Data Descriptor: Comprehensive analyses of somatic TP53 mutation in tumors with variable mutant allele frequency

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Somatic mutation of the tumor suppressor gene TP53 is reported in at least 50% of human malignancies. Most high-grade serous ovarian cancers (HGSC) have a mutant TP53 allele. Accurate detection of these mutants in heterogeneous tumor tissue is paramount as therapies emerge to target mutant p53. We used a Fluidigm Access Array™ System with Massively Parallel Sequencing (MPS) to analyze DNA extracted from 76 serous ovarian tumors. This dataset has been made available to researchers through the European Genome-phenome Archive (EGA; EGAS00001002200). Herein, we present analyses of this dataset using HaplotypeCaller and MuTect2 through the Broad Institute’s Genome Analysis Toolkit (GATK). We anticipate that this TP53 mutation dataset will be useful to researchers developing and testing new software to accurately determine high and low frequency variant alleles in heterogeneous aneuploid tumor tissue. Furthermore, the analysis pipeline we present provides a valuable framework for determining somatic variants more broadly in tumor tissue.

Design Type(s): parallel group design • individual genetic characteristics comparison design
Measurement Type(s): Disruptive TP53 Mutation
Technology Type(s): DNA sequencing
Factor Type(s): diagnosis
Sample Characteristic(s): Homo sapiens • female gonad

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Background & Summary
The tumor suppressor gene TP53 is the most frequently mutated gene in somatic cells of human cancers, with mutant TP53 identified in over 50% of tumors. While wild-type p53 acts to suppress a tumorigenic phenotype, both loss-of-function and oncogenic gain-of-function (GOF) TP53 mutations promote tumorigenesis. In some tumors, such as high-grade serous ovarian cancers (HGSCs), TP53 mutation is an early event, likely occurring in precursor lesions. In colorectal cancer, mutation of TP53 can occur as a relatively late event in a multistep tumorigenetic pathway that progresses from hyperproliferative cells in colon epithelium, through colorectal adenomas and finally metastatic colorectal cancer. Germline mutation of TP53 is associated with Li-Fraumeni syndrome where carriers are predisposed to develop malignancies including early onset breast cancer, brain and adrenocortical tumours, leukemia and soft tissue sarcoma. Whether a mutation occurs in a single allele in the germline associated with increased risk of familial syndromes, or in sporadic cancers in somatic tissue where timing of its emergence may be different along the tumor progression pathway, has the potential to influence its frequency in tumor tissue.

There is a large and growing interest in targeting mutant p53 for cancer therapy, resulting in a concomitant need to accurately detect the presence of a TP53 mutation. This was the driving motivation for the original manuscript, i.e., to develop methodologies to accurately identify somatic TP53 mutation in HGSC that could be used to triage women with this malignancy into appropriate trials targeting specific forms of mutant p53. While the detection of a germline mutation in DNA extracted from a blood sample is relatively straightforward using the established method of Sanger sequencing, the detection of somatic DNA variants in tumor tissue, especially those occurring at low frequency, can pose challenges. Reasons for this include the heterogeneous nature of tumor tissue as the result of expansion of clonal populations and factors such as the presence of non-neoplastic cells, as well as aneuploidy, originating from tumor-associated phenomenon such as chromosomal instability.

Massively parallel sequencing (MPS) of tumor tissue for variant detection in single genes of interest to the exclusion of either a cohort of other genes or the entire genome, is not broadly supported by current technologies in a cost effective manner. The Fluidigm Access Array System, specifically the Access Array BRCA1/BRCA2/TP53 Target-Specific Panel (Fluidigm, South San Francisco, CA, USA) coupled with MPS, was a cost effective way to achieve our goal of generating comprehensive MPS data for TP53 in DNA extracted from a moderately sized cohort of primary ovarian tumors.

Here, we present a detailed analysis of TP53 MPS data using two software programs embedded within the Broad Institute’s Genome Analysis Toolkit (GATK), specifically HaplotypeCaller and MuTect2. HaplotypeCaller was specifically designed for the detection of germline mutations, although numerous papers have used this software for somatic variant calling. MuTect2 has been designed to detect a range of variant allele frequencies, as described below. TP53 variants identified by HaplotypeCaller were also screened for using Sanger sequencing and this data is presented. A schematic overview of this study, including bioinformatic pipelines, is presented (Fig. 1).

