germline variants in 36 genes that were previously determined to be strongly associated hereditary cancer syndromes that were

present on the somatic sequencing panel. Based on current knowledge about the spectrum of cancers associated with these hereditary cancer syndromes, 24 of the 36 genes were considered concordant with the cancer types of the patients being analyzed (Table 1). These cancers included colorectal, ovarian, breast, musculoskeletal, lung, kidney, brain/CNS, melanoma, hematologic, and pancreatic cancers[12, 15-20]. Of all cases examined, 19/439 (4.3%) had pathogenic germline variants in a hereditary cancer predisposing gene. Of these, 12 were in genes concordant with the presenting cancer at enrollment and 7 were in other hereditary cancer genes (Figure 4, Table 2). The majority of these findings occurred in patients with colorectal, ovarian, breast, and pancreatic cancers; very few such findings occurred in patients with musculoskeletal, lung, kidney, brain, skin, or hematologic malignancies.

Overall, *BRCA1* and *BRCA2* harbored 11/19 (57.9%) of the pathogenic variants, the majority of which were classified Likely Pathogenic because they were novel variants expected to result in an early truncation or for which existing evidence suggested a pathogenic role based on classification guidelines[10] (Figure 5). As would be expected in an unselected cancer patient population, a small percentage had previously undergone clinical genetic assessment for hereditary cancer predisposition. Medical record review following variant classification revealed that the *BRCA1/2* variants identified in breast and ovarian cancer patients in this study had all been previously identified through routine clinical genetic testing, indicated based on medical and family history[21]. The *ATM* and *CDKN2A* pathogenic variants identified in breast cancer patients were not previously known (Table 2), reinforcing the idea that additional variants may exist in

breast cancer patients that would be missed in individuals whose clinical testing was restricted to *BRCA1* and *BRCA2*[22-23].

The table in Appendix 1 shows a clinical summary of UNCseq™ patients with germline pathogenic variants. Some patients had a personal history of cancer consistent with the variant identified in the germline analysis, but had been enrolled for cancers that were presumably unrelated (Table 2). For example, Patient 11 was previously diagnosed with breast cancer at age 41 and was enrolled in UNCseq™ when diagnosed with colorectal cancer at age 49. She was found to have a pathogenic canonical splice site variant in *BRCA1* (NM_007294.3:c.594-2A>C) that provides an explanation for her breast cancer (Appendix 1), but is not likely to have any relevance to her colorectal cancer diagnosis. Similarly, Patient 17 was previously diagnosed with breast cancer at age 52, but was enrolled in the UNCseq™ study for non-small cell lung cancer. She was found to have a pathogenic nonsense variant in *ATM* (NM_000051.3:c.352C>T, (p.Gln118Ter)) (Appendix 1) that provides a plausible explanation for her breast cancer and could potentially suggest a role of *ATM* in lung cancer, although previous studies have found limited evidence to support this role or suggested that environmental factors may contribute much more significantly.

Although relatively few patients had clearly pathogenic variants, 178/439 (40.5%) had a germline Variant of Uncertain Significance (VUS) (Figure 6). In 24 patients, a VUS was found in a gene relevant to the presenting cancer type, while 143 patients had a VUS in hereditary cancer genes unrelated to their cancer type. Not surprisingly, 11 patients had a VUS in both pertinent and non-pertinent genes (Figure 6).

