A pedigree-based prediction model identifies carriers of deleterious *de novo* mutations in families with Li-Fraumeni syndrome

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Abstract

De novo mutations (DNMs) are being increasingly recognized as causal factors for rare diseases. Identifying rare DNM carriers will allow researchers to study the likely distinct molecular mechanisms of DNMs. We developed *Famdenovo* to predict DNM status (DNM or familial mutation, FM) of deleterious autosomal dominant germline mutations for any syndrome. We introduce *Famdenovo.TP53* for Li-Fraumeni syndrome (LFS) and analyze 324 LFS family pedigrees from four US cohorts: a validation set of 186 pedigrees and a discovery set of 138 pedigrees. The concordance index for *Famdenovo.TP53* prediction was 0.95 (95% CI: [0.92, 0.98]). Forty individuals (95% CI: [30, 50]) were predicted as DNM carriers, increasing the total number from 42 to 82. We compared clinical and biological features of FM versus DNM carriers: 1) cancer and mutation spectra along with parental ages were similarly distributed; 2) ascertainment criteria like early-onset breast cancer (age 20 to 35 years) provides a condition for an unbiased estimate of the DNM rate: 48% (23 DNMs vs. 25 FMs); 3) Hotspot mutation R248W was not observed in DNMs, although it is as prevalent as hotspot mutation R248Q in FMs. Furthermore, we introduce *Famdenovo.brca* for hereditary breast and ovarian cancer syndrome, and apply it to a small set of family data from the Cancer Genetics Network. In summary, we introduce a new study design to systematically evaluate deleterious DNMs in inherited cancer syndromes. Our findings will facilitate future studies on how new deleterious mutations are established in the germline and how rare genetic diseases prevail.

Introduction

De novo mutations (DNMs) are defined as germline variants (mutations) occurring in the carrier, but not in either of the carrier's parents (Rahbari et al. 2016). In humans, dozens of DNMs (Conrad et al. 2006; Kondrashov 2003; Kong et al. 2012; Lipson et al. 2015; Lynch 2010; Nachman and Crowell 2000) occur throughout the genome of each newborn. DNMs are considered a major cause for the occurrence of rare early-onset, reproductively lethal diseases (Acuna-Hidalgo et al. 2016). Initial analyses have shown that the rate of germline DNMs increases with paternal (Kong et al. 2012) and maternal age (Wong et al. 2016); plus, DNMs are correlated to a number of factors (Battle and Montgomery 2014), including the time of replication (Francioli et al. 2015), the rate of recombination (Francioli et al. 2015), GC content (Michaelson et al. 2012), and DNA hyper-sensitivity (Michaelson et al. 2012). DNM carriers and their associated factors have always been a focus of genetics research because of their crucial role in the evolution of species (Goldmann et al. 2019).

Not only are DNMs the raw materials of evolution, but they may also shape and determine disease susceptibility (De Ligt et al. 2012; De Rubeis et al. 2014; Iossifov et al. 2014; Krumm et al. 2015; Robinson et al. 2014). DNMs have been recognized as causal factors for rare diseases, such as hereditary retinoblastoma (Dryja et al. 1997), epileptic encephalopathies (Allen et al. 2013), Bosma arhinia microphthalmia syndrome (Gordon et al. 2017), ubiquitin-proteasome system-dependent disorders (Küry et al. 2017), and neurodevelopmental diseases, including intellectual disability, autism and schizophrenia (Katrancha et al. 2017). In the near future, as more whole-genome or whole-exome sequencing of individuals with various disorders and their relatives in different populations become available, it is likely that more DNMs will be identified as associated with disease outcomes. These mutations, although individually rare, may capture a significant part of the heritability for complex genetic diseases that is not detectable by genome-wide association studies.

DNMs play an important role in hereditary cancer syndromes, such as retinoblastoma and Neurofibromatosis, where the *de novo* rates are as high as ~90% (Dryja et al. 1997) and ~50% (Evans et al. 2010; Jett and Friedman 2010), respectively. Other hereditary syndromes have lower *de novo* rates. For example, Li-Fraumeni syndrome (LFS) is an autosomal dominant cancer predisposition syndrome that commonly manifests as soft tissue and bone sarcomas, breast cancer, brain tumors,

leukemia, and adrenal cortical carcinomas (Olivier et al. 2003; Nichols et al. 2001). LFS is associated with germline mutations in the *TP53* tumor suppressor gene (Malkin et al. 1990; Correa 2016), of which 7% - 20% are *de novo* (Gonzalez et al. 2009). Clinically, it is of interest to identify *de novo* carriers of a *TP53* germline mutation, who may otherwise go unnoticed until they or their offspring develop multiple cancers. Identifying these carriers allows them to undergo LFS screening and surveillance protocols. Understanding the cause of *de novo* mutations in cancer genes like *TP53* will help develop strategies for early identification and will have a novel and significant impact on the field of human genetics.

Predicting a DNM in a gene is not trivial when the parental mutation statuses are unknown. Incomplete family history may skew observational criteria and the prediction may suffer from low sensitivity and specificity. Mendelian risk prediction models (Chen et al. 2004) have been used successfully for accurate prediction of deleterious mutation status in familial breast and ovarian (Euhus et al. 2002), bowel (Chen et al. 2006b), pancreatic (Wang et al. 2007) cancers, melanoma (Wang et al. 2010), and most recently in LFS (Peng et al. 2017; Renaux-Petel et al. 2018). Using LFS family pedigree cohorts that include cancer history as a case study, we developed a novel statistical approach, *Famdenovo*, to predict the DNM status, i.e., whether the carrier's deleterious mutation is familial or alternatively arose from a new mutation during meiosis or mitosis. *Famdenovo* was developed as a freely available R package and Shiny web app (http://bioinformatics.mdanderson.org/main/Famdenovo).

DNMs are rare, and therefore require a large amount of high-quality data to be studied thoroughly. We introduce *Famdenovo.TP53*, which was modeled and validated on a large cohort consisting of 324 LFS family cohorts that were collected from four major cancer centers in the US, with *TP53* genetic testing results available in at least one trio per family. Our method consisted of a large-scale validation of the performance of *Famdenovo.TP53* using families with LFS, in which the *de novo* status of *TP53* mutations are known, as well as an application of *Famdenovo.TP53* to identify potential DNMs in *TP53.* The computer-based and data-driven identification of deleterious DNM carriers in *TP53,* who are otherwise hidden in a wide population, enabled a new perspective of DNMs that is gene and disease-centric. In addition, we introduce *Famdenovo.brca* for hereditary breast and ovarian cancer syndrome (HBOC), and apply it to a small set of family data from the Cancer Genetics Network (CGN).

Results

***Famdenovo* for predicting the deleterious DNM status among mutation carriers**

Famdenovo is based on widely-used Mendelian models (Chen et al. 2004, 2006b; Parmigiani et al. 1998; Wang et al. 2007, 2010) and calculates the probability that a deleterious germline mutation is *de novo*. The *de novo* probability of interest can be written as the Pr(father is a noncarrier, mother is a noncarrier | child is a mutation carrier, family history). We calculate this probability as a function, g(*c*, *f*, *m*), of pedigree-based carrier probabilities for the child (*c*), father (*f*) and mother (*m*), respectively. We derived the g() function using Bayes' rule (as detailed in the Methods section), and obtained input values using Mendelian models as the corresponding marginal probabilities for one individual's *c*, *f*, and *m*, respectively. Each of the marginal probabilities is calculated using the Elston-Stewart algorithm (Fernando et al. 1993). *Famdenovo* then applies Bayes' rules to estimate *de novo* probabilities in deleterious mutations. Similar to previous developed Mendelian models, *Famdenovo* can be adapted for specific disease-gene associations by setting its input parameters: the penetrance of the disease-gene, the allele frequency of the gene(s), and the *de novo* mutation rate. We have incorporated well-validated parameter estimates in *Famdenovo.TP53* for LFS-*TP53* (Peng et al. 2017) and *Famdenovo.brca* for HBOC-*BRCA1/2* (Chen et al. 2006a; Parmigiani et al. 2007).

