High-throughput functional evaluation of BRCA2 variants of unknown significance

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Numerous nontruncating missense variants of the BRCA2 gene have been identified, but there is a lack of convincing evidence, such as familial data, demonstrating their clinical relevance and they thus remain unactionable. To assess the pathogenicity of variants of unknown significance (VUSs) within BRCA2, here we develop a method, the MANO-B method, for high-throughput functional evaluation utilizing BRCA2-deficient cells and poly (ADP-ribose) polymerase (PARP) inhibitors. The estimated sensitivity and specificity of this assay compared to those of the International Agency for Research on Cancer classification system is 95% and 95% (95% confidence intervals: 77–100% and 82–99%), respectively. We classify the functional impact of 186 BRCA2 VUSs with our computational pipeline, resulting in the classification of 126 variants as normal/likely normal, 23 as intermediate, and 37 as abnormal/likely abnormal. We further describe a simplified, on-demand annotation system that could be used as a companion diagnostic for PARP inhibitors in patients with unknown BRCA2 VUSs.

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The BRCA1 and BRCA2 (BRCA) genes encode key proteins in the homology-directed DNA break repair (HDR) pathway, and their inactivation predisposes individuals to cancer development. Germline loss-of-function variants in BRCA markedly increase the risk of early-onset breast and ovarian cancer; in such cases, prophylactic oophorectomy and mastectomy and genetic testing for at-risk relatives must be considered. Tumors with pathogenic variants within BRCA and defective HDR have been shown to be particularly sensitive to platinum-based chemotherapies and poly (ADP-ribose) polymerase (PARP) inhibitors, the efficacy of which is mediated through synthetic lethality in cancer cells with BRCA loss-of-function.

The American College of Medical Genetics and Genomics (ACMG) standards and guidelines for the interpretation of sequence variants recommend a process for variant classification based on criteria using population, computational, functional, and segregation data. The ACMG guidelines assign a categorical strength to each evidence: supporting, moderate, strong, very strong, or stand-alone. Then, each variant is assigned to five categories using combining criteria: benign, likely benign, uncertain significance, likely pathogenic, and pathogenic. Family-based studies including a multifactorial model of pathology, a cosegregation profile, and the cooccurrence and family history of cancer may exemplify the most reliable methods for classifying BRCA2 gene variants. Nonsense or frameshift variants within the coding exons of BRCA markedly alter the structures of the protein products and are presumed to confer loss-of-function. However, the vast majority of missense variants are individually rare in both general populations and cancer patients, and case-control studies may not have sufficient statistical significance to classify these variants as pathogenic or benign. No current in silico computational prediction algorithm for missense variants is accurate enough when used alone. In the framework of the ACMG guidelines, the functional impact of a variant, which is determined by a well-established functional assay, is regarded as strong evidence for benign/pathogenic status. Thus, the functional evaluation of missense variants of unknown significance (VUSs) is urgently required to improve the interpretation of variants identified by genetic testing and to support clinical decision-making for their carriers.

While thousands of BRCA1 VUSs have been assessed by recently developed high-throughput functional assays, a few hundred BRCA2 variants have been evaluated by a conventional functional assay for BRCA2, the HDR assay. The HDR assay has three issues requiring improvement: (i) the throughput is quite low, (ii) it uses a hamster cell line with transient expression of BRCA2 cDNA, and most importantly, (iii) it can evaluate only variants in the DNA-binding domain. To overcome these limitations, we propose herein a high-throughput method using a human cell line stably expressing BRCA2 variants that enables the evaluation of all exonic variants of the BRCA2 gene.

Establishment of MANO-B method. We next performed a cell viability assay to evaluate the drug sensitivity of the BRCA2 variants. Cells treated with PARP inhibitors (olaparib, niraparib, and rucaparib) or carboplatin (CBDCA) at various concentrations for 6 days were assessed with PrestoBlue reagent (Supplementary Fig. 2). Wild-type BRCA2 and benign variants showed greater tolerance to these drugs than pathogenic variants. However, the threshold between the benign and pathogenic variants was unclear. It is important to note that wild-type BRCA2-induced cells were significantly more sensitive than parental DLD1 cells; therefore, we should establish a sensitive method in which all comparisons could be made with wild-type-induced cells rather than parental cells. We thus modified the mixed-all-nominated-in-one (MANO) method that was originally developed to enable high-throughput evaluation of VUSs in transforming genes and invented the MANO-BRCA (MANO-B) method for the functional evaluation of BRCA2 variants (Fig. 1).

In the MANO-B method, DLD1 BRCA2 (∼/∼) cells were transfected with the individual BRCA2 variant cDNAs and the variant-specific barcodes, mixed, and cultured with or without drugs. Genomic DNA was extracted from each cell mixture after the drug treatments, and the barcode sequences were amplified by PCR and deep sequenced. The barcode read count, which was linearly correlated to the number of viable cells harboring the corresponding variant, was normalized to that of the vehicle control to evaluate the relative cell viability. Based on these profiles, the drug sensitivity and pathogenicity of each variant were determined (Fig. 1).
The relative viability of the cells treated with each drug was first calculated by comparing the viability with that of vehicle-treated cells, then normalized to that of cells expressing wild-type BRCA2. All variants were sorted by relative viability at the optimal concentrations. A scatter plot of the relative viability of olaparib and the other three drugs revealed an intermediate group including the P2329L, P2639L, D2913H, and S3291C mutants between the benign and pathogenic groups (Supplementary Fig. 3). With this intermediate group as a boundary, all known pathogenic variants showed lower relative viability, whereas all but two known benign variants—R2842H and V2908G—showed higher relative viability. The two variants were indeed shown to be hypomorphic by HDR assay in a previous study. In addition, we found that one likely pathogenic variant (N3187K) defined by ClinVar exhibited normal function as evaluated by the MANO-B method. This discordance between the functional classification and the clinical classification suggests potential errors in the clinical annotations for rare variants.

We believe that the discrepancy in the classification of IARC Class 1/2 variants (R2842H and V2908G) that were evaluated as hypomorphic using the MANO-B method was because of the unreliable evidence which the IARC criteria was based on. The IARC classification is based on epidemiological evidence such as cosegregation data and other family-based genetic analyses. According to the Genome Aggregation Database, both R2842H and V2908G (minor allele frequency $=6.34 \times 10^{-5}$ and $1.69 \times 10^{-5}$) were rare variants. Therefore, it might be difficult to obtain solid evidence for these variants.