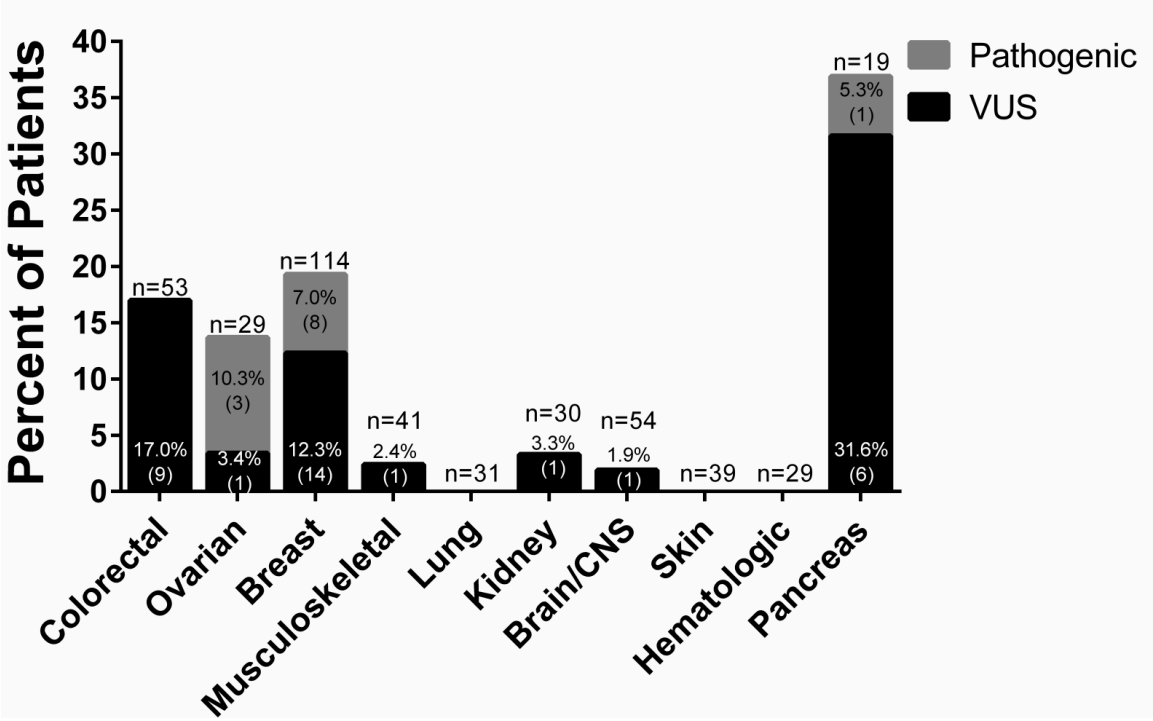

Figure 4. Germline findings across all UNCseq™ patients. The percentages of patients with Pathogenic variants (light shading) or Variants of Uncertain Significance (VUS, dark shading) in genes that are concordant with the tumor type are depicted as stacked bar graphs. Numbers above the bars represent the sample size for the specific tumor type. Numbers in parentheses represent the number of patients with Variants of Uncertain Significance or Pathogenic variants. Here, Pathogenic variants include both Likely Pathogenic (LP) and Known Pathogenic (KP) variants. In both the ovarian and breast cancer groups, one patient in each group had a Variant of Uncertain Significance and a Pathogenic variant. Hence, 8 cases were found to have pathogenic variants in genes concordant with breast cancer, 3 cases were found with pathogenic variants in genes concordant with ovarian cancer, and 1 case was found to have a pathogenic variant in a gene concordant with pancreatic cancer.