Based on the validation study and a sensitivity analysis in the LFS cohort data, we recommend classifying individuals with *de novo* estimated probability greater than 0.2 as DNM carriers. The family-wise *de novo* probability is defined to be the largest *de novo* probability of all the family members, accounting for the fact that children of *de novo* carriers may carry familial mutations. We classify a family as *de novo* if they present a family member with a *de novo* probability that is above the 0.2 cutoff.

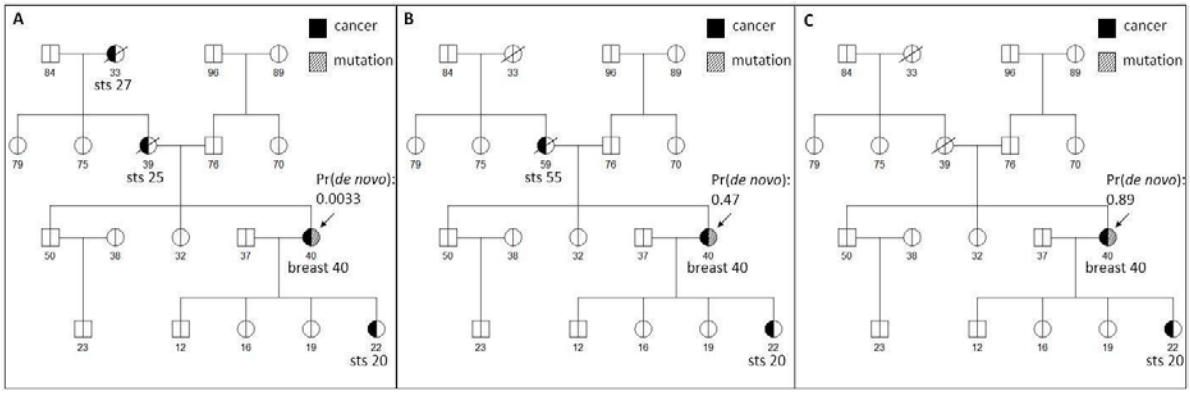


Figure 1. Illustration of clinical counseling using *Famdenovo* based on family history. The number below each individual is the age at last contact (healthy or affected with cancer). The arrow points to the counselee who is known to be a *TP53* mutation carrier. The *de novo* probabilities shown are given by *Famdenovo.TP53*. **(A)** A pedigree with four cancer patients. **(B)** A pedigree with three cancer patients. **(C)** A pedigree with two cancer patients.

Figure 1 depicts three scenarios of a hypothetical pedigree resulting in different DNM probabilities in *TP53* given by *Famdenovo.TP53*. **Figure 1A** shows a pedigree with four cancer cases: two soft tissue sarcoma (STS) at 25 and 27 years old, one breast cancer at 40 years old, and another STS at 20 years old. The arrow points to the counselee who is a *TP53* mutation carrier, and the probability of the mutation being *de novo* for the counselee is 0.0033, which is below the 0.2 cutoff. As the probability is below the cutoff, *Famdenovo.TP53* predicted that this mutation is not *de novo*. Based on pedigree analysis, it is likely that this is a familial mutation inherited from the proband's mother. **Figure 1B** shows a similar pedigree with three cancer patients, with one less affected relative in the maternal branch of the counselee compared with **Figure 1A**, and with an older age of diagnosis for the counselee's mother, which increases the predicted probability of the mutation being *de novo*. The *de novo* probability given by *Famdenovo.TP53* is 0.47, which is greater than the 0.2 cutoff. As the probability is above the cutoff, *Famdenovo.TP53* predicted this mutation to be a DNM. **Figure 1C** shows a similar pedigree with only two cancer cases in the counselee and counselee's offspring, compared with **Figure 1B**. Because there were no longer any cancer patients in the older generations and the counselee's siblings were all healthy, it was very likely that this mutation was *de novo*. The probability of the mutation being *de novo* was 0.89, which was much greater than the 0.2 cutoff, so *Famdenovo.TP53* predicted this mutation to be a DNM.

Validation of *Famdenovo.TP53* in individuals with known DNM status in *TP53*

We evaluated the performance of *Famdenovo.TP53* based on germline *TP53* tested individuals with known DNM status from the four cohorts (**Figure 2**). Among the 324 LFS family pedigrees we collected, 186 families had a known DNM status and 138 had an unknown DNM status (**Figure 2**). **Table 1** provides a summary of the demographics for all four study cohorts. The largest set of DNM carriers was contributed by the MD

Anderson (MDA) cohort. In total, there were 42 known DNM carriers and 144 families with known FM carriers.

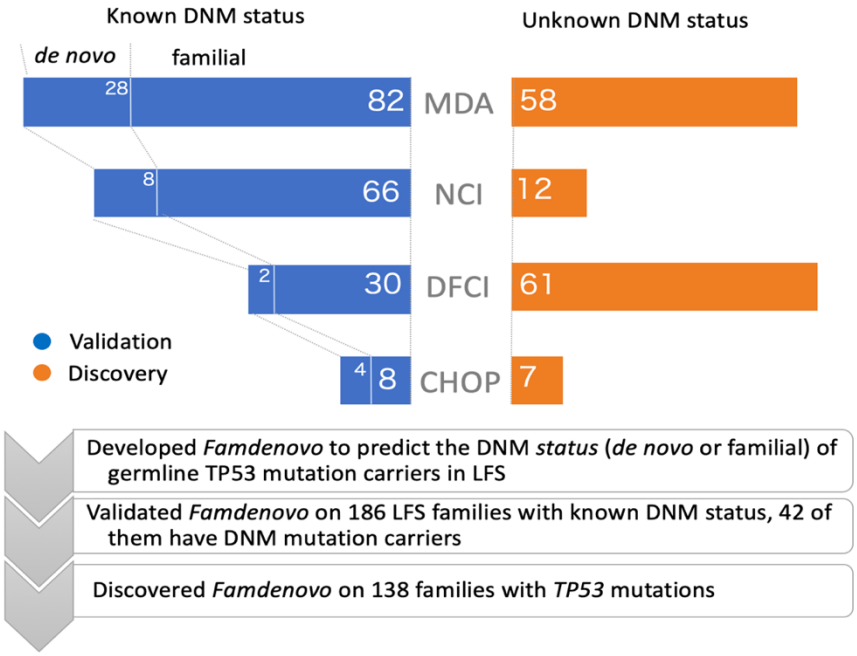


Figure 2: Study design and methods. The study included four cohorts (from MDA, National Cancer Institute or NCI, Dana Farber Cancer Institute or DFCI and Children Hospital of Philadelphia or CHOP) in our study. A subset of families within each cohort have mutation testing results from the parents, and therefore the true DNM status is known. These families form the validation set of our study. In the other families, the family members' DNM statuses are unknown, and they form the discovery set.

Table 1. Summary of demographic information for all the families with *TP53* mutation carriers from the four cohorts

Cohort			Individuals per families		Age at first diagnosis	
MDA	No. of Families	140	Mean	45	Mean	42.3
	Max Family Size	151	Standard Deviation	31	Standard Deviation	22.2
	Min Family Size	4				

NCI	No. of Families	78	Mean	24	Mean	33.9
	Max Family Size	130	Standard Deviation	17	Standard Deviation	22.1
	Min Family Size	3				
DFCI	No. of Families	91	Mean	34	Mean	38.9
	Max Family Size	107	Standard Deviation	25	Standard Deviation	20.5
	Min Family Size	3				
CHOP	No. of Families	15	Mean	26	Mean	43.4
	Max Family Size	45	Standard Deviation	12	Standard Deviation	25.2
	Min Family Size	13				

We used observed:estimated ratios (OEs) and receiver operating characteristic (ROC) curves to evaluate the calibration and discrimination of our model. **Figure 3** shows the ROC curves for the validation set from the four cohorts combined. The total sample size for the validation was 186 families; the corresponding area under the curve (AUC, or concordance index) was 0.95 (95% CI: [0.92, 0.98]), which demonstrated that *Famdenovo.TP53* performed well in discriminating DNM carriers from FM carriers. The calibration was satisfactory, with some under-prediction and an OE of 1.33 (95% CI: [1.09, 1.63]) (**Table 2**).