ClinVar, a clinical database curated by experts, also classified R2842H and V2908G as benign/likely benign, mainly based on co-occurrence analysis and functional data. R2842H was reported to co-occur with Q3066X, a truncating variant in BRCA2. According to the ACMG standards and guidelines, evidence that a variant is observed in trans with a pathogenic variant for a fully penetrant dominant gene/disorder is a supporting evidence indicating that the variant is benign. However, because a pathogenic BRCA2 variant is not fully penetrant, we believe that co-occurrence with a pathogenic variant cannot exclude the possibility that the variant is hypomorphic. For example, another hypomorphic variant R2784Q was reported to co-occur with pathogenic variants in BRCA1 and BRCA2.

Multiple and diverse functional assays for V2908G were reported by Wu et al. in 2005 and Farrugia et al. in 2008, who both belonged to the same research group. However, ClinVar does not include two important studies reported by the same group in 2013 and 2018. The HDR activity of V2908G in their recent papers was decreased, indicating hypomorph of the variant. No other laboratories have replicated their experiments performed in 2005 and 2008. Thus, we considered V2908G a hypomorphic variant based on their latest reports and our result.

To assess the pathogenicity of such variants accurately, the classification method must be based on a wide variety of evidence. Therefore, we reinterpreted all the 59 Class 1/2/4/5 variants (37 benign and 22 pathogenic) used in the MANO-B method according to the ACMG 2015 guidelines as previously described. As a result, 18 variants (31%), including R2842H and V2908G, were classified into VUSs because of insufficient evidence (Supplementary Data 2). Furthermore, N3187K, defined as a likely pathogenic variant by ClinVar, is also defined as VUS according to both IARC and ACMG criteria. The criteria of the ACMG guidelines are more conservative and stringent than those of other classification methods because ACMG uses multiple...
Fig. 2 Comparative results of the MANO-B method with the IARC, ClinVar, and Align-GVGD classifications for 107 variants. DLD1 BRCA2 (--/-) cells transfected separately with the 107 BRCA2 variants or the empty vector were treated with PARP inhibitors (olaparib, niraparib, and rucaparib), CBDCA, or DMSO at the indicated concentrations. The relative viability of the cells treated with each drug was calculated based on the viability of vehicle-treated cells, then normalized to that of cells expressing wild-type BRCA2. All variants were ordered by relative viability at the optimal concentration. The classification of the MANO-B method was based on the relative viability at the optimal concentration of each drug and defined as normal (relative viability of 1–82), intermediate (83–87), and abnormal (88–108). The IARC, ClinVar, and Align-GVGD classifications are color-coded in each column. The ClinVar classifications of five variants (V159E, M1168I, K1530N, G1696V, and A2911E) without assertion criteria were considered as VUSs.
criteria from different points of view and perspectives. This discrepancy among clinical databases implies potential errors in the interpretation of clinical variants. Moreover, the IARC and ACMG classification methods were designed for detecting high-risk pathogenic variants, and therefore, hypomorphic function variants or moderate cancer risk variants could be classified inappropriately. Hence, we regard the discrepancy between functional and epidemiological evidence as reasonable.

To evaluate the evenness of the pooled variant ratio, we collected cell pellets at day 0 (the day the drug treatment was started) to count the barcode reads by MiSeq. Regarding the raw data obtained from batch #1, the minimum, maximum, and median read counts were 778, 32394, and 5763 for N55S, R3385H, and Y3098H, respectively. The distribution of each variant ratio is shown as a histogram (Supplementary Fig. 4a). A total of 97 variants (90%) were within the range of 0.5–2-fold average. We also calculated the fold change from day 0 to day 12 with dimethyl sulfoxide (DMSO) treatment (Supplementary Fig. 4b). The majority of cells expressing these variants generally exhibited a uniform growth, and the functions of the induced BRCA2 variants did not have much impact on cell growth. The unevenness of the variant ratio probably has a small effect on the result of the MANO-B method because this method is based on fold-change calculation and the abundance bias is corrected through analysis.

Evaluation of 244 BRCA2 variants by Bayesian inference. We expanded the experiment to encompass 244 variants, including 186 VUSs, at the optimal concentration of each drug, and obtained an assay dataset comprising 7344 individual viability values (Supplementary Data 3 and 4). Using the optimal threshold for receiver operating characteristic (ROC) analysis, the estimated sensitivity and specificity of the MANO-B method for variant pathogenicity compared with those of the IARC classification system were 100% and 95% (95% confidence intervals: 85–100% and 82–99%), respectively (Supplementary Fig. 5).

Given that the existence of hypomorphic variants was suggested, a nondichotomous mathematical approach was explored to precisely determine the pathogenicity of each variant. Relative viability data were log normalized and compensated using the values of the wild-type and the D2723H variant as standard values for benign and pathogenic variants, respectively. The resulting viability data followed a two-component Gaussian mixture model by the expectation–maximization algorithm (Supplementary Fig. 6).

In the following analysis, we adopted the Bayes factor (BF) as the strength of evidence in favor of pathogenicity. To calculate the BF, we utilized a Bayesian hierarchical two-component Gaussian mixture model based on the VarCall model with a noninformative prior probability. In this setting, the BF was calculated as the probability ratio of a variant being functionally abnormal to it being normal. A five-tiered functional classification based on the BF was assigned to each variant according to established criteria consistent with the ACMG variant evaluation guidelines as follows: iClass 1 (normal; BF ≤ 0.003), iClass 2 (likely normal; 0.003 < BF ≤ 0.053), iClass 3 (intermediate; 0.053 < BF < 18.7), iClass 4 (likely abnormal; 18.7 ≤ BF < 350), or iClass 5 (abnormal; 350 ≤ BF).

The function of a variant, v, was estimated by a variant-specific effect, \( \eta_v \). Using 22 known pathogenic and 37 known benign variants as the training set, the \( \eta_v \) values of benign and pathogenic variants were gradually distributed (Supplementary Fig. 7). The sensitivity and specificity of the Bayesian inference for detecting IARC class 4/5 variants were 95% and 95% (95% confidence intervals: 77–100% and 82–99%), respectively. When a variant is classified as iClass 4/5, the odds ratio of pathogenicity come out a 17.7:1 in favor of pathogenicity. We also analyzed the concordance between the ACMG classification and the MANO-B classification. All the 41 benign/pathogenic variants (22 benign and 19 pathogenic) used in the MANO-B method were clearly divided into two groups, and the odds ratio of pathogenicity is infinity (Supplementary Fig. 8). Thus, the BF-based functional classification by the MANO-B method provides strong evidence of pathogenicity in the framework of the ACMG guidelines.