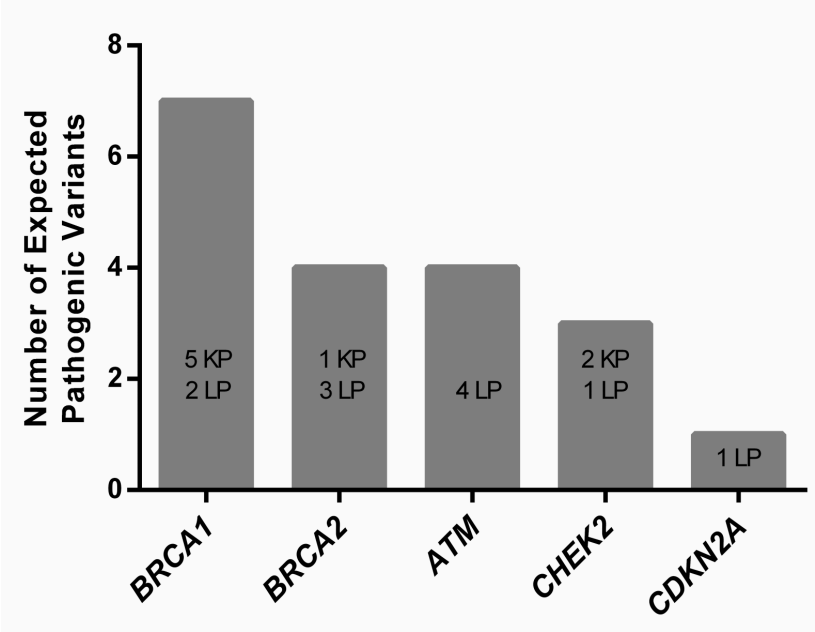

Figure 5. Germline pathogenic variants identified in all UNCseq™ patients. The numbers of Known Pathogenic (KP) and Likely Pathogenic (LP) variants across all UNCseq™ patients analyzed are depicted as a bar graph, divided by gene.

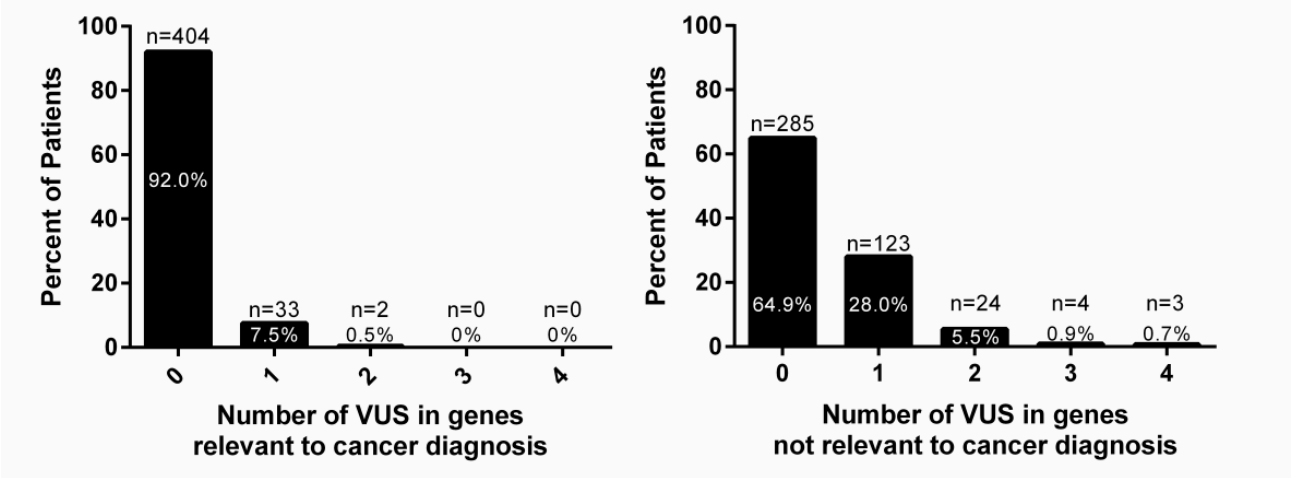

Figure 6A Variants occurring in genes relevant to the patient's cancer diagnosis.

Figure 6B Variants occurring in genes unrelated to the patient's cancer diagnosis.

Numbers above the bars represent the frequency of patients with 0,1,2,3 or 4 variants.

Percentages represent the percent of all UNCseq™ patients analyzed.