The dataset described herein offers a cohort on which to further develop robust methodologies and pipelines for determining a range of frequencies of somatic variants in tumor tissue that, by its very nature, is often heterogeneous and driven by genomic events resulting in aneuploidy. Data has been generated using DNA extracted from a cohort of HGSC (N=72) that is recognized as a genomically complex malignancy with extensive chromosomal abnormalities. Given that a large study from The Cancer Genome Atlas (TCGA) reported over 96% of HGSC with a somatic TP53 mutation, there was an expectation that TP53 mutation should be identified in the vast majority of HGSCs in this cohort. Somatic TP53 mutation is not a feature of low-grade serous ovarian cancers (LGSC), of which four are included here and in the original study.

Methods
This section includes, and expands upon, the Methods outlined in our earlier manuscript. When reference is made to previously published figures or tables (including Supplementary Data), the identifier is preceded by ‘OM’ denoting from the ‘Original Manuscript’. Methods, samples and datasets are outlined in the Experimental Study Table.

Study cohort
Seventy-two HGSCs and four LGSCs collected from between 2004–2014 at three hospitals (Royal North Shore Hospital, North Shore Private and The Mater Hospital—North Sydney, Sydney, Australia) were analyzed for this study (Supplementary Table OM-S3). Advanced stage HGSCs (Stage III or IV) made up the majority of this cohort (82%; 59/72). Written informed patient consent was obtained as per our ethics protocol (Protocol: 108–243 M, approved by the Northern Sydney Local Health District Human Research Ethics Committee). All tumors were snap frozen in liquid nitrogen and stored in the Kolling Institute of Medical Research (KIMR) Gynecological Tumor Bank until required.

Tumor DNA preparation
DNA was extracted from approximately 30 mg of fresh frozen tumor tissue. Tissue was homogenised in 50 μl phosphate buffered saline (PBS) until liquefied using two glass beads with shaking three times for 90 s each time at the highest frequency in a Retsch MM 301 Mixer Mill (MEP Instruments Pty. Ltd.,...
NSW, Australia). Protein was digested at 56 °C overnight with 20 μl of proteinase K (20 mg ml\(^{-1}\)) (Qiagen Pty Ltd, Chadstone, VIC, Australia). DNA was extracted using the DNeasy Blood and Tissue Kit in an automated system (QIAcube; Qiagen Pty Ltd, Chadstone, VIC, Australia). DNA concentration was determined using Qubit Fluorometric Quantitation, specifically using the Qubit dsDNA BR Assay Kit (Life Technologies Australia Pty. Ltd., Mulgrave, VIC, Australia). A NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Australia, Scoresby, VIC, Australia) was used to determine 260:280 and 260:230 ratios.

Fluidigm access array and massively parallel sequencing (MPS) of tumor DNA to identify somatic TP53 mutations

As described in the original manuscript\(^16\), DNA extracted from tumors was processed for MPS using the Access Array BRCA1/BRCA2/TP53 Target-Specific Panel (Fluidigm, South San Francisco, CA, USA). The 48.48 Access Array integrated fluidic circuits (IFC) was used, including target specific primers containing a common sequence tag (CS1 or CS2) and Illumina adaptors PE1 and PE2. Samples were identified by a sample specific barcode located on the reverse sequence (PE1_CS1 Forward Primer, 5’-AATGATACGGCGACCACCGAGATCTACACTGACGACATGGTTCTACA-3’, 47 bp; PE2_BC_CS2 Reverse primer, 5’-CAAGCAGAAGACGGCATACGAGAT [sample specific barcode] TACGGTAGCAGAGACTTGGTCT-3’, 56 bp). This system uses 16 primer pairs generating amplicons of between 191–209 base pairs to enable 92% coverage of TP53 exons.

Five μl of DNA (50 ng μl\(^{-1}\)) was added to the array and processed on the Fluidigm Biomark HD Real-Time PCR fluids system according to the manufacturer’s guidelines by the Ramaciotti Centre for Genomics (University of New South Wales, Randwick, Australia). Amplicon libraries were pooled and a single MPS run was performed on a MiSeq platform using Miseq Control Software (MCS) version 2.4.1 (Illumina Inc., San Diego, CA, USA).

