Ultra-deep sequencing detects ovarian cancer cells in peritoneal fluid and reveals somatic *TP53* mutations in noncancerous tissues

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Edited by Philip C. Hanawalt, Stanford University, Stanford, CA, and approved March 31, 2016 (received for review January 25, 2016)

Current sequencing methods are error-prone, which precludes the identification of low frequency mutations for early cancer detection. Duplex sequencing is a sequencing technology that decreases errors by scoring mutations present only in both strands of DNA. Our aim was to determine whether duplex sequencing could detect extremely rare cancer cells present in peritoneal fluid from women with high-grade serous ovarian carcinomas (HGSOCs). These aggressive cancers are typically diagnosed at a late stage and are characterized by *TP53* mutations and peritoneal dissemination. We used duplex sequencing to analyze *TP53* mutations in 17 peritoneal fluid samples from women with HGSOC and 20 from women without cancer. The tumor *TP53* mutation was detected in 94% (16/17) of peritoneal fluid samples from women with HGSOC (frequency as low as 1 mutant per 24,736 normal genomes). Additionally, we detected extremely low frequency *TP53* mutations (median mutant fraction 1/13,139) in peritoneal fluid from nearly all patients with and without cancer (35/37). These mutations were mostly deleterious, clustered in hotspots, increased with age, and were more abundant in women with cancer than in controls. The total burden of *TP53* mutations in peritoneal fluid distinguished cancers from controls with 82% sensitivity (14/17) and 90% specificity (18/20). Age-associated, low frequency *TP53* mutations were also found in 100% of peripheral blood samples from 15 women with and without ovarian cancer (none with hematologic disorder). Our results demonstrate the ability of duplex sequencing to detect rare cancer cells and provide evidence of widespread, low frequency, age-associated somatic *TP53* mutation in noncancerous tissue.

TP53 mutations | ultra-deep sequencing | ovarian cancer | clonal hematopoiesis | premalignant mutations

The detection of tumor-specific mutations in clinically accessible samples has enormous potential to transform cancer diagnostics, monitoring, and screening. However, a major limitation is insufficiently accurate sequencing methods. Conventional next-generation sequencing (NGS) technologies have a high false positive error rate, which precludes reliable detection of mutations at frequencies <1/100 (1). "Molecular tagging" of single-stranded DNA decreases the rate of false mutations to less than 1 per 10,000 sequenced nucleotides and has been successfully applied to the detection of mutant cancer DNA in a variety of clinical samples (2–6). However, this false positive error rate limits the specificity of this method in challenging situations in which ultra-deep sequencing is needed to detect extremely low frequency mutant molecules (e.g., <1/10,000), as is the case of ovarian cancer DNA in Pap smears (5). Because true mutations are indistinguishable from artifacts, compromised specificity leads to lower sensitivity and overall low diagnostic accuracy. Duplex sequencing is an NGS technology that employs molecular tagging of both strands of DNA independently. True mutations are defined as mutations that are present at the same position in both strands of DNA and that are complementary (7). This internal error correction effectively reduces false positive mutations because PCR and sequencing artifacts are very unlikely to occur at both strands of DNA at the same position and with complementary nucleotide changes (theoretical false positive rate is $\sim 4 \times 10^{-10}$) (7). Previous studies have demonstrated that duplex sequencing is able to detect a single point mutation among $>10^7$ sequenced nucleotides (7, 8), an unprecedented accuracy that holds significant potential for early cancer detection.

High-grade serous ovarian carcinoma (HGSOC) is the most common and most aggressive type of ovarian cancer, with a dismal 5-y survival rate of 10–30% (9). A main cause of poor prognosis is the absence of effective screening tools to detect early-stage disease. HGSOC frequently metastasizes through the peritoneal cavity, the anatomic potential space between abdominal and pelvic organs and the abdominal walls. The putative premalignant lesion to HGSOC is intraepithelial neoplasia in the distal fallopian tube (also known as serous tubal intraepithelial carcinoma), which is in direct continuity with the peritoneal cavity (10–12). Even in the absence of gross metastasis to the peritoneum, ovarian cancer cells can frequently be found in peritoneal fluid upon cytopathological examination, and the presence of these cells has prognostic value in the current clinical staging system (13). Thus, peritoneal fluid is routinely collected during surgery for women with suspected ovarian cancer. We reasoned that peritoneal fluid is an optimal clinical biopsy for high-sensitivity early detection of ovarian cancer because

Significance

The detection of rare tumor-specific somatic mutations in "liquid biopsies" is limited by the high error rate of DNA sequencing technologies. By sequencing peritoneal fluid from women with high-grade serous ovarian cancer, we demonstrate that duplex sequencing, currently the most accurate sequencing technology, is able to detect one cancer cell among tens of thousands of normal cells. This unprecedented sensitivity also revealed a striking prevalence of extremely low frequency *TP53* mutations in normal tissue. Women with and without cancer harbored *TP53* mutations of pathogenic consequences, both in peritoneal fluid and peripheral blood. These mutations likely represent a premalignant mutational background that accumulates in cancer and aging.