To validate the BF-based classification from the functional perspective, we set two functional classification thresholds determined by the ACMG benign/pathogenic variants to classify the following three components according to a previous report: functionally normal, intermediate, and abnormal. The higher threshold between normal and intermediate components is defined as the minimal effect of benign variants according to the ACMG criteria (R18H, \( \eta_v = -1.38 \)), whereas the lower threshold between intermediate and abnormal components is defined as the maximal effect of pathogenic variants (C2535X truncation variant, \( \eta_v = -2.17 \)). As a result, all the functional effects of iClass 1/2 variants were above the higher threshold, whereas all those of iClass 4/5 variants were below the lower threshold. Now, iClass 4/5 variants can be regarded as pathogenic because their probability is in the abnormal component and their functional levels are equivalent to those of the known pathogenic variants.

Notably, the iClass values of the individual variants for the four drugs were concordant (Fig. 3a–c). The data for 0.5 \( \mu \text{M} \) niraparib exhibited the highest resolution in the evaluation of the variants of intermediate function. We assume that the difference in functional estimation among drugs in the MANO-B method was primarily because the drug concentrations of olaparib and rucaparib were not as optimal as that of niraparib. The concentration of 2.0 \( \mu \text{M} \) of olaparib, rucaparib, and CBDCa was probably too high for DLD1 cells expressing hypomorphic variants to survive, considering that the cell viability assay with 1 \( \mu \text{M} \) of olaparib, rucaparib, and carboplatin (Supplementary Fig. 2b) demonstrated broader transitional zones between benign and pathogenic variants. Among the 186 VUSs analyzed in the full dataset with niraparib, 98, 28, 23, 6, and 31 variants were classified as iClass 1–5, respectively (Fig. 3d, Supplementary Data 5). All variants outside functional domains belonged to iClass 1/2. Most abnormal variants were located in the DNA-binding domain (DBD). Other intermediate or abnormal variants were W31C/G/L in the transactivation domain (TAD) (Supplementary Fig. 9). However, this combinational classification for variants outside the DBD should be interpreted with caution because no reliable prior probability data have been established for this region.

Model validation. The internal validation of our model was confirmed by posterior predictive checks. The posterior predictive distribution of the log-normalized relative viability data with prior probability, such as the Align-GVGD classification based on the evolutionary conservation and biophysical properties of amino acids (Supplementary Fig. 9). However, this combinational classification for variants outside the DBD should be interpreted with caution because no reliable prior probability data have been established for this region.
Fig. 3 Evaluation of 244 BRCA2 variants by applying Bayesian inference to the MANO-B method. a–c Functional classification by the MANO-B method with four drugs. A total of 244 BRCA2 variants were classified by Bayesian inference. The number of variants classified for each drug as fClass 1/2 (a), fClass 3 (b), and fClass 4/5 (c) is shown. No variant was classified as fClass 1/2 with one drug and fClass 4/5 with another drug. 

Bar plots of functional variant effects and functional diagnosis of 244 BRCA2 variants mapped against the BRCA2 full-length sequence and domains. The key domains of BRCA2 consist of a PALB2 interaction domain encompassing amino acids (a.a.) 10-40, a transactivation domain (TAD) encompassing a.a. 18-105, a RAD51-binding domain including eight BRC repeats encompassing a.a. 1008-2082, an MEILB2-binding domain (MBD) encompassing a.a. 2117-2339, a DNA-binding domain encompassing a.a. 2402-3186 and containing a helical domain (HD) encompassing a.a. 2402-2669, oligonucleotide/oligosaccharide-binding domains (OBs) (OB) encompassing a.a. 2670-2803, 2809-3048, and 3056-3102, and a C-terminal RAD51-binding domain (CTD) encompassing a.a. 3270-3305. The function of each variant was estimated as a variant-specific effect value, $\eta_v$, based on a two-component model. Data of niraparib are shown as a representative case. The upper and lower components correspond to normal function and loss-of-function, respectively. The dotted lines indicate the median value of each component. The classification of each variant based on the Bayes factor (BF) is indicated by the color and shape of the lines and plots, as shown in the legend. Variant functions were classified into five classes by the MANO-B method: fClass 1 (normal) ($BF \leq 0.003$), fClass 2 (likely normal) ($0.003 < BF \leq 0.053$), fClass 3 (intermediate) ($0.053 < BF < 18.7$), fClass 4 (likely abnormal) ($18.7 \leq BF < 350$), and fClass 5 (abnormal) ($350 \leq BF$). N = 3 cells were examined over 2 or 3 independent experimental batches. Plots indicate mean values. Open error bars, 95% CIs. WT, wild-type; NS, nonsense variants.
variants was confirmed (Supplementary Fig. 11). The results of the two assays revealed a small discordance in some variants. The S3291C variant exhibited a relatively high value in the HDR assay compared to that in the MANO-B method, probably because S3291 is the phosphorylation site for CDK1/2 and the HDR assay cannot evaluate its function exactly.

Accurate BRCA Companion Diagnostic (ABCD) test. To rapidly evaluate the pathogenicity of BRCA2 VUSs in the clinic, the Accurate BRCA Companion Diagnostic (ABCD) test was developed as a potential companion diagnostic for PARP inhibitor treatment. Newly detected BRCA2 cDNAs, along with four control variants for benign (wild-type and T2515I) and pathogenic (Y2660D and D2723H) variants, were generated for assessment by a small-scale MANO-B method. The resulting data correlate with the data from previous MANO-B experiments, enabling batch effect adjustment for pathogenicity determination. When a BRCA2 VUS is believed to be related to splicing aberration, its mRNA should be evaluated by RNA sequencing of patient samples. Any aberrant BRCA2 transcripts detected should also be subjected to the ABCD test (Fig. 4).