DISCUSSION

Frequency of Pathogenic Germline Variants

This thesis explores the yield of clinically relevant findings from germline analysis in patients undergoing tumor-germline dyad sequencing. The proportion of patients found harboring a pathogenic germline mutation (4.3%) is consistent with the range reported in previous hereditary cancer studies[3, 24-25]. It should be noted that this frequency is only an estimate, and may vary based on which genes are included in the capture panel as well as the cancer types considered. For example, a similar exploratory study conducted by Schrader et al. 2016[4] reported a frequency of 12% of cancer patients sequenced via tumor-germline dyad sequencing harboring germline mutations. However, Schrader and colleagues[4] expanded the survey to include 187 genes associated with Mendelian diseases while also including a wider array of tumor types than this study (e.g. prostate cancer, thyroid cancer, liver cancer, etc.). If the data published by Schrader and colleagues[4] were limited to the same cancer types and hereditary cancer predisposition genes in our current analysis, a pathogenic variant would be found in 3.9% of cases, which is consistent with our findings. This also demonstrates a limitation in our analysis in that only hereditary cancer predisposition genes in patients from 10 major cancer types were considered. Future studies should aim to ascertain the frequency of incidental germline findings in other Mendelian disorders besides hereditary cancer syndromes, as these incidental findings may also reveal information critical diagnostic information about a patient.

Moreover, the distribution of pathogenic variants across cancer types (Figure 4) generally aligned with our expectations. Despite only accounting for roughly one-third of

the patient cohort, the majority of pathogenic variants were in patients with breast and ovarian cancer, which are tumor types that both have well-documented genetic etiologies. Perhaps more surprisingly, none of the patients diagnosed with colorectal cancer were found to have a mutation in a gene associated with Lynch Syndrome, which accounts for roughly 3 to 5% of all colorectal cancer cases. However, this is likely due to the relatively small number of colorectal cancer cases evaluated (N = 53). In tumor types with lesser-known genetic etiologies (i.e. musculoskeletal, lung, kidney, brain/CNS, skin, hematologic cancers), no pathogenic germline variants were found as expected.

Furthermore, while the majority of pathogenic germline findings occurred in genes that were concordant with the presenting cancer at enrollment, a significant portion (7/19, or 36.8%) of the pathogenic variants were found in discordant genes – meaning that either the mutation was found in a gene that was concordant with another cancer type or in one of the "Other hereditary cancer genes" listed in Table 1. For example, a *BRCA1* pathogenic variant was found in patient 11 with colorectal cancer, *BRCA2* in patient 15 with AML, and so on (Appendix 1). The vast majority of the discordant findings occurred in patients who had cancer types with lesser-known genetic etiologies, such as acute myeloid leukemia, non-small cell lung cancer, and musculoskeletal cancer. These incidental findings

Finally, among the 19 patients with positive findings in a hereditary cancer predisposition gene, half of the pathogenic variants had previously been identified through clinical evaluation. The other half, representing roughly ~2% of patients in the entire cohort, were not associated with any prior clinical cancer genetic evaluation. For this group of patients, the opportunistic germline analysis provides critical information

that was not otherwise known to the patient and their family, enabling potential lifesaving interventions[26-27]. Identifying pathogenic germline variants could also provide important prognostic information, guiding surgical procedures and or targeted therapeutic options for the individual cancer patient, thereby providing immediate applications[2, 28]. However, we recognize that such unexpected germline susceptibility information might be unwelcome to some patients depending on their personal situation or preference for information. Therefore, further long-term follow-up is needed in order to assess what portion of patients would ultimately welcome and benefit from such information.

Variants of Uncertain Significance

Often, variants of uncertain significance (VUS) are returned to patients after the diagnostic evaluation of hereditary cancer risk[29]. However, when tumor-germline sequencing is performed for prognostic or therapeutic indications, the identification of germline variation would be considered an incidental or secondary finding. In this situation, as per the evidence-based guidelines published by the American College of Medical Genetics and Genomics[10], only Pathogenic or Likely Pathogenic findings should be reported to patients. This notion is supported by our data, in which we discovered at least one VUS in almost half (40.5%) of all patients. By definition, the clinical relevance of these variants remains to be determined. Based on the low prior probability of clinical relevance, the majority of these variants are likely to be inconsequential.