Author contributions: J.D.K., L.A.L., E.M.S., and R.A.R. designed research; J.D.K. and M.I.H. performed research; M.W.S., K.J.A., and S.R.K. contributed new reagents/analytic tools; J.D.K., M.J.E., and R.A.R. analyzed data; and J.D.K., L.A.L., E.M.S., and R.A.R. wrote the paper.

Conflict of interest statement: M.W.S. and L.A.L. declare leadership, consulting role, and stock ownership at TwinStrand Biosciences, Inc.

This article is a PNAS Direct Submission.

Data deposition: Sequencing data from this paper have been deposited in the Sequence Read Archive [accession no. SRP072370 (BioProject ID: PRJNA316476)].

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1601311113/-/DCSupplemental.

HGSOC disseminates early and preferentially to the peritoneal cavity, suggesting that cancer cells may be more abundant in peritoneal fluid than in the relatively distant uterine cavity or cervix, as previously attempted by other studies (5, 14).

An important feature of HGSOC, which may facilitate early detection by high-sensitivity sequencing, is the high prevalence of tumor protein p53 gene (*TP53*) mutations (>96%) (15, 16), even in premalignant lesions (17). Moreover, >95% of *TP53* mutations cluster in exons 4–10 (15), which provides a relatively small target to perform cost-efficient ultra-deep sequencing (8). Thus, we used duplex sequencing to sequence *TP53* exons 4–10 in peritoneal fluid from patients with HGSOC with known *TP53* mutations and control patients without ovarian cancer. Our goal was to provide proof of principle of the ability of duplex sequencing to detect very rare cancer cells, and thus the study was enriched for subjects with early stage disease or negative peritoneal fluid by traditional cytological evaluation.

Results

Patients and Sequencing Information. Sixteen patients with HGSOC ("ovarian cancers") (*SI Appendix*, Table S1) and 20 patients without detected gynecologic malignancy ("controls") (*SI Appendix*, Table S2) were included in this study. Seven ovarian cancer patients and 10 control patients had germline *BRCA1* or *BRCA2* breast cancer genes mutations. Half of the cancers were early stage (0–II). *TP53* mutations were determined by NGS in all primary tumors except three occult microscopic cancers, for which Sanger sequencing was used due to the limited amount of DNA (*SI Appendix*, *SI Materials and Methods*) (18–20). A single clonal *TP53* mutation was found in all tumors (*SI Appendix*, Table S3). No additional clonal or subclonal *TP53* mutations were found in any of the primary tumors (NGS average depth was ~300×). Peritoneal fluid was centrifuged, and DNA was extracted from the cell pellet, obtaining an average of 11.9 μg of DNA (range 1.1–112 μg). Duplex sequencing for *TP53* exons 4–10, which cover >95% of mutations in HGSOC (15), was performed. Molecular tagging of both strands of DNA allowed us to group raw reads sharing the same molecular tag into a single-strand consensus sequence (SSCS) and to collapse the two SSCSs with complementary tags into a single, highly accurate duplex consensus sequence (DCS) (*SI Appendix*, Fig. S1). The median DCS depth was calculated as the median DCS coverage at each genomic position in the capture target, and this value essentially corresponds to the total number of unique haploid genomes sequenced. The median DCS depth for the 37 peritoneal fluid samples ranged from 1,689 to 36,133. Sequencing information is presented in Dataset S1.

Duplex Sequencing Detected Tumor-Specific *TP53* Mutations in Almost All Ovarian Cancer Peritoneal Fluid Samples. The *TP53* mutation identified in the primary tumor was detected in 94% (16/17) of HGSOC peritoneal fluid samples (Fig. 1), including 9 peritoneal fluids without malignant cytopathology. In one of the cases (case 16), the mutation was not detected in DCS reads but was present in two SSCS reads well above background. The only cancer missed was an occult stage IA carcinoma with negative cytopathology. Importantly, half of the tumor mutations (8/16) were found at a frequency at or below 0.001 (3/16 at a frequency at or below 0.0001) and would not be reliably discernible from technical errors with less accurate sequencing techniques.