An experiment was designed to confirm the accuracy of the ABCD test, in which I1929V, D3095E, Y3035S, and S3291C were assessed as if they were novel variants. The classification of normal and abnormal variants was almost reproducible and consistent with that observed for previous large-scale batches; the classification of the D3095E pathogenic variant in batch #1 was underdiagnosed (from fClass 5 to fClass 4, Table 1). One misclassification is crucial in case we judge the clinical application based on the result of a single batch. Because transfection efficiency might cause inconsistent results in the ABCD test, we recognized the importance of multiple independent transfections using different batches of mutants to ensure the accuracy of the assay. Therefore, we combined the results from three batches with different transfections and performed a statistical analysis to annotate the pathogenicity. This approach improved the consistency (4/4, 100%) between the ABCD test and the large-scale MANO-B method, although the validation study remains to be performed in a larger scale.

Discussion

Several functional assays for BRCA2 variants have been developed to date based on the various functions of BRCA2 protein. However, it should be noted that loss of a certain function does not directly lead to cancer predisposition. For example, an established R3052W pathogenic variant did not show pathogenicity in the centrosome amplification assay, whereas G2353R, once annotated as a benign variant, showed a pathogenic response in the spontaneous homologous recombination assay. The results of each functional assay should be carefully interpreted together with clinical genetic data.

It is remarkable that the distribution of the functional parameter \( \eta_v \) for the MANO-B method was continuous from the normal to the abnormal component and not clearly segregated into two components, in accordance with the HDR assay, suggesting the existence of variants of intermediate function.

For the nondichotomous classification, a high-resolution quantitative assay together with an appropriate inference method are necessary. In the MANO-B method, all cell clones with individual variants are evenly mixed and cultured in one dish. Since each clone grows competitively under the same culture conditions, including pH, temperature, and drug concentration, we can minimize the technical error and bias and evaluate even small differences in BRCA function among the variants. This is an advantage over other methods such as the cell viability assay and the HDR assay, which test variants individually.

Recently, Findlay et al. reported a robust saturation genome editing method by the use of a haploid cell line, HAPI, that depends on the HDR pathway for growth. In contrast to the MANO-B method, their technology may accurately evaluate the function of variants at the splicing sites or the promoters of BRCA genes. On the other hand, our method uses a diploid cell line and, therefore, may better reflect HDR function in physiological conditions. The MANO-B method is, further, able to directly...
interrogate the sensitivity of each VUS to different PARP inhibitors. The MANO-B method and the saturation genome editing method may thus be complementary to each other.

While clinical gene testing has identified plenty of BRCA2 variants, most VUSs are rare and thus there is a lack of substantial familial data. Recently, the uncommon (but not rare) variants G2508S (IARC Class 4) and Y3035S (IARC Class 2) were reported in a large case–control study showing indecisive cancer predisposition

Table 1 Results of an experimental ABCD test.

| Variant | IARC classification | ABCD test | $\eta_v$ | Bayes factor | fClass |
|---------|---------------------|-----------|----------|--------------|--------|
| I1929V  | Class 1             | Previous batches | 0.37 (−0.02 to 0.79) | 1.05 × 10⁻⁵ | fClass 1 |
|         |                     | New batch-1   | 0.19 (−0.33 to 0.73) | 1.02 × 10⁻⁵ | fClass 1 |
|         |                     | New batch-2   | −0.23 (−0.73 to 0.28) | 1.63 × 10⁻⁴ | fClass 1 |
|         |                     | New batch-3   | 0.08 (−0.43 to 0.6)  | 3.71 × 10⁻⁵ | fClass 1 |
|         | Three batches combined | 0.11 (−0.23 to 0.44) | 1.40 × 10⁻⁵ | fClass 1 |
| Y3035S  | Class 2             | Previous batches | −0.63 (−0.101 to −0.25) | 1.11 × 10⁻³ | fClass 1 |
|         |                     | New batch-1   | −0.55 (−1.05 to −0.04) | 1.12 × 10⁻³ | fClass 1 |
|         |                     | New batch-2   | −0.53 (−1.04 to −0.02) | 9.57 × 10⁻⁴ | fClass 1 |
|         |                     | New batch-3   | −0.36 (−0.87 to 0.16)  | 3.01 × 10⁻⁴ | fClass 1 |
|         | Three batches combined | 0.47 (−0.79 to −0.15) | 2.79 × 10⁻⁴ | fClass 1 |
| D3095E  | Class 5             | Previous batches | −3 (−3.41 to −2.62) | 1.33 × 10⁴  | fClass 5 |
|         |                     | New batch-1   | −2.31 (−2.8 to −1.78)  | 4.45 × 10¹  | fClass 4 |
|         |                     | New batch-2   | −2.63 (−3.12 to −2.15) | 5.75 × 10²  | fClass 5 |
|         |                     | New batch-3   | −2.69 (−3.2 to −2.19)  | 9.28 × 10²  | fClass 5 |
|         | Three batches combined | −2.52 (−2.85 to −2.19) | 7.85 × 10² | fClass 5 |
| S3291C  | Unclassified        | Previous batches | −2.07 (−2.43 to −1.69) | 1.15 × 10¹  | fClass 3 |
|         |                     | New batch-1   | −1.06 (−1.7 to −0.53)  | 6.15 × 10⁻² | fClass 3 |
|         |                     | New batch-2   | −2.06 (−2.6 to −1.34)  | 7.05 × 10⁰  | fClass 3 |
|         |                     | New batch-3   | −1.35 (−2.12 to −0.74) | 3.17 × 10⁻¹ | fClass 3 |
|         | Three batches combined | −1.46 (−1.86 to −1.11) | 3.39 × 10⁻¹ | fClass 3 |

Data are presented as means (95% credible interval).

predicting the effectiveness of PARP inhibitors for patients with cancer harboring BRCA2 VUSs, and the other is for cancer risk stratification for considering prophylactic surgery to prevent cancer in patients who at the time are healthy. In the former scenario, clinicians could decide on the use of PARP inhibitors for patients with cancer harboring BRCA2 VUSs to prevent tumor progression. Therefore, an evaluation of BRCA function using the ABCD test could be a companion diagnostic method for PARP inhibitor treatment, although the clear threshold of fClass as a marker to predict which variant is actually beneficial for PARP inhibitor treatment in the clinical setting should be determined by clinical trials. In the latter scenario, healthy patients with hereditary BRCA2 VUSs can wait and observe for several years for the tumor to emerge. After receiving positive genetic test results, approximately half of the women wait more than 12 months before undergoing prophylactic salpingo-oophorectomy. For patients with fClass 5 variant evaluated by MANO-B method, the application of prophylactic surgery could be considered carefully along with genetic counseling because the possibility of these patients to develop cancer may be high. The penetration rate of cancer likely depends on the extent of BRCA function deficiency; healthy patients with a BRCA variant of fClass 3 or 4 might be referred to careful check-up rather than prophylactic surgery.