CONCLUSION

Though it is a major focus of precision medicine efforts, whether next generation sequencing should be applied on a routine basis for tumor mutation profiling remains to be determined[30]. Here, we demonstrate that utilizing an incidental/secondary variant analysis approach for germline sequence data in unselected patients undergoing tumor-germline sequencing may provide a small but important benefit with regard to the detection of clinically relevant, highly penetrant variants in hereditary cancer predisposition genes. Most of these findings can be ascertained through cancer genetics evaluation recommended on the basis of family history, age at presentation, ancestry or tumor phenotype. However, some of these patients may not be referred to a cancer genetic service[31-34] and a minority will be missed due to lack of typical clinical and/or family history indications[35-36].

Potentially unsuspected pathogenic variants have now been reported in a small, but not insignificant, proportion of cancer patients undergoing therapeutically indicated tumor-germline testing[4,6], and our data provide further support to this scenario. Disclosing the identification of a hereditary cancer predisposition would be highly relevant to the clinical care of these cancer patients and have important implications for their relatives' medical guidance. Providers who obtain tumor sequencing will need to be cognizant of the implications of tumor-germline analysis with respect to potential incidental findings[37], understand the differences between tumor sequencing and clinical genetic testing for hereditary cancer susceptibility, and be able to effectively communicate these issues to their patients.

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APPENDIX 1: Clinical summary of UNCSeq™ patients with germline pathogenic variants

Patient	Cancer type at Enrollment	Sex	Age[a]	Gene	cDNA change[b]	Protein change	Variant type	Classification	Clinical Genetics Evaluation	Personal and/or Family History	Prior Clinical Testing	Clinical Test Result[c]	Concordance with Cancer at Enrollment[d]
1	Ovarian	F	48	*BRCA1*	NM_007294.3: c.5266_5267insC	p.(Gln1756fs)	Frameshifting indel	KP	Yes	Ashkenazi Jewish F:Lung, Bladder MA: Pancreas, 72	Yes	(+)	Yes
2	Ovarian	F	41	*BRCA1*	NM_007294.3: c.5193+1G>T	N/A	Splice-site	KP	Yes	No Cancer History	Yes	(+)	Yes
3	Breast	F	37	*CDKN2A*	NM_000077.4: c.35C>A	p.(Ser12Ter)	Nonsense	LP	Yes	Personal: Melanoma, 21,31 Family: Adopted	Yes	(-)	No
4	Breast	F	55	*BRCA1*	NM_007294.3: c.2457_2457delC	p.(Asp821fs)	Frameshifting indel	KP	Yes	MMaR:Breast, Ovarian, Pancreas M:Breast, 36	Yes	(+)	Yes
5	Breast	F	29	*BRCA1*	NM_007294.3: c.211A>G	p.(Arg71Gly)	Missense	KP	Yes	S:Breast, 28; M:Breast,42 MA:Breast,33; MA:Breast,37	Yes	(+)	Yes
6	Breast	F	63	*ATM*	NM_000051.3: c.1561_1562delAG	p.(Glu522fs)	Frameshifting indel	LP	Yes	M:Breast,55; MA:Breast,30s& 40s 3MU:Blood MA:Cancer,60s	Yes	(-)	Yes
7	Breast	F	29	*BRCA2*	NM_000059.3: c.7538_7539insA	p.(Thr2515fs)	Frameshifting indel	LP	Yes	M:Breast, 39&49, and Brain/CNS, 58	Yes	(+)	Yes
8	Breast	F	35	*BRCA1*	NM_007294.3: c.131G>A	p.(Cys44Tyr)	Missense	KP	Yes	PA: Bilateral Breast, 45	Yes	(+)	Yes
9	Breast	F	37	*BRCA2*	NM_000059.3: c.8575delC	p.(Gln2859fs)	Frameshifting indel	KP	Unknown	Unknown (adopted)	Yes	(+)	Yes