In one patient, peritoneal fluid samples were available from two different surgeries: primary surgery and subsequent surgery for recurrent disease 2 y later. The exact same tumor-specific *TP53* mutation was found in both. The tumor mutant allele frequency (TMAF), which should approximate the fraction of tumor cells in the sample, increased dramatically from 0.000039 to 0.685 from the initial peritoneal wash to the recurrent ascites.

In univariate analyses of dependent variables vs. TMAF in peritoneal fluid, TMAF was not significantly associated with preoperative CA-125 (Spearman test), germline *BRCA* status, clinical stage, or future recurrence (Mann–Whitney test). Higher TMAF

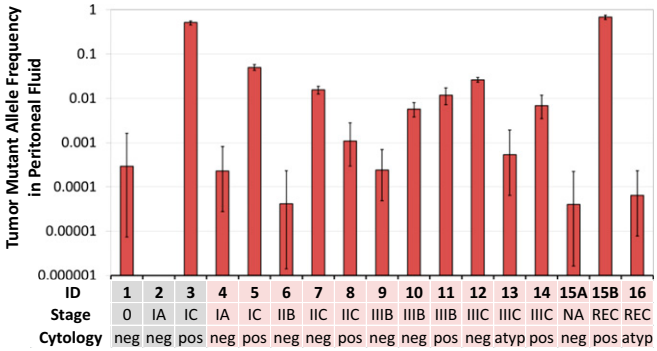

Fig. 1. *TP53* mutations detected in ovarian tumors are also present in peritoneal fluid. Sample ID, Fédération Internationale de Gynécologie et d'Obstétrique (FIGO) stage, and cytopathological results are indicated in the *x* axis. Samples 1–3 correspond to cancers incidentally discovered at prophylactic surgery in women with hereditary *BRCA* mutations. Samples 15A and 15B correspond to primary and recurrence surgeries from the same patient. Sample 15A was unstaged because this patient was undergoing chemotherapy for a previously diagnosed breast malignancy. Tumor mutant allele frequency was calculated as the number of Duplex Consensus Sequence (DCS) reads with the given mutation divided by the total DCS nucleotides sequenced at the mutation coordinate. Tumor 16 was the only one with an indel, and matching peritoneal fluid showed the indel mutations in single-strand consensus sequences, but not DCS. Errors bars represent the exact/Clopper–Pearson 95% confidence interval. atyp, atypical; NA, not available; neg, negative; pos, positive; REC, recurrence.

was, however, significantly associated with positive peritoneal fluid cytology (mean ± SD, 0.212 ± 0.30 in positive cytology vs. 0.004 ± 0.009 in negative or atypical cytology, Mann–Whitney $P = 0.009$) and ascites as opposed to peritoneal washes (mean ± SD, 0.132 ± 0.27 in ascites vs. 0.048 ± 0.15 in peritoneal washes, Mann–Whitney $P = 0.009$). After adjusting for possible confounding of one variable by another in multivariate models, higher TMAF remained associated with ascites but not with positive cytology. In addition to ascites, older age also associated with higher TMAF in all models (*SI Appendix*, Table S4).

Low Frequency *TP53* Mutations Were Found in Peritoneal Fluid from Nearly All Patients. We detected low frequency (<0.001) *TP53* mutations in nearly all of the peritoneal fluid samples from women with and without cancer (Fig. 2) (16/17 cancers and 19/20 controls). A total of 197 mutations were found (Dataset S2): 97 mutations in ovarian cancer patients and 100 mutations in controls. It was previously demonstrated that duplex sequencing is able to uniquely detect a single mutation among $>10^7$ sequenced nucleotides, which places the false positive rate of this technology below 10^{-7} (7). Using this figure as a conservative estimate of the false positive error rate and given the fact that a total of $\sim 3.8 \times 10^8$ DCS nucleotides were sequenced in this study, we calculated that potentially ~38 mutations could be artifacts. This figure represents only 20% of all of the mutations found and leaves >150 mutations unlikely to be explained as technological error. To distinguish them from the tumor mutations, we termed these highly prevalent low frequency *TP53* mutations "biological background."

The mean number of biological background mutations per sample was 5.3 (range, 0–14). The number of mutations was directly proportional to the DCS depth for each sample (i.e., the deeper a sample was sequenced, the more mutations were found) (*SI Appendix*, Fig. S2). For cancer patients, the tumor mutation was more abundant than biological background mutations in the majority of peritoneal fluid samples (11/16) and was at least 10-fold more abundant than biological background mutations in 50% (8/16) of samples (Fig. 2*A*).