Tumor response to PARP inhibitor therapy has several biomarkers other than BRCA1/2 inactivation, such as EMMY amplification, Fanconi anemia pathway inactivation, and other HR gene defects. Therefore, it is a good method to evaluate BRCA status by whole-genome sequencing of tumors based on mutation and rearrangement signatures without the knowledge of the precise causative mutations. Nones et al. demonstrated that 21 breast cancers from 22 BRCA2 pathogenic variant carriers (95%) in their study exhibited reduced BRCA function, whereas only one was BRCA-proficient. In contrast to the test of BRCA function, the ABCD test is a simple functional method for evaluating BRCA2 variant pathogenicity. Although the ABCD test cannot explain the BRCA status of non-BRCA1/2 tumors, it is a rapid and precise method that directly evaluates the PARP inhibitor sensitivity of cancers harboring BRCA2 VUSs. In
addition, an advantage of the ABCD test over BRCA functional analysis is that the ABCD test can evaluate whether germline BRCA variants found in people without cancer are pathogenic variants predisposing to cancer. Therefore, both the ABCD test and the whole-genome sequencing of tumors could be used in parallel for the assessment of BRCA status. As the ABCD test reported in this study was limited to BRCA functional analysis, we sought to apply this test to other HR genes in the future.

There are several limitations regarding the use of the MANO-B method for clinical annotation. The MANO-B method evaluates HDR function of BRCA2 variants via PARP inhibitor sensitivity and estimates their pathogenicity indirectly. This method does not directly consider other BRCA2 functions, such as the regulation of the G2−M transition or transcriptional elongation. Although the high concordance of the MANO-B method with the IARC and ACMG classifications suggest that BRCA2 pathogenicity can be evaluated primarily by investigating HDR activity, we would like to note that BFs for abnormal do not have a direct correlation with the likelihood ratios for pathogenicity, as the normal or abnormal variants defined by this functional assay are not necessarily benign or pathogenic variants in the clinical setting. In fact, there are discrepancies between the functional interpretation by drug sensitivity and clinical annotation of ClinVar in some variants, such as R2842H and V2908G. Those variants were mostly hypomorphic variants or very rare variants that have not been reviewed by expert panels in ClinVar. Therefore, the accurate cancer risks of these variants need to be evaluated carefully. For these reasons, we expect that the functional classification should be used as evidence of pathogenicity and combined with other evidence in a comprehensive framework of ACMG guidelines. We believe that our functional evaluation by drug sensitivity might adjust the interpretation of cancer risks, especially of those variants, although further validation studies are needed.

There are also several limitations of our cDNA-based assay. This approach cannot rule out potential effects on splicing. Importantly, although there are some computational predictors for possible splicing aberrations, either algorithms could not demonstrate adequate reliability for clinical usage, especially for variants outside of the consensual splice sites. Comprehensive splicing functional assays such as hybrid minigene assay and saturation genome editing technique would reinforce the evidence. It is also recommended to perform RNA-seq of patient blood and tumor to detect aberrantly spliced mRNA. In addition, the MANO-B method was performed in this study using only one colorectal cancer cell line. There is no evidence of the increased risk of colorectal cancer in BRCA2 pathogenic mutation carriers, and it is unclear whether the result of the MANO-B method is reproducible in other cell lines. To evaluate the variant functions precisely and robustly, replication studies with multiple cell lines with various homologous recombination statuses are desired. Although it is noteworthy that the high copy number of integrated BRCA2 constructs was necessary to obtain induced mRNA expression at levels similar to endogenous expression, the regulation of CMV promoter-driven exogenous expression of BRCA2 would not be physiologically relevant.

It is possible that the MANO-B method may lead to a better understanding of cancer biology and provide a concept of clinical functional annotation to improve cancer diagnosis and treatment.

Methods

**Cell lines and culture conditions.** Human colorectal adenocarcinoma cell line DLD1 parental cells and homozygous BRCA2 (−/−) variant cells were purchased from Horizon Discovery, Inc. Cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum and 2 mM l-glutamine at 37 °C in 5% CO2 and passed at 70–90% confluency with TrypLE Express (all from Thermo Fisher Scientific).

**Choice of variants.** A total of 239 BRCA2 missense variants identified in the BRCA Exchange database were selected. Sequence nomenclature was based on Human Genome Variant Society (HGVS) and National Center for Biotechnology Information (NCBI) reference NP_0005050.2: p.V2466A. We randomly selected 155 variants from functional domains and an additional 89 variants outside functional domains throughout the coding sequence. Variants in functional domains were selected considering Align-GVGD in silico prediction. Between 14 and 19 VUSs were randomly selected from each of the five Align-GVGD C15–C55 categories, and 62 variants were randomly selected from the C65 category. The proportion of each category was defined as previously reported. An additional five novel missense variants thought to be definitely pathogenic variants were evaluated (Supplementary Data 3). Eight VUSs located in the last 3 exonic bases of the splice donor site and five VUSs located in the first 2 exonic bases of the splice acceptor site were possible candidates for disrupting splice consensus sequences.

**Plasmid construction.** The piggyBac transposon vector was constructed by inserting a random 10-bp DNA barcode sequence between the CMV promoter and the multiple cloning site of the piggyBac dual promoter vector (PB513B-1; System Biosciences). The GFP sequence in the piggyBac vector was deleted by a site-directed mutagenesis technique for the direct repeat-green fluorescent protein (DR-GFP) assay. The full-length wild-type cDNA of human BRCA2 was subcloned from pcdNA3 236HSC WT (Addgene plasmid # 16246) into the piggyBac vector. Plasmids encoding BRCA2 variants were developed by a site-directed mutagenesis technique with variant-specific primers designed using an online tool (QuickChange Primer Design; https://www.agilent.com/store/primerDesignProgram.jsp). The primers used for mutagenesis are listed in Supplementary Data 3. For western blot analysis, the N-terminal FLAG-tag (DYKDDDK) was inserted in the piggyBac-BRCA2 wild-type by site-directed mutagenesis, and then the 19 BRCA2 variants were also subcloned into the FLAG-BRCA2-piggysBac vector. Plasmids were fragmented by an E220 Focused-ultrasonicator (Covaris, Inc.) to an average size of 300 bp, ligated with adaptors, and sequenced using MiSeq (Illumina). All the entire BRCA2 cDNA sequences, including the ones with point mutations generated by site-directed mutagenesis, the unique barcode sequences, and the piggyBac vector backbone sequences of every plasmid were confirmed by our original pipeline. Next-generation sequencing (NGS) data were aligned by Bowtie2 v2.3.4.2 against the reference sequence composed of wild-type BRCA2 cDNA and the piggyBac vector.