10	Breast	M	53	*BRCA2*	NM_000059.3: c.5718_5719delCT	p.(Leu1908fs)	Frameshifting indel	LP	Yes	S:Breast,50; B:Colon,53 PA:Breast, 55; PC:Colon, 35 Known Familial Mutation	Yes	(+)	Yes
11	Colorectal	F	49	*BRCA1*	NM_007294.3: c.594-2A>C	N/A	Splice-site	LP	Yes	Personal: Breast, 41 Family: PA: Breast, 29; PC: Breast, 50	Yes	(+)	No
12	AML	M	54	*BRCA1*	NM_007294.3: c.594-2A>C	N/A	Splice-site	LP	Unknown	Not Reported	No	N/A	No
13	GI-other	M	54	*ATM*	NM_000051.3: c.8545C>T	p.(Arg2849Ter)	Nonsense	LP	No	No Cancer History	No	N/A	No
14	Breast	F	59	*CHEK2*	NM_007194.3: c.1100delC	p.(Thr367fs)	Frameshifting indel	KP	No	Non-Contributory M:Lymph node, 80 U: Liver	No	N/A	Yes
15	AML	M	57	*BRCA2*	NM_000059.3: c.5233_5233delA	p.(Met1745fs)	Frameshifting indel	LP	No	F:Pancreas, 72	No	N/A	No
16	Pancreas	M	61	*ATM*	NM_000051.3: c.170G>A	p.(Trp57Ter)	Nonsense	LP	Yes	B:Pancreas, 52	No	N/A	Yes
17	NSCLC	F	66	*ATM*	NM_000051.3: c.352C>T	p.(Gln118Ter)	Nonsense	LP	No	Personal: Breast, 52 Family: S:Breast, F: Bone (myeloma), B:Amyloidosis	No	N/A	No
18	Musculoskeletal	F	57	*CHEK2*[e]	NM_007194.3: c.1100delC	p.(Thr367fs)	Frameshifting indel	KP	No	F:Kidney PGM:Lung, B:CNS (2)	No	N/A	No
19	Ovarian	F	52	*CHEK2*	NM_007194.3: c.1486C>T	p.(Gln496Ter)	Nonsense	LP	No	Personal: Melanoma, 57 Family: PU: Stomach	No	N/A	Yes

[a] Age at the time of diagnosis

[b] Transcripts are listed according to the HGVS nomenclature recommendations or the commonly accepted transcript.

[c] Clinical Test Result: (+) = Same Mutation Reported, (-) = Gene was not included in the clinical genetic test and these negative results indicate new diagnostic results.

[d] Concordance with cancer at enrollment: Yes = Pathogenic variant is in a gene that is concordant with the presenting cancer at enrollment. No = Pathogenic variant is in a gene that is discordant with the presenting cancer at enrollment.
[e] CHEK2 has been implicated as a susceptibility gene for a Li-Fraumeni-like cancer syndrome. However, the current evidence for this association is disputed(50).

Abbreviations:
Cancer type: AML=Acute Myelogenous Leukemia; NSCLC= Non-small cell lung cancer.
Gender: F= female; M= male. Classification: KP= Known Pathogenic; LP= Likely Pathogenic; VUS= Variant of Uncertain Significance.
Family History: M=Mother; F=Father; S=Sister; B=Brother; MA= Maternal Aunt; MU= Maternal Uncle; MGM= Maternal Grandmother; MGF= Maternal Grandfather; MMaR=Multiple Maternal Relatives; PA= Paternal Aunt; PU= Paternal Uncle; PGF=Paternal Grandfather; PGM=Paternal Grandmother; PC= Paternal Cousin; U=Uncle.