Low Frequency *TP53* Mutations in Peritoneal Fluid Are Similar to Cancer-Specific *TP53* Mutations. To explore whether these low

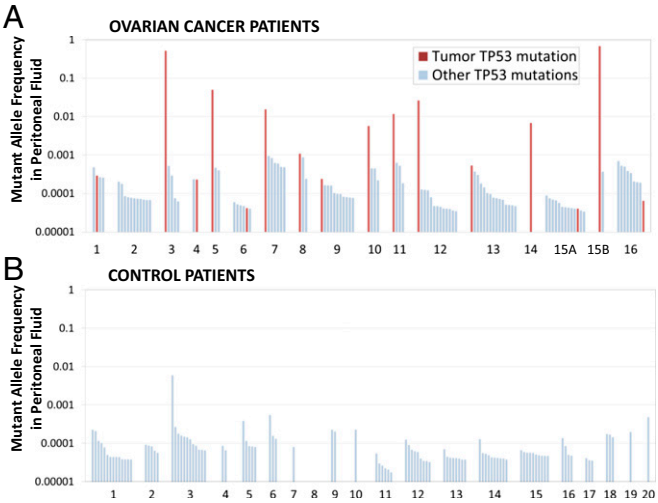

Fig. 2. Mutant allele frequency of *TP53* mutations (exons 4–10) in peritoneal fluid from patients with ovarian cancer (*A*) and controls (*B*). Each bar represents a unique mutation observed at least once. Mutations are ordered by descending mutant allele frequency within each patient. Magenta bars indicate tumor mutations.

frequency *TP53* mutations were biologically relevant, we first analyzed their type. Most of the background mutations were missense, both in women with (67/97) and without cancer (72/100) (Fig. 3*A*). The prevalence of missense mutations is consistent with clonal *TP53* mutations most commonly seen in human cancers in general and in ovarian cancers in particular, according to the International Agency for Research on Cancer (IARC) ovarian cancer database (21, 22) (*SI Appendix*, Fig. S3). Specifically, within exons 5–8, which encode the protein's DNA-binding domain and harbor the majority of *TP53* mutations found in human cancers, there are 1,567 possible single nucleotide substitutions, which are expected to produce 73.4% missense, 3.7% nonsense, and 22.9% silent mutations (23). However, in our study, silent mutations were less than half the expected frequency, in both cases and controls (*SI Appendix*, Fig. S4). Nonsynonymous mutations, which include missense and nonsense, represented 89.8% (53/59) and 90.0% (63/70) of all single nucleotide substitutions in these exons in cases and controls, respectively, significantly above the 77.1% expected in the absence of selection ($P = 0.027$ in cancers; $P = 0.013$ in controls, by Fisher's exact test). Approximately 80% of these missense mutations were projected to generate an inactive or partially inactive p53 protein (Fig. 3*B*) as determined by *TP53*-MUT 2.00, an online tool that predicts the biological activity of known *TP53* mutations (24). In addition, missense mutations were enriched in the 9 most commonly mutated "hotspot" *TP53* codons (175, 179, 195, 220, 237, 245, 248, 273, and 282) in ovarian carcinomas according to the IARC database (21, 22). These codons represent only 2.7% of the codons in our capture set; however, 10/67 missense mutations in cancers (14.9%) and 13/72 missense mutations in controls (18.1%) clustered in those codons ($P = 2 \times 10^{-4}$ and $P < 1 \times 10^{-5}$, respectively, by Fisher's exact test). Importantly, when cancers and controls were subdivided by *BRCA* germline status, the distribution of mutation type and the functional impact of missense mutations remained very similar for each of the groups (*SI Appendix*, Fig. S5). To demonstrate that the findings were not driven by outlier individuals, we also plotted the distribution of mutation type (*SI Appendix*, Fig. S6) and predicted functional impact of missense mutations (*SI Appendix*, Fig. S7) for each patient in the study. The overwhelming majority of patients (including 19/20 controls) harbored at least one deleterious mutation. Next, we analyzed the mutational spectra of *TP53* mutations found in ovarian cancers and controls (Fig. 3*C*). The spectrum was similar in both groups and showed an abundance of C:G→T:A transitions. Furthermore, 38.6% of C:G→T:A mutations occurred at CpG sites, which are known to be highly mutable and prone to deamination. These data are consistent with known mutational signatures in cancer associated with age, specifically signatures 1A and 1B reported in Alexandrov et al. (25, 26).