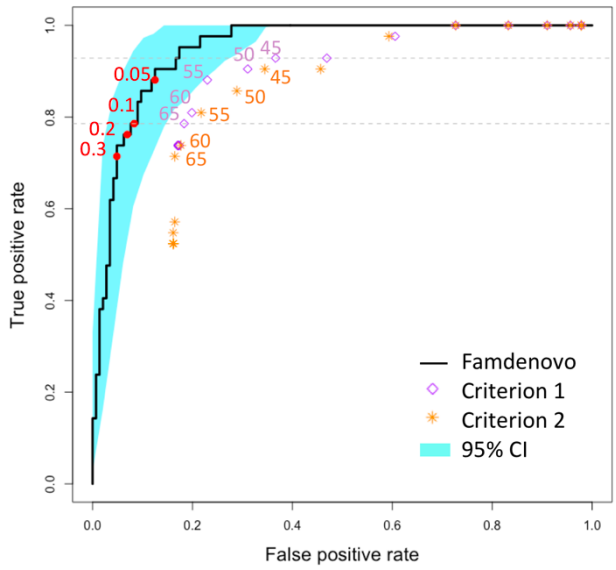


Figure 3. ROC curves for the validation of *Famdenovo.TP53*. The x axis is the false positive rate (i.e., 1 – specificity) and the y axis is the true positive rate (i.e., sensitivity). The black curve corresponds to the results of *Famdenovo.TP53* on the 186 families in the validation set and the blue shade shows the 95% confidence interval. The red circles represent the different *de novo* cutoff probabilities for the model. The purple diamonds correspond to the result of Criterion 1 at a cut-off age (provided next to the diamonds). The orange stars represent the result of Criterion 2 at a cut-off age (provided next to the stars). The gray dotted lines show the range of cut-off ages, 45 to 65, where a partial AUC will be calculated for Criterion 1.

Table 2: Comparison of AUCs of *Famdenovo.TP53* and various criterion-based prediction models, where 95%CI were obtained by bootstrapping 1000 times. The criterion-based prediction models predict a mutation to be familial if the mutation carrier has a parent diagnosed with cancer before a cut-off age. The AUC of the criterion-based prediction model is calculated by converting the original partial AUC to the [0, 1] range (see the Methods section for details).

	Mean	95% CI
Famdenovo.TP53		
AUC	0.95	[0.92, 0.98]
Criterion 1 AUC	0.82	[0.78, 0.87]

Diff AUC of Criterion 1	0.14	[0.054, 0.20]
Criterion 2 AUC	0.80	[0.77, 0.84]
Diff AUC of Criterion 2	0.15	[0.087, 0.20]
OE	1.33	[1.09, 1.63]

We also compared our method with two simple criterion-based prediction models that do not require the collection of extended pedigree data. Criterion 1 predicts a mutation to be *de novo* if the mutation carrier does not have a parent diagnosed with cancer before a cut-off age, e.g., 45 or 60. Criterion 2 predicts a mutation to be *de novo* if, in addition to criterion 1, neither of the grandparents was diagnosed with cancer before the cut-off age. Criterion 1 is more relaxed than Criterion 2. **Figure 3** shows that both criteria (over a range of cut-off ages) fall outside of the 95% confidence interval band of our method. Neither of the criteria is able to reach the full range of the ROC curve. Criterion 2 follows the same trend as criterion 1, but it is less sensitive and only slightly more specific. Moreover, for each criterion we calculated a partial AUC area with cutoff ages from 45 to 65, which were then rescaled to the full range of 0 to 1. The rescaled AUC for Criterion 1, the better performing of the two criteria, is still much lower than *Famdenovo.TP53*, with a difference of 0.14 (95% CI: [0.054, 0.20]) (**Table 2**). Our results demonstrate that *Famdenovo.TP53* outperforms criterion-based prediction models and that extended pedigrees with cancer diagnosis information are needed for the accurate identification of DNM carriers.

We evaluated the sensitivity and specificity of *Famdenovo.TP53* under various prediction cutoff values using the validation set (**Supplementary Table 2**) in order to identify an optimal classification cutoff. If the predicted probability was equal to or above the prediction cutoff, the individual was classified as a DNM carrier. As the cutoff probability increases, the sensitivity decreases and specificity increases. We chose a cutoff of 0.2, which provided a good tradeoff of a sensitivity of 0.76 and a specificity of 0.93. Maintaining a high specificity is essential to allow for downstream clinical interpretation of *de novo* mutations. Using the observed 25% for deleterious DNMs in our *TP53* clinical cohorts, we estimated that *Famdenovo.TP53*, at a cutoff=0.2, will achieve a positive predictive value (PPV) of 0.78 and a negative predictive value (NPV)

of 0.92. Without using pedigree information and model-based analysis like *Famdenovo.TP53*, like Criterion 1, will only achieves a PPV of 0.63 and an NPV of 0.92 (cutoff age=65, sensitivity=0.8, and specificity=0.8).

We also conducted a sensitivity analysis to evaluate how the pedigree size affects the validation results. The range of pedigree sizes for each of the four cohorts is listed in **Table 1** (see also **Supplementary Figure 1**). We divided the 186 families in the validation dataset into two groups: large families and small families, using the median size of all families (n = 29) as the cut-off size. We obtained similar results under the two scenarios (small families: AUC 0.96, 95% CI [0.91, 0.99], large families: AUC 0.94, 95% CI [0.89, 0.98]). These results support the robustness of our model by showing that *Famdenovo* is capable of accurately predicting *de novo TP53* mutations in a range of family sizes.

The discriminating ability of *Famdenovo.TP53* was not affected by the two input parameters, one of which was the germline *TP53* mutation allele frequency in the general population, the other being the DNM rate in the *TP53* gene, which has not been well estimated. Tuning the two parameters barely influenced the AUC or OE (**Supplementary Table 3**).

Discovery in individuals with unknown DNM status using *Famdenovo*

We applied *Famdenovo.TP53* to the 138 families with unknown DNM status in the discovery set from the same four cohorts. **Figure 4** provides the number and percentage of predicted DNM carriers at a cutoff of 0.2. In total, 40 (95% CI: [30, 50]) out of the 138 families were predicted to be *de novo*. The number of predicted *de novo* families for MDA, NCI, DFCI and CHOP were 23 out of 58 (95% CI: [16, 30]), 7 out of 12 (95% CI: [4, 10]), 7 out of 61 (95% CI: [3, 12]) and 3 out of 7 (95% CI: [1, 5]), respectively. MDA and CHOP had higher percentages of DNMs, likely due to differences in ascertainment across cohorts.

As a sensitivity analysis, we evaluated the number and percentage of predicted DNM carriers using prediction cutoffs of 0.05, 0.1, and 0.3, results of which are shown in **Supplementary Table 4**.