Figure 1. Overview of workflow and bioinformatic pipelines employed in this study.
Sequencing data was received in FASTQ file format and adaptors trimmed using cutadapt (http://cutadapt.readthedocs.io/en/stable/guide.html). Trimmed FASTQ files were then aligned to the human genome (hg19) using Burrows-Wheeler Aligner (BWA) 0.7.10 and ‘known-indel’ realignment and recalibration which is embedded in the Broad Institute’s Genome Analysis Toolkit (GATK) Queue 3.2–2 data processing pipeline. The TP53 gene region (chr17:7,569,720–7,592,868) was extracted from BAM files using samtools (http://samtools.sourceforge.net). At the time of publication of the original manuscript16, HaplotypeCaller was the variant analysis software embedded into the GATK best practice pipeline (GATK 3.2–2; https://www.broadinstitute.org/gatk/guide/best-practices). HaplotypeCaller assumes that DNA is from a diploid organism. It is best suited to germline variant calling; however, is able to detect allele frequencies outside of an expected 50:50 ratio. Annotation of variant calls was performed using ANNOVAR, version 2013J 24.

Each sample summary was imported into Excel and filtered to display TP53 variants, excluding intronic variants other than the canonical splice sites. Filtering criteria were applied to remove reads with a quality (QUAL) score less than 100. TP53 variants were further filtered based on their frequency in the 1,000 Genome Database (Phase 3 integrated, all population, updated August2015) 25. If a particular variant occurred at a frequency greater than 10% in this database, the variant was deemed to be non-deleterious and excluded from the analysis. Lastly, variants were filtered based on SIFT scores (Sorting Intolerant From Tolerant; from dbNSFP v3.0 that amalgamates SIFT to the version based on Ensembl 66. For release 66, Ensembl ran SIFT version 4.0.5 using UniProtKB [release 2012_01, both the SwissProt and TrEMBL sets]). SIFT is an in silico tool for predicting the functional effects of a variant on the associated protein26. Variants predicted to be tolerated were excluded. All remaining variants were considered deleterious or did not have a SIFT score and were visualized using the Integrative Genomics Viewer (IGV, v2.3, www.broadinstitute.org)27,28. The allele frequency of each mutation was recorded upon visualization of the mutation via IGV. This analysis pipeline was previously summarized (Supplementary Fig. OM-S5).

MPS data analysis and processing with MuTect2 software
Since publication of the original manuscript16, MuTect2 has become available through GATK that combines aspects of the original MuTect29 and HaplotypeCaller for somatic genotyping. MuTect2 detects a range of allele frequencies, making it eminently more suitable for somatic genotyping in heterogeneous, often aneuploid, tumor tissue compared to HaplotypeCaller that was designed for germline variant calling where alleles are present in equal ratios. FASTQ files were trimmed and aligned as described for HaplotypeCaller. Somatic variant calling was performed using MuTect2 beta in GATK version 3.6. The four LGSCs (previously shown to be wild-type for TP53 using identical MPS and analysis pipelines to the HGSCs studied; Supplementary Table OM-S2) were combined into a Panel of Normals (PoN) variant cohort against the Single Nucelotide Polymorphism database current build 138 (dbsnp138) and COSMIC coding mutations. Tumor only variant-calling was then performed using the pre-generated PoN for each tumor sample. MuTect2 software requires a minimum of two samples to create a PoN variant call format (VCF) file. Each tumor VCF was annotated using ANNOVAR (2016Feb01; http://annovar.openbioinformatics.org/en/latest/) and merged into an Excel spreadsheet for downstream analyses.

The PoN calls were removed, as were low quality calls (defined as having a theta-logarithm of the Odds (TLOD) < 6.3). Synonymous variant calls were filtered out along with variants in intronic and
untranslated regions. Non-deleterious calls were filtered out based on SIFT scores as above. Variant calls not occurring within the full length transcript (TP53:NM_000546) or canonical splice sites were also removed. Lastly, a manual filter was applied to remove variant calls occurring at a frequency of less than 5%.

**Code availability**

All tools required for the analysis of this data are freely available. Instructions for downloading and installation are in scripts.sh (https://figshare.com/articles/GenomeAnalysisTK_jar/4541719) and ANNOVAR (2016Feb01). The file named 'script.sh' (https://figshare.com/articles/GenomeAnalysisTK_jar/4541719) will need to be downloaded in which the section uses '/path/to/' in order to indicate paths that should be modified by the user depending on the location the data files are to be downloaded to. Certain files will require download as compressed files that will need decompression and setting of a path to the executable file. Script pipelines may take 22 h to run on a 4 cores, 16 GB personal computer. All file downloads will require 34 GB of storage space.

**Data Record**

TP53 MPS data (Data Citation 1) is available in the European Genome-phenome Archive (EGA) with the study accession number EGAS00001002200 and dataset accession number EGAD00001003119 (Table 1 (available online only)). This dataset contains MPS information on 76 unique tumor samples from individual patients, of which four are LGSCs and 72 are HGSCs. All sample files are in the BAM format and have been extracted to have the p53 gene (TP53) region reads along with the unmapped reads.