Low Frequency *TP53* Mutations Increase with Cancer and with Age. Next, we quantified the frequency of biological background *TP53* mutations for each individual as the number of mutations divided by the total number of DCS nucleotides. Interestingly, women with ovarian cancer had a significantly higher frequency of *TP53* mutations in exons 4–10 than cancer-free women (8.4×10^{-7} in cancers vs. 4.23×10^{-7} in controls, Mann–Whitney $P = 0.02$) (*SI Appendix*, Fig. S8), and this significant difference remained after adjusting for age and *BRCA* status ($P = 0.004$). Although our study was focused on *TP53* coding mutations, the sequencing protocol resulted in the incidental capture of intronic regions contiguous with *TP53* exons 4–10, which also contain low-frequency biological background mutations. The frequency of mutations in introns was higher in cancers than in controls, but this comparison was not statistically significant (*SI Appendix*, Fig. S8). Notably, in control women, we found that the frequency of *TP53* biological background mutations increased with age for mutations found in exons (Fig. 4*A*) as well as in introns (Fig. 4*B*). The frequency of mutations increased by 2.2×10^{-7} for every 10-y increase in age for exons ($P = 0.001$ after adjusting for *BRCA* status) and by 1.1×10^{-7} for every 10-y increase in age for introns ($P = 0.01$ after adjusting for *BRCA* status). These regressions also were done separately for women with and without germline *BRCA* mutations because of the near complete age separation between those two groups and were statistically significant for mutations in exons and introns regardless of germline mutational status (Fig. 4 *A* and *B*) with near parallel slopes, as assumed in the common regression model. In women with ovarian cancer, there was no association of exonic *TP53* mutations with age, but the intronic mutation frequency showed a significant increase of 3.0×10^{-7} with every 10-y increase in age after adjusting for *BRCA* status ($P = 0.0004$) (Fig. 4 *C* and *D*).

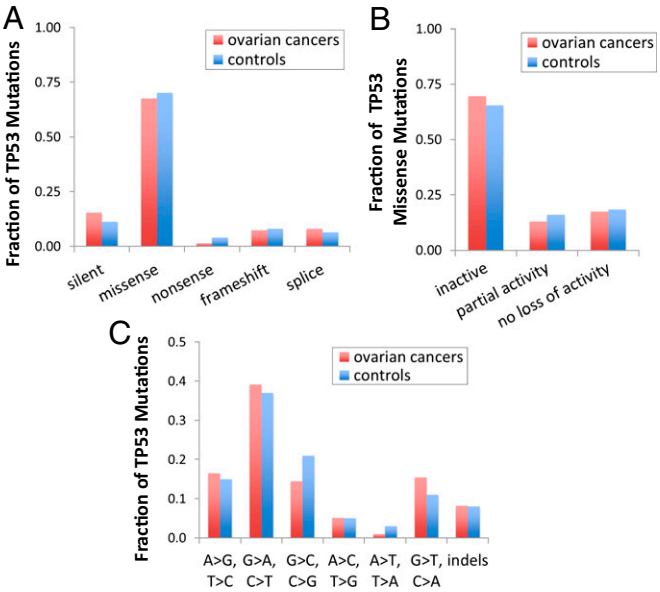

Fig. 3. Characterization of "biological background" *TP53* mutations found in peritoneal fluid of patients with ovarian cancer (97 mutations) and controls (100 mutations). The fraction of mutations is indicated for categories of mutation type (*A*), pathogenicity (*B*), and spectrum (*C*). *TP53* activity of missense mutations for *B* was determined via "MUT-*TP53* 2.00" (24).