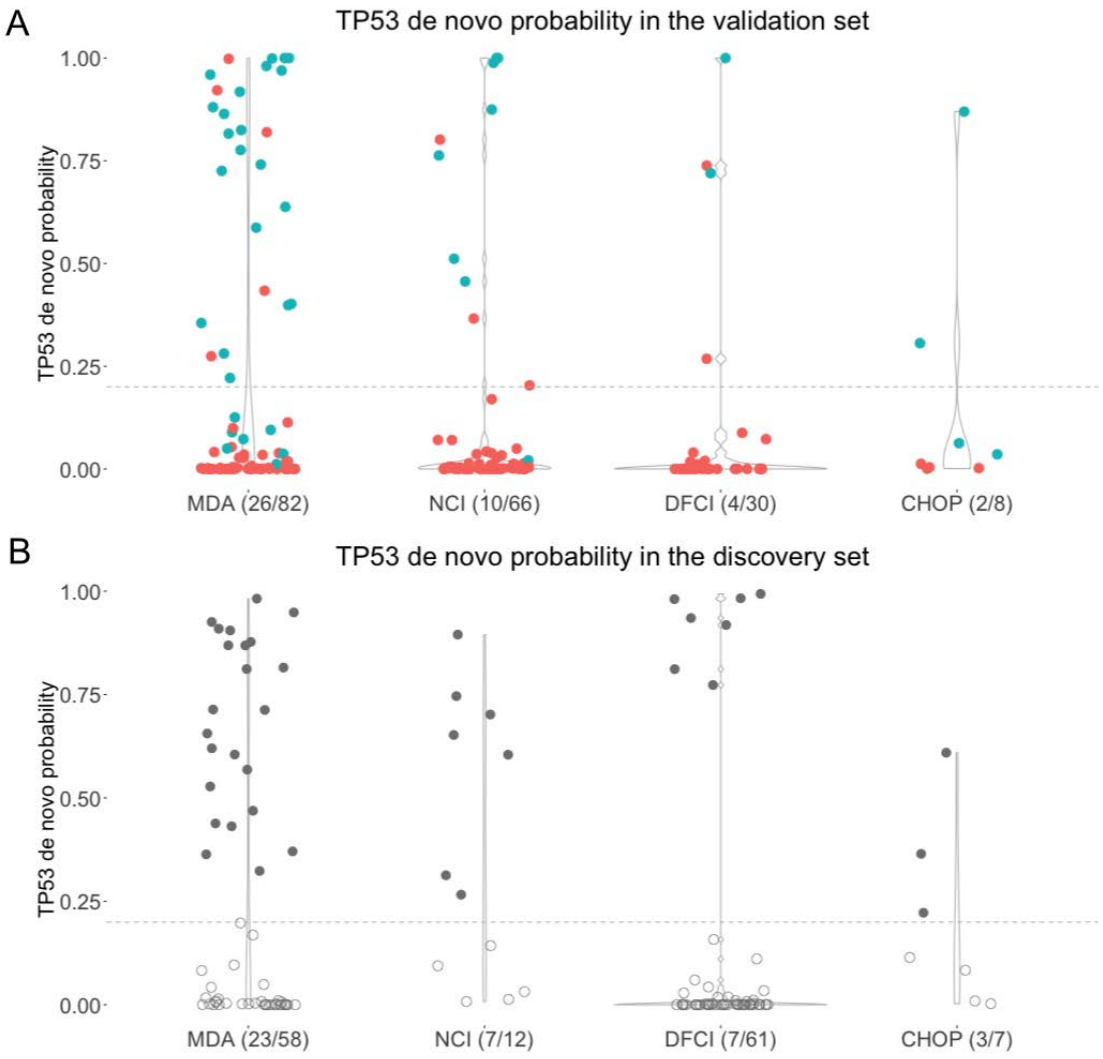


Figure 4: Distributions of the *de novo* probabilities in validation and discovery sets in the four cohorts. The y axis is the predicted *de novo* probability by *Famdenovo.TP53*. The width of each column changes over y axis, representing the percent of individuals who have the corresponding range of probability values. The individuals with points above the gray line (cutoff = 0.2) are predicted to be DNM carriers. The x axis gives the names of the cohorts and the corresponding predicted numbers of DNM carriers out of the total number of individuals. (A) In the validation set, the individuals with blue dots are true DNM carriers, and those with red dots are true FM carriers. (B) In the discovery set, the individuals with solid gray dots are predicted to be DNM carriers, and those with empty dots are predicted to be FM carriers.

Association of deleterious DNMs in *TP53* with clinical outcomes

Combining the validation and discovery sets resulted in a total of 82 DNMs from 82 families and 450 FMs from 242 families, which equipped our study with a sample size that was meaningful to evaluate associations between mutation status and a diverse range of individual outcomes: sex, cancer types, multiple primary cancers, age-of-cancer-diagnosis, parental ages, and types of mutation.

Sex and breast cancer. In order to scrutinize whether there is a difference in the biology of DNMs between males and females, we determined if the male:female ratio was significantly different in *de novo* and familial *TP53* mutation carriers. Comparing DNM to FM carriers in 397 cancer patients, we observed more females than males (Odds Ratio (OR) = 1.78, 95% CI: [1.01, 3.13]) (**Supplementary Table 5**). Considering the strong association between sex and breast cancer, we asked whether this significant finding comes from ascertainment bias. To investigate this, we performed another test within the probands, i.e., the index person that brings the family into the research cohort. This time, in contrast, we found no sex differences (34% DNMs in female and 32% DNMs in male, OR=1.08, 95% CI: [0.58, 2.03]) (**Supplementary Table 5**). All but two DNM carriers were probands. Therefore, we infer the previous significant finding was due to an imbalanced addition of FM carriers, who were relatives of the probands, to the female and male categories, i.e., a numerical artifact. In the following association analyses, we present comparisons within the probands only.

Next, we asked whether the DNM status was associated with breast cancer. Among 233 probands with cancer information (**Supplementary Table 6A-B**), we observed an OR = 1.9 (95% CI: [1.09, 3.33]) for DNMs in breast cancer as compared to other cancer types. We further divided the probands into three age categories: [0, 20), [20, 35) and 35+, based on the age of last contact. We observed within the [20, 35) age category an OR = 5.98 (95% CI: [1.81, 19.76]) for DNMs in breast cancer as compared to other cancer types, whereas a similar test in the age 35+ category was not statistically significant. This association is again due to ascertainment of DNMs through patients with early-onset breast cancer and not with other cancer types (**Supplementary Tables 7** (Li and Fraumeni Jr. 1969; Tinat et al. 2009)).

Within the [20, 35) age category and probands with breast cancer, where the ascertainment of each individual is consistent, we found 23 DNM carriers (29% of all proband DNM carriers) and 25 FM carriers (16% of all proband FM carriers), resulting in a 48% (sd=7.2%) DNM rate. This extremely high proportion of DNMs is also observed

within the validation set only (n=11 vs. 8, **Supplementary Table 6C**). Sensitivity analyses (**Supplementary Table 6D**) were performed using various thresholds of age of last contact. Our observations remain consistent, supporting the robustness of this finding.

Multiple primary cancers. We evaluated the association between the DNM status and the risk of developing subsequent multiple primary cancers (MPC) within the probands. **Supplementary Table 8A** shows that more patients with DNMs present multiple primary than single primary cancers (OR=1.77, 95% CI: [1.01, 3.11]). Similarly, we attribute such association to the ascertainment practice, i.e., ascertaining patients with a personal history of multiple primary cancers (at least one cancer is at an early onset) is part of the clinical criteria used for patient ascertainment in our study cohorts (**Supplementary Table 7** (Li and Fraumeni Jr. 1969; Tinat et al. 2009)). In an attempt to adjust for ascertainment to make the DNM and FM carriers comparable, we looked within the [20, 35) age category at probands with MPC. We found 17 DNM carriers and 22 FM carriers, resulting in 44% of DNMs within this combined cohort population (sd=7.9%, **Supplementary Table 8B**). This high proportion is also observed with the validation set (n=10 vs. 8, **Supplementary Table 8C**). This result is congruent with the previous observation of a high DNM rate in probands with early-onset breast cancer.