**Technical Validation**

**Quality control—assessment of percentage tumor cells in each sample**

A pathologist [AJG] reviewed all tumor tissue in order to confirm diagnosis, histological grade and pathological stage. Sequential sections from frozen tumors were analyzed to determine percent tumour cells after staining with hematoxylin and eosin. For inclusion in this study, tumors were required to contain a minimum of 5% tumor cells. The percent tumor cell composition in samples used in this study ranged from 5–90% (Supplementary Table OM-S1).

**Quality control—DNA integrity**

Prior to analysis on the Fluidigm Access Array, DNA integrity was assessed using the Qubit dsDNA BR Assay Kit for fluorometric quantitation. This assay is selective for double-stranded DNA (dsDNA) over RNA and is designed for optimal performance within a concentration range of 100 pg–1,000 ng μl⁻¹. Based on this quantitation, DNA was diluted to 50 ng μl⁻¹ using nuclease and PCR inhibitor free elution buffer EA from the QIAamp DNA Mini Kit (Qiagen Pty Ltd). DNA was confirmed to be clean by assessment of 260:280 and 260:230 ratios >1.8 using the NanoDrop.

**Quality control -massively parallel sequencing (MPS) data and analysis**

As described in the original manuscript, amplicon libraries for 72 samples were generated using the Access Array BRCA1/BRCA2/TP53 Target-Specific Panel (Fluidigm, South San Francisco, CA, USA), pooled and sequenced in a single run on a MiSeq platform using MiSeq Control Software (MCS) version 2.4.1 (Illumina Inc., San Diego, CA, USA).16. This single sequencing run produced a cluster density of 1,133±31 K/MM² (84.57% ± 1.89 passing filter) and 20,626,284 sequence reads (17,452,900 passing filter) with 95.42% ≥ Q30 (Read 1) and 92.61% ≥ Q30 (Read 2).

Described in methods, analysis of MPS data, using both HaploTypoCaller and MuTect2, required extensive filtering to remove reads of poor quality, variants that appeared in datasets of normal genomes and variants predicted to be non-pathogenic. Filtering protocols are summarized in Fig. 1 as part of our bioinformatics analysis pipeline. The allele frequency for TP53 mutations called by MPS using HaploTypoCaller ranged from 13–92% in the HGSC cohort (mean and median values 55 and 54% respectively; Supplementary Table OM-S1 and Fig. 2a). The TP53 mutant allele frequency for a single sample with a large in-frame insertion (#880–13 [c.723_724dupACCATCCACTACAACTACATGTG- TACACGTTCC]; Supplementary Table OM-S1) was unable to be determined with our analysis pipeline, although was detectable by Sanger sequencing.
We assessed whether the percent tumor cell composition was likely to influence the frequency of the mutant alleles that were detected. These two variables were graphed, a line of best fit plotted and the R² value calculated (Fig. 2b). This analysis demonstrated a small correlation (R² = 0.3917) between these two variables, suggesting that our minimum criteria of 5% tumor cell composition was adequate for detecting TP53 variants using our pipeline. Any concerns regarding potential influence of a low percentage of tumor cells could be circumvented by the use of tumor macro- or micro-dissection to ensure a more pure cancer cell population for analysis.30

Re-analysis of our data with MuTect2 (beta) resulted in identification of all of the variants detected by HaplotypeCaller, with one exception discussed below, and an additional five TP53 variants with allele frequencies ranging from approximately 2–3% (Table 2). We excluded these variants by setting a manual filter for all frequencies below 5%. It is unclear whether these low frequency variants are artefacts introduced by MuTect2 software, or indeed represent very low frequency somatic TP53 mutations in sporadic tumors. We do not have further access to these specimens to investigate them with alternative methodology such as digital PCR that may detect very low frequency variant alleles. If these low frequency variants are not artefacts, their biological significance in the tumor milieu is unclear. The possibility that MuTect2 software can detect very low frequency alleles in heterogeneous cell populations may be of relevance in some malignancies where active screening for early relapse and/or response to therapy is undertaken. Furthermore, analyses with MuTect2 showed the allele frequency of the large in-frame insertion (#880-13; Supplementary Table OM-S1) as 0.5%. Given that we could easily visualize this mutation using Sanger sequencing that we showed in the original manuscript16 could not reliably detect variants at allele frequencies less than 25%, it is not possible that this insertion is present at such a low frequency in this tumor. This data suggests that care should be taken when using MuTect2 to identify variants involving larger alterations.