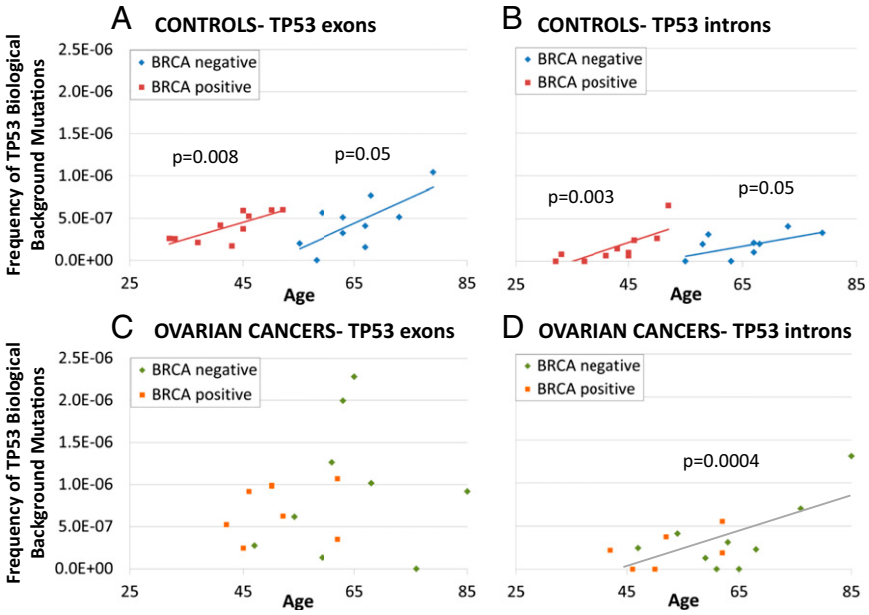

Fig. 4. Frequency of biological background mutations (number of mutations divided by total number of DCS nucleotides) detected in *TP53* exons 4–10 and corresponding introns in peritoneal fluid of women with and without ovarian cancer. Patients' age is indicated in the *x* axis, and germline *BRCA* status is color-coded for ovarian cancers and controls. Women without ovarian cancer showed significant associations between the frequency of biological background *TP53* mutations and age, both for mutations found in *TP53* exons (*A*) and introns (*B*). Because control women were segregated into young age and old age depending on germline *BRCA* status, the correlations with age (regression lines and Spearman's test *P* values) are presented separately for women with and without *BRCA* germline mutations. Women with cancer did not show a significant association between the frequency of biological background *TP53* mutations in exons and age (*C*). For introns, the association was significant after adjusting for *BRCA* status (*D*).

***TP53* Mutation Burden in Peritoneal Fluid Distinguishes Individuals With and Without Ovarian Cancer.** We sought to assess whether the *TP53* mutations detected in peritoneal fluid samples could distinguish individuals with ovarian cancer from controls without knowledge of the primary tumor *TP53* mutation. As previously shown, almost all cancer samples harbor the tumor mutation (some at relatively high allele frequency) (Fig. 1), and most cancer samples carry more *TP53* biological background mutations than controls (Fig. 4*C* vs. Fig. 4*A*). Thus, we reasoned that the total burden of mutated *TP53* molecules found in a sample could be a useful biomarker to distinguish individuals with and without cancer. For each sample, the mutation burden was calculated as the total number of mutant *TP53* molecules in exons 4–10 divided by the total number of DCS nucleotides sequenced. Fourteen out of 17 peritoneal fluid samples from cancer patients had a *TP53* mutation burden $>10^{-6}$ (Fig. 5). In contrast, only 2 out of 20 controls reached that threshold, and, interestingly, they were among the oldest controls in the study (Fig. 5). These frequencies correspond to 82% sensitivity and 90% specificity for distinguishing cancer patients from controls.

Low Frequency *TP53* Mutations Are also Found in Peripheral Blood. The observation that *TP53* biological background mutations are present in the peritoneal fluid of essentially all patients (with and without ovarian cancer) provides evidence for the emerging concept that somatic mutation in classically cancer-associated "driver" genes may occur in "normal" tissues (27). However, because peritoneal fluid consists of a heterogeneous mixture of cell types, we sought to assess whether rare *TP53*-mutated subclones were present in a different sample source. We chose peripheral blood because multiple studies have demonstrated that clonal hematopoiesis with mutations in driver genes occurs in a subset of normal individuals (28–33).

We applied duplex sequencing to whole blood samples from 15 women in our study (7 with ovarian cancer and 8 without cancer). No patients had known history of hematologic disease, and all but one were chemotherapy-naive at sample collection. We identified at least one low frequency *TP53* mutation in all patients (15/15), with a range of 1–8 mutations per sample (Fig. 6). A comprehensive list of these mutations can be found in Dataset S2. Similar to the findings in peritoneal fluid, these mutant clones were exclusively present at extremely low mutant allele frequency (≤0.001), and, thus, they are undetectable by less accurate sequencing methods. Importantly, 22% (13/58) of the biological background mutations in peripheral blood were also detected in the matched peritoneal fluid sample from the same patient, further proving the validity of these mutations. Leukocytes are common in peritoneal fluid (34), and, thus, overlap of mutations between matched blood and peritoneal fluid is expected. In one patient with ovarian cancer, the primary tumor *TP53* mutation was identified in blood DNA, consistent with the presence of circulating tumor cells or cell free tumor DNA in whole blood. In the other six ovarian cancer patients, the tumor-specific *TP53* mutation was not found in blood.