Age-of-diagnosis and cancer spectrum. **Figures 5A** shows the distributions of cancer diagnosis age as stratified by cancer type and sex for the combined set (**Supplementary Figure 3** shows the distributions for the validation and discovery sets, respectively). We observed DNMs in all of the eight LFS spectrum cancers: breast, brain, leukemia, choroid, adrenocortical carcinoma, osteosarcoma, soft tissue sarcoma, and lung, as well as other invasive cancers that are not within the LFS spectrum. Other than breast cancer, most cancer types have both female and male DNM carriers, with the exceptions of lung cancer and leukemia. For lung cancer, DNMs were only presented in females (n=4, mutation type: p.G245S, p.S127F, p.R337H, **Supplementary Table 10**) and not males. For leukemia, DNMs also were only presented in females (n=2, mutation type: R282W, **Supplementary Table 9**). We found no difference in ages of diagnosis between DNM and FM carriers across cancer types, including breast cancer (**Figure 5A**).

Parental ages. We also investigated whether the parental ages (paternal or maternal) were different among *TP53* DNM and FM carriers. Parental age refers to the age of the parent at the time of child birth. Literature on the origins of DNMs suggested

their occurrence rate across the genome was correlated with paternal age (Kong et al. 2012). Little is known about occurrence of deleterious DNMs within a given gene with regard to parental ages. Here parental age is obtained by calculating the difference between the birth year of the child and the parent. **Figures 5B and 5C** show the distributions for the combined set. We observed that both paternal age (mean=29, sd=5.8) and maternal age (mean=27, sd=5.0) were similarly distributed between DNMs and FM carriers. This is consistent with what was reported recently in another study (Renaux-Petel et al. 2018) for deleterious DNMs within *TP53*.

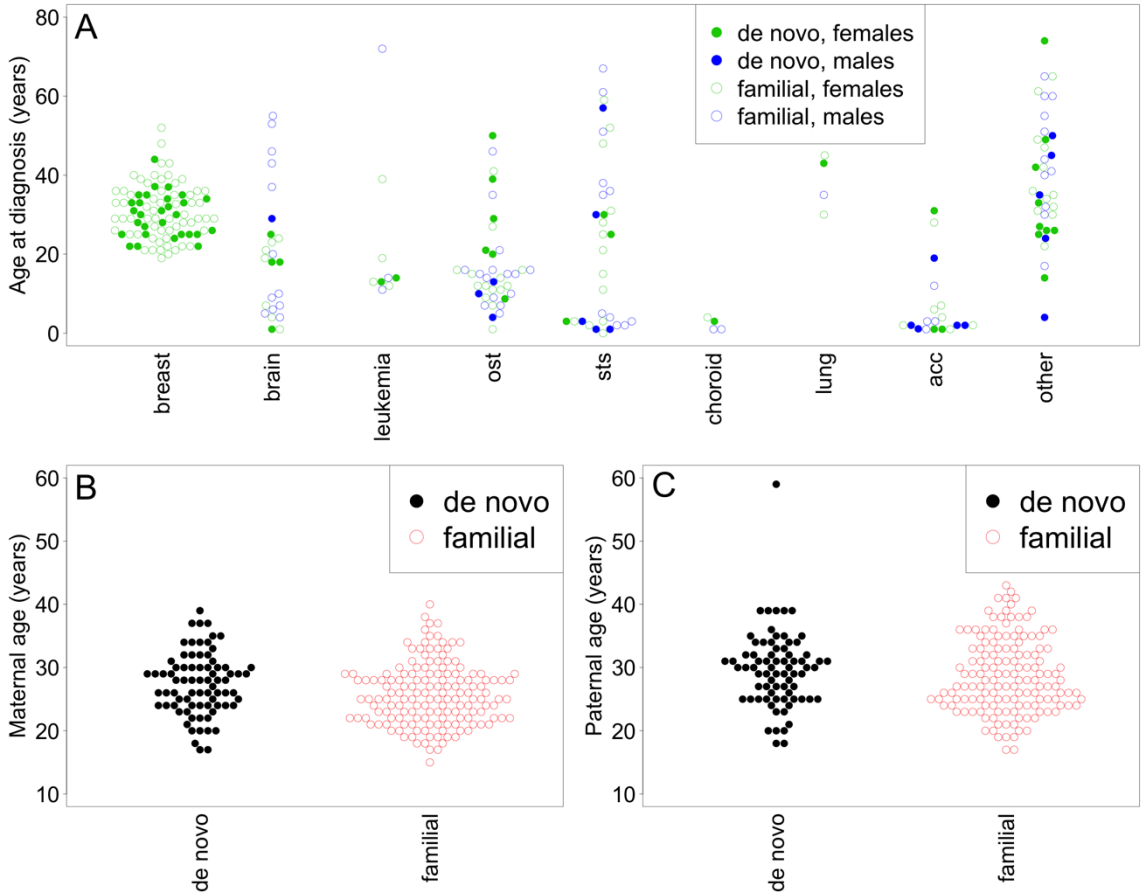


Figure 5. Ages of cancer diagnosis and parental ages in DNM and FM carriers. (A) Age of cancer diagnosis for all patients. The x axis represents types of different cancer types. Cancers were classified by site: LFS spectrum (osteosarcomas, soft-tissue sarcomas, breast, brain, adrenal, lung cancers and leukemia) and non-LFS spectrum (prostate, colon, kidney, or thyroid cancers and others). The green dots are for females,

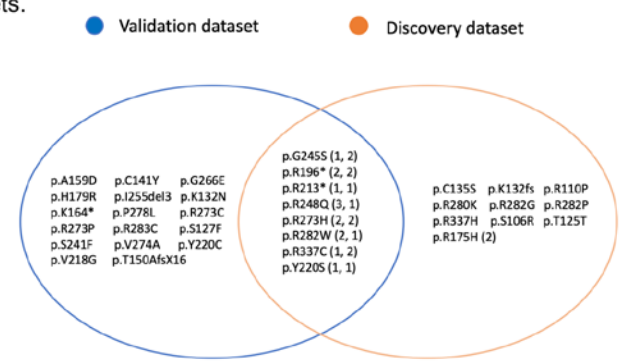
while the blue dots are for males. All solid dots represent the DNM carriers. **(B)** Distribution of paternal ages. **(C)** Distribution of maternal ages.

Mutation spectrum. **Supplementary Table 10** summarizes the frequencies of DNMs in each of the four cohorts MDA, NCI, DFCI, and CHOP within the validation and discovery sets. In this study of *TP53* mutations, there were a total of 35 variants that occurred as DNMs, and a total of 82 variants that occurred as FMs. **Figures 6A and 6B** summarize the occurrences of DNM and FM variants in the validation and discovery sets as well as their overlap in mutation types (22.9% and 34.1%). Multiple hotspot mutations were presented in FMs. Among them, two *TP53* hotspot mutations (Walerych et al. 2012), p.R248Q and p.R273H, were the most enriched in DNMs (p.R248Q, n=4, cancer types: acc, breast, ost, sts, **Supplementary Table 10**). In particular, p.R248W mutations were missing among the DNMs list for this study. Interestingly, among the FMs, p.R248W was as prevalent as p.R248Q (n=7 vs. 9 out of 242, **Figure 6B**). The observation of n=0 out of 82 DNMs for p.R248W was marginally significant (p-value=0.09, based on a Poisson distribution). The cancer types of FM-p.R248W carriers encompass the cancer spectrum of LFS, including breast cancers. The frequencies of DNMs at different amino acid positions (**Figure 6C and Figure 6D**) did not differ significantly in the validation and discovery sets, supporting the validity of predicted DNMs in the discovery set.

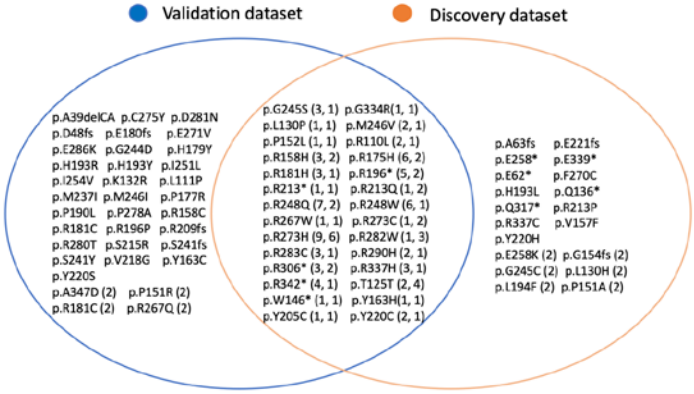
Deleterious DNM status among mutation carriers in BRCA1/2.