Table 2. Additional TP53 variants identified by MuTect2 at low frequency. ^D, Deleterious; N/A, no SIFT call; *IARC, International Agency for Research on Cancer.

| Sample ID | Genomic position (chr:start-end) | Reference: Variant allele (%) | Exon | CDNA change | Protein effect | Reference: Variant allele read count | Tumor variant allele ratio | SIFT call | Database Presence (IARC) | % Tumor cell composition |
|-----------|---------------------------------|-----------------------------|------|-------------|---------------|-------------------------------------|--------------------------|-----------|--------------------------|-------------------------|
| 10-04     | 17:7578440–7578440 T(98%); G(2%) | 5 c.490A>G                   | Lys164Glu | 2929:58     | 0.02          | D                                   | Yes                      | 70        |
| 198-08    | 17:7577541–7577541 T(97%); G(3%) | 7 c.740A>G                   | Asn247Ser | 2336:60     | 0.026         | D                                   | Yes                      | 10        |
| 198-08    | 17:7579471–7579471 G(97%); T(3%) | 4 c.216delC                  | Pro72Argfs*49 | 2116:56     | 0.026         | N/A                                 | No                       | 10        |
| 206-08    | 17:7577556–7577556 C(98%); G(2%) | 7 c.725G>C                   | Cys242Ser | 2334:53     | 0.023         | D                                   | Yes                      | 50        |
| 427-09    | 17:7577574–7577574 T(97%); G(3%) | 7 c.707T>A                   | Try236Cys  | 2003:59     | 0.029         | D                                   | Yes                      | 80        |

Usage Notes

The following gives clear instructions as to how to apply to access the dataset described in this manuscript.

Use the search bar on the front page of the European Genome-Phenome Archive website (https://ega-archive.org/) to search for this study with a keyword such as “TP53” or the study ID number that is EGAS00001002200. This will bring you to a screen where you can view information on datasets, data providers, data access committees (DACs) and any other documentation associated with this study. A description of this study is located under the heading ‘Study Description’. There is a single dataset associated with this study (Study ‘Datasets 1 dataset’ and its data ID number is EGAD00001003119. Click on this dataset ID to take you to information about who to contact regarding access to this data.

Each dataset in EGA is affiliated to a Data Access Committee (DAC), which is the group responsible for data access decisions following a formal application process. Access to actual data files is not managed by the EGA. You must apply to this DAC to gain access to this controlled dataset using your EGA account. Upon clicking on the dataset ID, you will come to the heading ‘Who controls access to this dataset’. For requests to access this dataset, please contact:

DAC: Functional Genomics Laboratory, Kolling Institute of Medical Research DAC—TP53 mutation data in ovarian cancer.

Contact Person: Deborah Marsh
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More details: EGAC0000100589

A Data Access Agreement (DAA) will be required. The DAA is a contract between the proposed user of the data and the DAC. This will contain information such as details of data use, publication embargoes and storage of data. Completion of a DAA by the applicant(s) should be considered as part of the application process to the DAC. A template DAA can be found on the EGA website under ‘Policy documentation—Data Access Agreement (DAA)’. A modified template specific for this dataset is provided on the EGA website.
provided as Supplementary Data. The completed EGA DAA signed by both parties (the data provider and those wishing to access the data) should be emailed to ega-helpdesk@ebi.ac.uk.

Upon receiving the completed DAA approved by the DAC, EGA will arrange a one-time login to set a password for your EGA account that will be sent to your email address. Following authorisation of your password, you will receive email notification that your EGA account is ready for your use. A list of the datasets you have been granted access to will appear on your ‘My Datasets’ page in EGA. From here, you will be able to download the data.

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Author Contributions
A.J.C. performed all experiments, analyzed data and wrote the paper. Y.Z. undertook bioinformatics analyses and contributed to writing the paper. T.D. advised on bioinformatics analyses and contributed to writing the paper. B.Y. advised on specific bioinformatic pipelines used and contributed to writing the paper. K.-A.D. assisted with experiments and data analysis, and contributed to writing the paper. G.B.G. collected the majority of specimens used in this study and contributed to writing the paper. J.M. collected specimens used in this study and contributed to writing the paper. S.V. collected specimens used in this study and contributed to writing the paper. A.J.G. confirmed fitness for purpose of samples used in this study and contributed to writing the paper. R.C.-B. advised on experimental plan and contributed to writing the paper. D.J.M. conceived the study, analyzed data and wrote the paper.

Additional Information
Table 1 is only available in the online version of this paper.

Competing interests: The authors declare no competing financial interests.

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