The type, pathogenicity, and spectrum of the *TP53* mutations found in blood were very similar to mutations found in peritoneal fluid (*SI Appendix*, Fig. S9). In addition, similar to peritoneal fluid, 21.6% (8/37) of the nontumor missense mutations were present at one of the nine previously mentioned *TP53* hotspot codons although these codons represent only 2.7% of the total codons in *TP53* exons 4–10 ($P = 6 \times 10^{-5}$). Furthermore, consistent with our findings in peritoneal fluid and reported age-related clonal hematopoiesis (28–30, 32, 33), the frequency of *TP53* biological background mutations in peripheral blood increased significantly with patient age ($P = 0.011$).

Discussion

This study demonstrates that duplex sequencing—the most accurate NGS technique currently available—is able to identify tumor DNA at mutant allele frequencies as low as ~1/25,000, which is

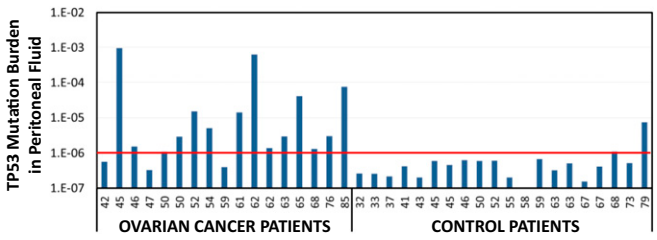

Fig. 5. *TP53* mutation burden in peritoneal fluid distinguishes women with ovarian cancer from controls. Within each group, patients are sorted by ascending age, indicated in the *x* axis. For each sample, the mutation burden was calculated as the total number of mutant *TP53* molecules (exons 4–10) divided by total DCS nucleotides sequenced. A threshold of 10^{-6} (red line, corresponding to one mutation for one million nucleotides) distinguishes cancers and controls with 82% (14/17) sensitivity and 90% specificity (18/20).

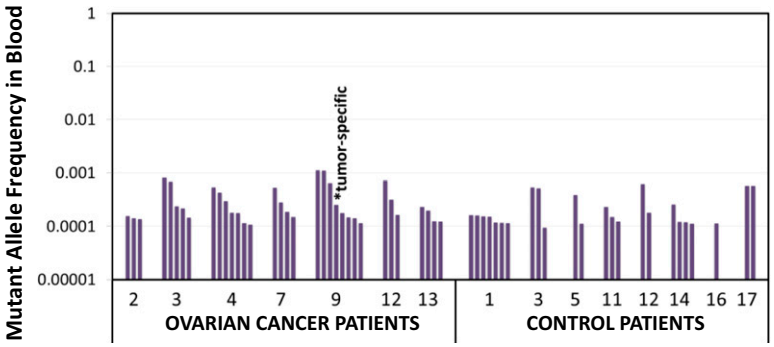

Fig. 6. *TP53* mutations in peripheral blood. Patient ID is indicated in the *x* axis. Each bar represents a unique mutation observed in at least one DCS. Mutations are ordered by descending mutant allele frequency within each sample. The tumor *TP53* mutation was detected in peripheral blood of case 9 but was not found in any other cases.

beyond the capabilities of other sequencing technologies. Ultradeep sequencing of *TP53* by duplex sequencing enabled the detection of tumor DNA in peritoneal fluid from women with ovarian cancer with 94% sensitivity, despite enriching cases for early stage and negative peritoneal cytology. Tumor DNA was present in some cases that would otherwise challenge clinical detection, in particular two cancers that were microscopic and identified only at risk-reducing surgery. These results provide evidence that ovarian cancer cells are present in the peritoneum even at the earliest detectable stages of disease and before clinically apparent metastasis.