We used a dataset with 39 extended pedigrees from Cancer Genetics Network (Anton-Culver et al. 2003): 23 families had *BRCA1* mutation carriers, and 16 families had *BRCA2* mutation carriers. Among them, we predicted a total of 7 (17.9%) DMNs using a cutoff of 0.2. Among the 7 predicted DMNs, 4 people (17.4%) were *BRCA1* mutation carriers, and 3 people (18.8%) were *BRCA2* mutation carriers. Visual inspection of the family cancer history of these 7 families (**Supplementary Figure 2**) supports the validity of the *Famdenovo* predictions. Due to lack of genetic testing information of both parents, we were limited to positive testing results from some parents, which provided us with a small set of true negatives for validation. A total of 8 people from 6 families were confirmed to be FMs. *Famdenovo.brca* also predicted all of them as FMs (all with DNM probabilities < 0.13), which demonstrated the specificity of *Famdenovo.brca*.

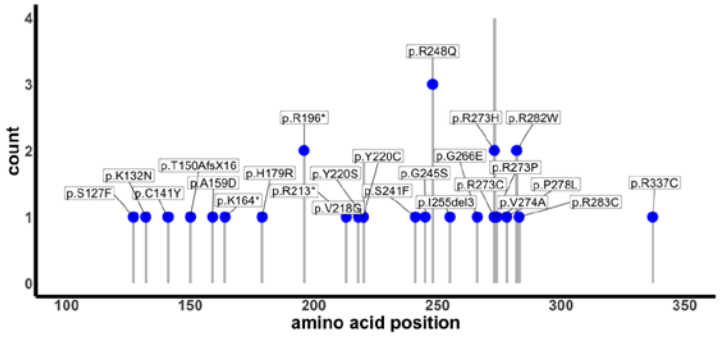
A. Venn diagram of *de novo* mutations in validation and discovery sets.



B. Venn diagram of familial mutations in validation and discovery sets.



C. Histogram of *de novo* mutations at different amino acid positions in the validation set.



D. Histogram of *de novo* mutations at different amino acid positions in the discovery set.

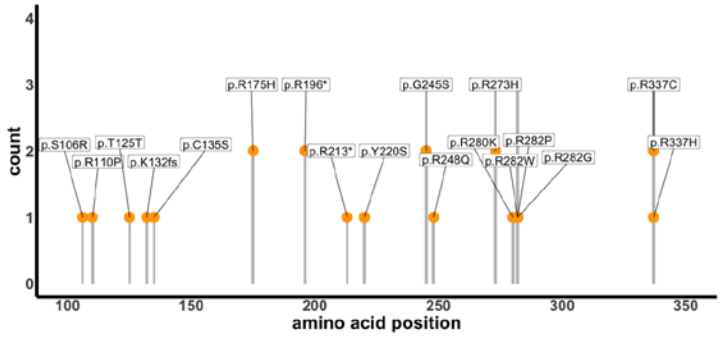


Figure 6. Mutation frequencies in the validation and discovery sets. The blue circles and dots represent the validation data set, and the yellow circles and dots represent the discovery data set. **(A-B)** Venn diagram of DNMs **(A)** and FMs **(B)** in the validation and discovery sets. The numbers in the parenthesis represent the occurrences of DNMs or FMs in the two sets. For example, p.G245S (1,2) means the mutation p.G245S appeared once in the validation set and twice in the discovery set. **(C-D)** Histogram of DNM frequency at different amino acid positions in the validation and discovery sets. The y axis is the count of DNMs. The x axis represents the amino acid positions.

Discussion

Deleterious germline DNMs are more prevalent than previously thought. For cancer genes such as *TP53*, we estimated a ~1:1 ratio of deleterious DNMs compared to FMs in our LFS cohorts, supporting the importance to study its etiology. To enable future genomic studies, we have developed a statistical method and tool, *Famdenovo*, to predict the deleterious DNM status in cancer genes using family history data. *Famdenovo.TP53* showed excellent performance, an AUC of 0.95, in discriminating DNM carriers from FM carriers of *TP53* deleterious germline mutations in 186 families collected from four clinical cohorts with varying ascertainment criteria. In our case study using 324 germline *TP53* pedigrees, *Famdenovo.TP53* identified a total of 82 deleterious DNM carriers (increased from only 42 with trio-based genetic testing results, 80 of which were cancer patients) and 450 deleterious FM carriers (317 of which were cancer patients), contributing to a sample size that is large enough to test for associations with patient outcomes: sex, cancer types (>9 different types), multiple primary cancers, age-of-cancer-diagnosis, parental ages, and mutation types. We provide a reference of cancer spectrum and mutation spectrum contrasting *TP53* DNM and FM carriers as a resource for future studies, complementing the ongoing efforts to accurately annotate *TP53* variants (Leroy et al. 2017; Tikkanen et al. 2018). Hotspot mutations p.R248Q and p.R273H, the most commonly mutated residues in breast cancer (Walerych et al. 2012), were the most frequently observed mutation types among DNMs. In contrast, p.R248W was not observed in DNMs, but was prevalent in FMs.

The observed discrepancy in frequency of p.R248W in DNMs versus FMs, but not in p.R248Q, is consistent with the literature on functional differences in the two mutations (Walerych et al. 2012). When mutated in cancer cells, p.R248W has recently been reported to induce a much stronger response from T cells than p.R248Q (Malekzadeh et

al. 2019). Germ cells containing DNMs are sustained in a microenvironment consisting of parental cells that do not carry such DNMs, hence mimicking heterogeneous cell-cell interactions that are similar to those presenting somatic mutations. In our data, it is plausible that a neoantigen-like response to p.R248W mutated germ cells has helped destroy these cells so that they are not viable for reproduction. If these germ cells have escaped such force, however, then their offspring may no longer incur this immune-like effect as all cells now contain this mutation. This hypothesis is supported by our observation of a high prevalence of p.R248W in FMs. An alternative hypothesis to explain this observation is that DNMs in p.R248W were simply not ascertained into this study, for unknown reasons, as discussed further below.

DNM carriers do not manifest family history and are likely to be identified in clinic only when they have developed multiple cancers, very early-onset breast cancer, or their offspring(s) have developed cancer. They are hence currently under-represented in cancer genetics studies. We estimate that our four study cohorts may miss up to 45% of deleterious DNMs in *TP53,* due to the lack of genetic testing for cancer patients with no family history or healthy individuals who carry these mutations. Correspondingly, we estimate a population prevalence of 0.00076 for deleterious *TP53* mutations, which is 26% higher than shown in previous studies (Peng et al. 2017). Our finding, that deleterious DNMs of *TP53* are as frequent as FMs in early-onset breast cancer, is strong evidence to support the NCCN guideline for women with early onset breast cancer to be offered testing for *TP53*, regardless of family history, to capture the DNM carriers who are not as rare as previously thought. Our results provide a quantitative evaluation on DNMs to genetic counselors so that they may tailor sessions to carefully explain the new knowledge. More importantly, DNMs are sparse in the general population although their downstream effect is detrimental for families. Among the four cohorts, there were 138/324 probands with missing parental genotypes. In these cases, *Famdenovo.TP53* would be useful to predict DNMs. Identification of patients with DNMs in order to study them collectively will further our understanding of the molecular mechanism underlying deleterious DNMs and is essential for future identification of the hidden DNMs for genetic testing.