**Stable transfection with the piggyBac transposon.** The lipid-based method was used to transfect DLD1 cells with the plasmid constructs. Transfections were performed with exactly one unique plasmid per well. This is essential to guarantee that cells were transduced with only one variant. The same clone of each plasmid harboring a variant was used for all independent batches. Twenty-four hours before transfection, cells were seeded in 96-well plates at a density of 1 x 10^5 cells/well. Prior to transfection, the culture medium was replaced with 100 µl of fresh medium. The transfection mixture for individual wells comprised 100 ng of the piggyBac transposon vector, 50 ng of pCMV-hyPBase, 0.5 µl of Lipofectamine Stem Transfection Reagent (Thermo Fisher Scientific), and 50 µl of Opti-MEM I Reduced Serum Medium (Thermo Fisher Scientific). The transfection mixture was added to each well after 20 min of incubation at room temperature. Two days after transfection, cells were cultured with antibiotics (3 µg/ml puromycin for piggyBac-BRCA2 and 100 µg/ml zeocin for piggysBac-DRGFP) for 7 days. Puromycin-resistant polyclonal cell populations harboring piggyBac-BRCA2 were used directly for assays, while a genetically homogeneous cloned cell line harboring piggyBac-DRGFP was generated from a single cell.

**Digital droplet PCR.** Digital droplet PCR was performed using a QX100 Droplet Digital PCR system (Bio-Rad Laboratories) with BRCA2 cDNA primers and probe and BRCA2 intron primers and probe, both at a final concentration of 900 nM containing 10% fetal bovine serum and 2 mM l-glutamine at 37 °C in 5% CO2 and passed at 70–90% confluency with TrypLE Express (all from Thermo Fisher Scientific).
primers and 250 nM probe. The sequences are shown in Supplementary Table 1. Aqueous droplets with a 20-µl volume containing final concentrations of 1x Droplet Digital PCR Supermix for probes (Bio-Rad Laboratories) and 100 ng of the genomic DNA template were generated. PCR amplification was conducted using a T100 Thermal Cycler under the manufacturer-recommended conditions. After amplification, the digital PCR data were collected and analyzed using a Bio-Rad QX100 droplet reader and QuantaSoft v1.6.0.0320 software. Crosshair gating was used to automatically split the data into four quadrants by the software’s normal setting. Approximately 15,000 droplets were analyzed per well. The induced BRCA2 cDNA copy number was calculated based on the observation that DLD1 cells are a pseudodiploid human cell line and the copy number of the endogenous BRCA2 gene is considered to be 2. The 95% confidence intervals were estimated by a Poisson distribution model.

Real-time quantitative RT-PCR. freshly recovered DLD1 BRCA2(+/−) cells harboring 20 variants of BRCA2, untransduced DLD1 BRCA2(−/−) cells, and DLD1 parental cells were pelleted. Total RNA was extracted from these pellets with RNA-Beet reagent following the manufacturer’s instructions. In each experiment, the cDNA copy number was calculated based on the observation that BRCA2 cDNA copy number was derived from the synthesized cDNA. The relative expression levels in DLD1 parental cells. The results are shown as the averages of biological triplicate experiments. The fold change in the barcode count for variants treated with each drug relative to that for variants treated with the DMSO control was calculated. These values were log normal to the standard values of log2(1.0) for wild-type and log2(0.003) for D273H as anchors. The remaining 242 variants were unbalanced for pathogenicity in this analysis. We hypothesized that the functional variant-specific effect ƞ followed a 2-component Gaussian mixture distribution. The prior probability of the variants’ pathogenicity inside key domains was noninformative or based on the Align-GVGD classification obtained from the H胸 Cancer Genes Prior probabilities website as follows: the PALB2 interaction domain (amino acid residues 10–40), DNA-binding domain (2481–3186), and TR2 RAD51-binding domain (3269–3305). The prior probability outside key domains was estimated at 0.02 by the HCI and the BRCA Exchange, independent of the Align-GVGD classification.

The equations and parameters of the model are as follows:

\[
\begin{align*}
&\prod_{v} \Pr(f_1|D, X, \theta) = \prod_{\{D_1 \ldots D_N\}} \Pr(f_1|D, X, \theta) \prod_{\{D_2 \ldots D_N\}} \Pr(f_2|D, X, \theta) \quad \text{for } \theta \neq 0;
&\prod_{\{D_i \text{ abnormal}\}} \Pr(f_1|D, X, \theta) \quad \text{for } 0 \leq \theta < 0.003;
&\prod_{\{D_i \text{ normal}\}} \Pr(f_1|D, X, \theta) \quad \text{for } \theta \geq 0.003.
\end{align*}
\]

The terms in the equation above are addressed below.

Pr(Data [Parameters]) = likelihood of data observation with parameters

\(f_1\) : measurements of functional experiments for \(v\)

\(D_1\) : \(D_1 = A\) (\(v\) = abnormal)

\(D_2\) : \(D_2 = N\) (\(v\) = normal)

\(X\) : batch and experimental indices for each measurement

\(\pi_{v, A(N)}\) : prior probability that is abnormal/normal

The true distributions of the data and parameters were estimated by the formula below.

\[
\Pr(f_1|D_1, X, \theta) = \prod_{\{(x, b) \in \text{domain}\}} \Pr(f_1|D_1, b, \theta)
\]

We established some constraints and weakly informative prior distributions as described below.