The high resolution afforded by duplex sequencing uncovered the presence of extremely low frequency, cancer-like *TP53* mutations in peritoneal fluid and peripheral blood from nearly all patients with and without ovarian cancer. We termed these mutations "biological background" to distinguish them from tumor-specific *TP53* mutations. Previous studies have reported somatic mutation of cancer-associated genes in normal tissue (blood and skin), but generally in relatively small subsets of older individuals (27–33, 35). Here, we show that low frequency, cancer-like *TP53* mutations in noncancerous tissue are very common, but almost exclusively occur at allele frequencies <0.001, which is below the limit of detection of conventional sequencing methods. Remarkably, the only biological background mutation at frequency >0.001 was found in the oldest woman in the control group. Thus, our results demonstrate widespread, low frequency *TP53* mutagenesis, in nearly all individuals, that increases with aging and cancer. These mutations are likely present in both the mesothelial lining of the peritoneal cavity (the dominant cell type in peritoneal fluid samples) as well as in leukocytes. We speculate that biological background mutation may be a phenomenon common to all healthy replicative tissues, but larger studies including multiple tissue samples from healthy individuals across a wide range of ages will be necessary to fully explore this concept.

Several lines of evidence confirm that the majority of these biological background mutations are functional and not technical artifacts. First, they closely resemble *TP53* mutations in cancers: nearly all of them are predicted to partially or completely inactivate *TP53*, they are predominantly C:G→T:A transitions, and they cluster in *TP53* hotspot codons. Second, they are more abundant in women with cancer than in women cancer-free, and, in the latter, they increase with age. The age effect was observed for mutations detected in both exons and introns, and independently in the analysis of peritoneal fluid and peripheral blood. This age dependency is in concordance with prior studies of somatic mutations in noncancerous tissues (28–30, 32, 33) and supports the notion that "clock-like mutations" commonly found in cancers accumulate in normal cells with aging before the development of cancer (25). Third, a proportion (13/58) of the low frequency mutations found in blood were also detected in peritoneal fluid from the same patient. Because peritoneal fluid is known to contain a variable number of leukocytes (34), this overlapping is expected and demonstrates the reproducibility of our approach.

Our results indicate that noncancerous tissue carries clones with positively selected driver *TP53* mutations. This finding is in agreement with a recent report of somatic cancer mutations in normal human skin (36). Multiple cancer-associated driver genes, including *TP53*, seemed to be mutated and clonally expanded in large patches of morphologically normal skin cells. Although the high prevalence of these clones was surprising, it is consistent with the concept that cancer is the result of clonal evolution over the lifespan of an individual. It is conceivable for multiple competing clones with cancer driver mutations to coexist and remain untransformed within normal tissue, and it is expected that the number and size of these clones would increase with age. It is also expected that the individuals that harbor more of these clones have higher probability of developing cancer. Indeed, individuals in previous studies carrying detectable somatic mutations in blood were at increased risk of developing hematopoietic malignancies (28–31, 33). Elevated mutagenesis is thought to be a characteristic feature of premalignant and malignant tissues (37), and our finding that women with HGSOC have a significantly higher burden of *TP53* mutations in peritoneal fluid is consistent with this concept. Although the allele frequency of tumor-specific mutations was not always distinguishable from the level of biological background in cases, the total burden of mutated *TP53* molecules in peritoneal fluid (including tumor and biological background mutations) discriminated women with and without ovarian cancer with 82% sensitivity and 90% specificity. Importantly, these estimates are based on a small number of patients and should be validated in larger studies.

In summary, this proof-of-principle study demonstrates that duplex sequencing shows promise for the detection of rare tumor alleles in "liquid biopsies." Peritoneal lavage is an invasive procedure that is unsuitable for screening, but duplex sequencing could be applied to a wide range of clinical samples. Whereas previous studies using other sequencing approaches to identify tumor-specific mutations in Pap smear (5) or uterine lavage DNA (14) had suboptimal sensitivities, the coupling of duplex sequencing with these minimally invasive biopsies may generate more useful tools for early ovarian cancer screening. The exquisite sensitivity of duplex sequencing demonstrated here could also be applicable to the identification of cancer cells from other cancer types in liquid biopsies, a promising approach to improve early cancer detection, monitoring, and prognosis. Finally, we have also demonstrated that cancer mutations are found in noncancerous tissue in association with aging and cancer. This phenomenon may challenge diagnostic strategies that hinge on the identification of tumor-specific *TP53* alleles above the biological background in a given tissue. However, the finding that individuals with cancer had higher burdens of *TP53* mutation in peritoneal fluid samples suggests that mutational burden should be considered in future cancer biomarker studies.

Materials and Methods

Patients and Samples. Blood, tumor, and peritoneal fluid were obtained from the University of Washington Gynecologic Oncology Tissue Bank, which collected specimens and clinical information after informed consent under protocol number 27077 approved by the University of Washington Human Subjects Division institutional review board. The study included 16 patients with HGSOC ("cancers") (*SI Appendix*, Table S1) and 20 patients who had gynecologic

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