Missing data in parental genotypes is more prevalent in HBOC cohorts, hence restricting us from conducting an extensive validation of *Famdenovo.brca*. Genetic counselors suggested that it is likely because the parents of *BRCA1/2* mutation carriers are generally at much older ages when they entered a study, hence at a higher

frequency of loss to follow-up, as compared to parents of *TP53* mutation carriers (commonly childhood cancer patients).

We remain cautious with the downstream interpretations of *Famdenovo* predicted DNMs, or to a larger extent any other biological factors that may confound our interpretation. We enumerate the following four considerations in the context of *TP53*, which are applicable to other genes of interest in future. First, mosaicism in parents could be partially contributing to DNMs in the children (Renaux-Petel et al. 2018). If a parent presents an LFS-like phenotype due to a mosaic *TP53* mutation, *Famdenovo.TP53* would consider the parent as a germline mutation carrier, hence deducing the child to be an FM carrier. The DNM status of a child under this condition would then be missed by *Famdenovo.TP53*. Such a condition is rare, at ~5% (Renaux-Petel et al. 2018), and of less concern to clinical practice as this population is already targeted by genetic testing. Patients with an LFS-like phenotype are often tested, first with Sanger sequencing, then with NGS at high depth, until mosaicism is confirmed. Second, somatic clonal expansion in cancer patients may create a look-alike germline *TP53* mutation, resulting in a false positive in DNM status. However, such observation was reported in a general panel-testing population, which is supposedly >99.9% without *TP53* mutations, hence presenting an expected low positive predictive value (Weitzel et al. 2018). By focusing on clinically ascertained families, we do not expect aberrant clonal expansion to be the cause of the identified DNMs. Furthermore, we only observed 2 DNM carriers who have ever had leukemia, a cancer type known to present clonal hematopoiesis. Third, *Famdenovo.TP53* assumes the mutations are deleterious and their effect on cancer outcomes follow the penetrance curves previously estimated from a set of families with LFS. Variants with unknown significance in *TP53* were excluded from the study. Last, DNMs across the genome were reported to be shared in siblings (Jónsson et al. 2018). Within a single gene like *TP53,* however, we did not observe sibling sharing.

Because of our study design, distinct from existing DNM studies that are genome-wide and across many individuals, we are limited to a small number of mutations, which even with *Famdenovo'*s discovery set is still too small to perform sequence context-based analyses that would follow those existing DNM studies. In contrast, what existing studies cannot provide is a laser-beam view of the impact of *de novo* mutations in a single gene and a single disease syndrome. Both study designs should be carried out in order to provide a well-rounded understanding of *de novo* mutations.

In summary, we present a new epidemiological study design, enabled by a statistical method called *Famdenovo*, to evaluate the clinical impact of deleterious *de novo* mutations in cancer genes. We introduce both *Famdenovo.TP53* and *Famdenovo.brca* and illustrate both models using family history cohorts. This study design is important for future genetic research, as well as the clinical management of patients and their families as we learn new biology and associated clinical risk for DNM carriers. *Famdenovo* is freely available at http://bioinformatics.mdanderson.org/main/Famdenovo.

Methods

Model development

Famdenovo estimates the probability of *de novo* mutation in any designated family member who carries a deleterious germline mutation in a given gene, based on a detailed family disease history and values of genetic parameters, such as the penetrance and prevalence, for the gene and the corresponding diseases. The prevalence for gene mutations is expressed as *Pr(G)*, where *G* denotes genotype, which could be wildtype (denoted as 0), a heterozygous mutation (denoted as 1), or a homozygous mutation (denoted as 2). *Pr(G)* for three genotypes can be derived from the prevalence of the mutated alleles using the Hardy-Weinberg equilibrium. The penetrance is the probability of developing the disease at a given age for individuals who carry the germline mutations. The disease of interest is an inherited syndrome, such as Li-Fraumeni syndrome or HBOC. The genes (one or more) of interest are those that are known to be the major cause of the corresponding inherited syndrome when they are mutated. The germline mutations here correspond to deleterious mutations that are accepted in clinics as mutations that would categorize the carriers as high risk for developing the disease in the future and who will be suggested to adhere to a series of cancer prevention protocols. Other mutations in these genes, including those that are variants of unknown significance, are considered as wildtype in the model. Either dominant or recessive mode of inheritance can be assumed in *Famdenovo*.

Let ***P*** be the pedigree information of the family (e.g., the pedigree drawn in Figure 1), let ***D*** be the disease phenotype information of all family members, and G_C be the genotype of the carrier, then the probability of the mutation being *de novo* can be written as $\Pr(G_c \text{ is } de\ novo \mid G_c \text{ is germline}, \mathbf{D}, \mathbf{P})$, referred to as the "*de novo* probability."

To calculate $\Pr(G_c \text{ is } de\ novo \mid G_c \text{ is germline}, \mathbf{D}, \mathbf{P})$, we have the following equation:

$$\Pr(G_c \text{ is } de\ novo \mid G_c \text{ is germline}, \mathbf{D}, \mathbf{P})$$
$$= \Pr\big(G_m = 0 \ \cap \ G_f = 0 \ \big| \ G_c = 1, \mathbf{D}, \mathbf{P})$$
$$= \frac{\Pr(G_c = 1 \mid G_m = 0, G_f = 0, \mathbf{D}, \mathbf{P}) \times \Pr\big(G_f = 0 \mid \mathbf{D}, \mathbf{P}\big) \times \Pr(G_m = 0 \mid \mathbf{D}, \mathbf{P})}{\Pr(G_c = 1 \mid \mathbf{D}, \mathbf{P})} \qquad (1)$$

where G_m is the genotype of the mother and G_f is the genotype of the father. In equation (1), all four probabilities are usually difficult to obtain through direct calculations. Hence, we apply Mendelian models to derive them. Let the family cancer history $\mathbf{H} = (\mathbf{P}, \mathbf{D})$. In a Mendelian model, the probability of the person's genotype given the family cancer history $\Pr(G_0 \mid \mathbf{H})$ is the updated population prevalence $\Pr(G_0)$ by incorporating family cancer history ***H***. Here, G_0 denotes the genotype of the person of interest, which can be G_m, G_f or G_c. We can estimate it via the following formula.

$$\Pr(G_0 \mid \mathbf{H}) = \Pr(G_0 \mid \mathrm{H}_0, \mathrm{H}_1, \dots, \mathrm{H}_\mathrm{n}) = \frac{\Pr(G_0)\Pr(\mathbf{H} \mid G_0)}{\sum G_0 \Pr(G_0)\Pr(\mathbf{H} \mid G_0)} \quad (2)$$

$$\Pr(\mathbf{H} \mid G_0) = \sum\nolimits_{G_1, G_2, \dots, G_n} \Pr(\mathbf{H} \mid \boldsymbol{G}) \Pr(G_1, G_2, \dots, G_n \mid G_0) =$$
$$\sum\nolimits_{G_1, G_2, \dots, G_n} \left[\prod\nolimits_{i=0}^{n} \Pr(\mathrm{H}_i \mid G_i)\right] \Pr(G_1, G_2, \dots, G_n \mid G_0) \qquad (3)$$

In equations 2 and 3, *n* is the total number of the counselee's relatives within a family. $\Pr(\mathbf{H} \mid G_0)$ is the probability of the phenotypes for the whole pedigree given the genotype of the counselee, which is the weighted average of the probabilities of family history given each possible genotype configuration of all relatives $\Pr(\mathbf{H} \mid \mathbf{G})$. The weights are the probabilities of the genotype configuration based on Mendelian transmission. $\Pr(\mathbf{H} \mid \mathbf{G})$ are products of the individual probability distributions of penetrance $\Pr(H_i \mid G_i)$ when we assume conditional independence. With the equations above for $\Pr(G_0 \mid \mathbf{H})$, we can then calculate the four probabilities in equation (1), respectively, by assigning the right person as G_0 and assuming known genotype status in the parents, when needed.