\(b\) : batch index;
\(v\) : experimental index;
\(\beta_i\) : batch-specific random intercept effect;
\(\tau_{b}\) : batch-specific random slope effect;
\(\kappa_i\) : center of the distribution \(\beta_i\);
\(\kappa_{c}\) : center of the distribution \(\tau_{b}\);
\(\lambda_{c}\) : standard deviation of the distribution \(\beta_i\);
\(\lambda_{c}\) : standard deviation of the distribution \(\tau_{b}\).
In the above model, the fixed value $\eta_{\text{muc}}$ was arbitrarily estimated by the mclust v5.4.5 package with the training set data. In the full analysis, $\eta_{\text{muc}}$ was fixed such that the value of $\beta_0 + \lambda D_{\text{muc}}$ was the same as that in the training set data. Normal(0,0) denotes the normal distribution with mean 0 and standard deviation 0. $X$ is normally distributed with a mean of 0 and a standard deviation of 5 but is constrained to be nonnegative. The parameters of the Beta distributions were determined such that their 2.5th and 97.5th percentiles fit the upper and lower 95% confidence limits of the Align-GVGD prediction for each class. The ranges of the normal distributions for the parameter prior distributions were set to adequately exceed the range of values expected for those parameters. The half-Cauchy distribution was thought to be superior to other weakly informative prior distributions for variance component parameters, but the Cauchy distribution might be too broad for our model because very large values could not occur. Thus, a half-normal distribution was adopted for variance component parameters.

References
1. Wooster, R. & Weber, B. L. Breast and ovarian cancer. *N. Engl. J. Med.* 348, 2339–2347 (2003).
2. Finch, A. P. et al. Impact of oophorectomy on cancer incidence and mortality in women with a BRCA1 or BRCA2 mutation. *J. Clin. Oncol.* 32, 1547–1553 (2014).
3. Heemskerk-Gerritsen, B. A. et al. Prophylactic mastectomy in BRCA1/2 mutation carriers and women at risk of hereditary breast cancer: long-term experiences at the Rotterdam Family Cancer Clinic. *Ann. Surg. Oncol.* 14, 3335–3344 (2007).
4. Paluch-Shimon, S. et al. Prevention and screening in BRCA mutation carriers and other breast/ovarian hereditary cancer syndromes: ESMO Clinical Practice Guidelines for cancer prevention and screening. *Ann. Oncol.* 27, v103–v110 (2016).
5. Farmer, H. et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature* 434, 917–921 (2005).
6. Bryant, H. E. et al. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature* 434, 913–917 (2005).
7. Richards, S. et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet. Med.* 17, 405–424 (2015).
8. Plon, S. E. et al. Sequence variant classification and reporting: recommendations for improving the interpretation of cancer susceptibility genetic test results. *Hum. Mutat.* 29, 1282–1291 (2008).
9. Tavtigan, S. V., Greenblatt, M. S., Goldgar, D. E. & Boffetta, P., Group IUGVW. Assessing pathogenicity: overview of results from the IARC Unclassified Genetic Variants Working Group. *Hum. Mutat.* 29, 1261–1264 (2008).
10. Easton, D. F. et al. A systematic genetic assessment of 1,433 sequence variants of unknown clinical significance in the BRCA1 and BRCA2 breast cancer-predisposition genes. *Am. J. Hum. Genet.* 81, 873–883 (2007).
11. Lindor, N. M. et al. A review of a multifactorial probability-based model for classification of BRCA1 and BRCA2 variants of uncertain significance (VUS). *Hum. Mutat.* 33, 8–21 (2012).
12. Momozawa, Y. et al. Germline pathogenic variants of 11 breast cancer genes in 7,051 Japanese patients and 11,241 controls. *Nat. Commun.* 9, 4083 (2018).
13. Ernst, C. et al. Performance of in silico prediction tools for the classification of rare BRCA1/2 missense variants in clinical diagnostics. *BMC Med. Genomics* 11, 35 (2018).
14. Brnich, S. E. et al. Recommendations for application of the functional evidence PS3/B3 criterion using the ACMG/AMP sequence variant interpretation framework. Genome Med. 12, 3 (2019).

15. Toland, A. E. & Andreassen, P. R. DNA repair-related functional assays for the classification of BRCA1 and BRCA2 variants: a critical review and needs assessment. J. Med. Genet. 54, 721–731 (2017).

16. Findlay, G. M. et al. Accurate classification of BRCA1 variants with saturation genotyping editing. Hum. Mutat. 36, 5023–5030 (2008).

17. Starita, L. M. et al. Massively parallel functional analysis of BRCA1 RING domain variants. Genetics 200, 413–422 (2015).

18. Starita, L. M. et al. A multiplex homology-directed DNA repair assay reveals the impact of more than 1,000 BRCA1 missense substitution variants on protein function. Am. J. Hum. Genet. 103, 498–508 (2018).

19. Guidugli, L. et al. Assessment of the clinical relevance of BRCA2 missense variants by functional and computational approaches. Am. J. Hum. Genet. 102, 233–248 (2018).

20. Hart, S. N. et al. Comprehensive annotation of BRCA1 and BRCA2 missense variants by functionally validated sequence-based computational prediction models. Genet. Med. 21, 71–80 (2019).

21. Guidugli, L. et al. Functional assays for analysis of variants of uncertain significance in BRCA2. Hum. Mutat. 35, 151–164 (2014).

22. Ding, S. et al. Efficient transposition of the piggyBac (PB) transposon in mammalian cells and mice. Cell 122, 473–483 (2005).

23. Hucl, T. et al. A syngeneic variance library for functional annotation of human protein domains: application to BRCA2. Cancer Res. 68, 5023–5030 (2008).

24. Drean, A. et al. Modeling therapy resistance in BRCA1/2-mutant cancers. Mol. Cancer Ther. 16, 2022–2034 (2017).

25. Cline, M. S. et al. BRCA Challenge: BRCA exchange as a global resource for functionally annotated human BRCA2 variants: an ENIGMA resource to support clinical variant classification. Hum. Mutat. 40, 1557–1578 (2019).

26. Lee, J. S. et al. Reclassification of BRCA1 and BRCA2 variants of uncertain significance: a multifactorial analysis of multicentre prospective cohort. J. Med. Genet. 55, 794–802 (2018).

27. Yusa, K., Zhou, L., Li, M. A., Bradley, A. & Craig, N. L. A hyperactive piggyBac transposase for mammalian applications. Proc. Natl. Acad. Sci. USA 108, 1531–1536 (2011).

28. Kohsaka, S. et al. A method of high-throughput functional evaluation of EGFR gene variants of unknown significance in cancer. Sci. Transl. Med. 9, eaan6566 (2017).