The posterior probability calculation is performed using the Elston-Stewart, or peeling algorithm. This algorithm characterizes Mendelian transmission using a transmission matrix of the probability of the genotype for an individual given the genotypes of the father and mother, $\Pr(G_i \mid G_{fi}, G_{mi})$ (Fernando et al. 1993).

With the calculated probabilities, we classify the DNM status based on a cutoff on the probability. $\Pr(G_c \text{ is } de\ novo \mid G_c \text{ is germline}, \mathbf{D}, \mathbf{P}) \geq cutoff$ are identified as DNM carriers. We used the validation data in this study to determine an ideal cutoff.

Parameter settings: penetrance and prevalence. *Famdenovo.TP53*: We used a previous penetrance estimate for *TP53* mutation carriers and noncarriers from six large pediatric sarcoma families at The University of Texas MD Anderson Cancer Center, which are not included in this study (Wu et al. 2006). We assumed the *TP53* mutation follows Hardy-Weinberg equilibrium, but this could be modified by user's input when homozygous genotype information is published. The mutation prevalence, i.e., allele frequency, is specified as 0.0006 for pathogenic *TP53* mutations, which was derived in our previous study (Peng et al. 2017). The assumed frequencies for the three genotypes (homozygous reference, heterozygous, and homozygous variant) were 0.9988, 0.001199, and 3.6e-07, respectively. We used 20% as percent DNMs among all mutations. The allele frequency and DNM rate are priors that are then updated by family history to generate a posterior probability. Both penetrance and prevalence estimates were previously validated using external study cohorts that are different from the study cohorts in this study (Peng et al. 2017).

Famdenovo.brca: We used previous externally validated penetrance estimates of *BRCA1* and *BRCA2* mutations of the US population for both Ashkenazi Jewish (AJ) and non-AJ families (Parmigiani et al. 2007; Chen et al. 2006a). We used 0.00305 and 0.0034 as the mutation allele frequencies for BRCA1 and BRCA2, respectively. We assumed a 10% DNM rate among all mutations. The allele frequency and DNM rate are priors that are then updated by family history to generate a posterior probability. The same cutoff value 0.2 was used to classify DNMs in *BRCA1/2*.

Study cohorts

We evaluated our method on *TP53* using four cohorts (**Supplementary Table 1**). (A) Families with LFS primarily ascertained through clinical criteria were included in the MDA cohort. Details on data collection, specific cancers, and germline testing for this cohort have been published (Shin et al. 2020). This cohort has 82 families with known *de novo* status and 58 families with unknown *de novo* status. (B) The National Cancer Institute (NCI) LFS cohort (NCT01443468), our second cohort, is from a long-term prospective, natural history study that started in 2011 (Mai et al. 2016). The cohort

includes individuals meeting classic or Li-Fraumeni-like diagnostic criteria, having a pathogenic germline *TP53* mutation or a first- or second-degree relative with a *TP53* mutation, or having a personal history of choroid plexus carcinoma, adrenocortical carcinoma, or at least three primary cancers (Birch et al. 1994). This cohort includes 66 families with known *de novo* status and 12 families with unknown *de novo* status. (C) The Dana Farber Cancer Institute's (DFCI) LFS cohort, our third cohort, is comprised of patients identified in the course of clinical genetics practice. The early years of the cohort were the result of single gene testing of individuals with features suggestive of LFS. Since 2012, the cohort includes individuals with *TP53* mutations identified on multigene panel testing, and individuals referred for clinical consultation of enrollment in a whole-body MRI surveillance protocol. This cohort has 30 families with known *de novo* status and 61 families with unknown *de novo* status. (D) Data from the Children's Hospital of Philadelphia (CHOP) were obtained through the Cancer Predisposition Program that provides care and counseling for children with genetic predisposition to cancer. This cohort has eight families with known *de novo* status and seven families with unknown *de novo* status.

We also tested *Famdenovo.brca* using a small set of family data that is from the Cancer Genetics Network (CGN) (Anton-Culver et al. 2003), http://www.cancergen.org/studies.shtml, a network established by the National Cancer Institute in 1999 to explore the impact of genetics on cancer susceptibility. Individuals with a family history of cancer were enrolled at clinical centers across the United States and completed questionnaires regarding family history of cancer and other relevant information. Most families were ascertained through high-risk clinics and have a family history of breast or ovarian cancers.

Mutation testing

For the MD Anderson cohort, peripheral blood samples were collected after informed consents were obtained. The probands' *TP53* mutation status was determined by PCR sequencing of exons 2-11 (Hwang et al. 2003). At MD Anderson Cancer Center, when a *TP53* mutation was identified, all first-degree relatives of the proband (affected and unaffected by cancer) and any other family member at risk of carrying the familial mutation were tested. Extending germline testing based on mutation status and not on phenotype of family members should not introduce ascertainment bias during analysis (Wu et al. 2006; Katki et al. 2008). Individuals unavailable for testing (largely deceased)

linked to mutation carriers were considered as obligate mutation carriers. No other family member was tested when the proband tested negative.

For the NCI cohort, copies of the clinical *TP53* test reports were obtained and verified by the study team for those tested prior to enrollment. For individuals actively participating in the protocol and not previously tested, clinical genetic testing was performed after enrollment. All at-risk family members of individuals who tested positive for a mutation (either prior to enrollment or on study) were offered the option of having site-specific testing through the study. No testing was offered to relatives if the proband tested negative for a *TP53* mutation. Additionally, high resolution melt analysis and multiplex ligation-dependent probe amplification were performed in the NCI cohort to detect large deletions or genomic rearrangements.

For the DFCI cohort, the patient information was collected by searching through the Clinical Operations and Research Information System (CORIS) database at the Dana Farber Cancer Institute. The classic (Li and Fraumeni Jr. 1969) and updated Chompret (Tinat et al. 2009) criteria were applied for selecting eligible families. The methods for *TP53* testing were further described in a previous study from DFCI (Rath et al. 2013).

For the CHOP cohort, pediatric oncology patients were evaluated in the Cancer Predisposition Program based on a primary tumor type concordant with the LFS tumor spectrum (e.g., adrenocortical carcinoma, choroid plexus carcinoma, early onset rhabdomyosarcoma, multiple primary cancers, etc.) and/or a suggestive family cancer history. Most patients met Classic or Chompret criteria. The laboratories used to complete clinical *TP53* testing included The Genetic Diagnostic Laboratory at The University of Pennsylvania, The Hospital for Sick Children, or Ambry Genetics.

Model evaluation and comparison

In four cohorts (described above) of *TP53* mutation carriers with known *de novo* status, we used *Famdenovo.TP53* to calculate the *de novo* probability for each individual. Because mutation carriers within the same family are not independent, we used family-wise *de novo* probability and status for model evaluation. If a family had at least one family member with a *de novo* probability that was over the cut-off, they were defined as a *de novo* family; otherwise they were defined as a familial family.

Validation was performed on 186 families with known DNM status from the four cohorts. We used OEs to evaluate the calibration and receiver operating characteristic (ROC) curves to evaluate our model's discrimination ability. The OE is the ratio between

the observed number of *de novo TP53* mutations and the summation of the estimated probabilities of *de novo TP53* mutations; ideally, the observed number of DNMs equals the estimated number (OE=1). The ROC curve is generated by plotting the true positive rate against the false positive rate at various prediction cutoffs using *Famdenovo.TP53*. A high area under the ROC curve (AUC), i.e., concordance index, indicates that we can find a point on the ROC curve for determining the *de novo* status with a high true positive rate and a low false positive rate.

We also compared our method with the other two criterion-based prediction models. The partial AUC is rescaled to the full range of 0 to 1 using the following equation:

$$Rescaled\ AUC\ \ of\ model = \frac{partial\ AUC\ of\ model}{Partial\ AUC\ \ of\ Famdenovo} * full\ AUC\ of\ Famdenovo \quad (4)$$

Finally, 95% CI were obtained by bootstrapping the AUC values of *Famdenovo* and the AUC or rescaled the AUC of the two criterion-based models.

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