29. Nagano, M. et al. High-throughput functional evaluation of variants of unknown significance in EBRB2. Clin. Cancer Res. 24, 5112–5122 (2018).

30. Wu, K. et al. Functional evaluation and cancer risk assessment of BRCA2 unclassified variants. Cancer Res. 65, 417–426 (2005).

31. Farrugia, D. J. et al. Functional assays for classification of BRCA2 variants of uncertain significance. Cancer Res. 68, 3523–3531 (2008).

32. Guidugli, L. et al. A classification model for BRCA2 DNA binding domain missense variants based on homology-directed repair activity. Cancer Res. 73, 265–275 (2013).

33. So, M. K. et al. Reinterpretation of BRCA1 and BRCA2 variants of uncertain significance in patients with hereditary breast/ovarian cancer using the ACMG/AMP 2015 guidelines. Breast Cancer 26, 510–519 (2019).

34. Woods, N. et al. Functional assays provide a robust tool for the clinical annotation of genetic variants of uncertain significance. npj Genomic Med. 1, 16001 (2016).

35. Iversen, E. S. Jr., Couch, F. J., Goldgärd, D. E., Tavtigian, S. V. & Monteiro, A. N. A computational method to classify variants of uncertain significance using functional assay data with application to BRCA1. Cancer Epidemiol. Biomark. Prev. 20, 1078–1088 (2011).

36. Tavtigian, S. V. et al. Modeling the ACMG/AMP variant classification guidelines as a Bayesian classification framework. Genet. Med. 20, 1054–1060 (2018).

37. Xia, B. et al. Control of BRCA2 cellular and clinical functions by a nuclear partner, PALB2. Mol. Cell. 22, 719–729 (2006).

38. Zhang, J., Fujiwara, Y., Yamamoto, S. & Shibuya, H. A meiosis-specific BRCA2 binding protein recruits recombinesis to DNA double-strand breaks to ensure homologous recombination. Nat. Commun. 10, 722 (2019).

39. Tavtigian, S. V., Byrnes, G. B., Goldgärd, D. E. & Thomas, A. Classification of rare missense substitutions, using risk surfaces, with genetic- and molecular- epidemiology applications. Hum. Mutat. 29, 1342–1354 (2008).

40. Easley, F. et al. CDK-dependent phosphorylation of BRCA2 as a regulatory mechanism for recombinational repair. Nature 434, 598–604 (2005).

41. Farber-Katz, S. et al. Quantitative analysis of BRCA1 and BRCA2 germline splicing variants using a novel RNA-massively parallel sequencing assay. Front. Oncol. 8, 286 (2018).

42. Balca, C., Galli, A. & Caligo, M. A. Effect of the overexpression of BRCA2 unclassified missense variants on spontaneous homologous recombination in human cells. Breast Cancer Res. Treat. 129, 1001–1009 (2011).

43. Shimelis, H. et al. BRCA2 hypomorphic missense variants confer moderate risks of breast cancer. Cancer Res. 77, 2798–2799 (2017).

44. Bradbury, A. R. et al. Uptake and timing of bilateral prophylactic salpingo-oophorectomy among BRCA1 and BRCA2 mutation carriers. Genet. Med. 10, 155–161 (2008).

45. Shimelis, H. et al. BRCA2 hypomorphic missense variants confer moderate risks of breast cancer. Cancer Res. 77, 2798–2799 (2017).

46. Lim, D. & Ngeow, J. Evaluation of the methods to identify patients who may benefit from PARP inhibitor use. Endocr. Relat. Cancer 23, R267–R285 (2016).

47. Davies, H. et al. HRDetect is a predictor of BRCA1 and BRCA2 deficiency based on mutational signatures. Nat. Med. 23, 517–525 (2017).

48. Nones, K. et al. Whole-genome sequencing reveals clinically relevant insights into the aetiologies of familial breast cancers. Ann. Oncol. 30, 1071–1079 (2019).

49. Daniels, M. J., Wang, Y., Lee, M. & Venkitaraman, A. R. Abnormal cytokinesis in cells deficient in the breast cancer susceptibility protein BRCA2. Science 306, 876–879 (2004).

50. Shioji, M. K. et al. BRCA2 hypomorphic missense variants confer moderate risks of breast cancer. Cancer Res. 77, 2798–2799 (2017).

51. Simeonov, A. et al. Modeling therapy resistance in BRCA1/2-mutant cancers. J. Comput. Graph. Stat. 9, 1–22 (2000).

52. Daniels, M. J. et al. Abnormal cytokinesis in cells deficient in the breast cancer susceptibility protein BRCA2. Cancer Res. 77, 2798–2799 (2017).

53. Acedo, A. et al. Comprehensive splicing functional analysis of DNA variants of the BRCA2 gene by hybrid minigenes. Breast Cancer Res. 14, R87 (2012).

54. Oh, M. et al. BRCA1 and BRCA2 gene mutations and colorectal cancer risk: systematic review and meta-analysis. J. Natl. Cancer Inst. 110, 1178–1189 (2018).

55. Lall, N., Henley-Smith, C. J., De Canha, M. N., Oosthuizen, C. B. & Berrington, D. Viability reagent, PrestoBlue, in comparison with other available reagents, utilized in cytotoxicity and antimicrobial assays. Int. J. Microbiol. 2013, 420601 (2013).

56. Scrucca, L., Fop, M., Murphy, T. B. & Raftery, A. E. mclust 5: clustering, classification and density estimation using Gaussian finite mixture models. R J. 8, 289–317 (2016).

57. Gelman, A. Prior distributions for variance parameters in hierarchical models. Bayesian Anal. 1, 515–534 (2006).

58. Ihaka, R. & Gentleman, R. A language for data analysis and graphics. J. Comput. Graph. Stat. 5, 299–314 (1996).

59. Gelman, A., Lee, D. & Guo, J. Stan: a probabilistic programming language for Bayesian inference and optimization. J. Educ. Behav. Stat. 40, 530–543 (2015).

60. Brooks, S. P. & Gelman, A. General methods for monitoring convergence of iterative simulations. J. Comput. Graph. Stat. 7, 434–455 (1997).

61. Fox, J. Weisberg, S. & An, R. Companion to Applied Regression. 2nd edn. Sage Publications: Thousand Oaks, 2011.
