Short communication

Effectiveness of a genetic test panel designed for gynecological cancer: An exploratory study

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Abstract

To increase diagnostic efficiency and cost-effectiveness, we performed an exploratory genetic test using a newly designed panel containing 28 actionable and druggable genes, alterations in which are frequently reported in gynecological cancers (TANRE-G, Targeted variants ANalysis RElated to Gynecological cancers). Samples consisted of the formalin-fixed, paraffin-embedded tissue of endometrial (4 cases), cervical (3 cases), and ovarian (4 cases) carcinomas. The sequencing procedure was performed using Ion-PGM in our institute with related sequencing kits, and data were analyzed using ClinVar. The present system achieved more than 2500 reads in all tumor samples, and enabled a copy number variation analysis. Results showed that actionable and druggable mutations were detected in 82% (9/11) and 64% (7/11) of cases, respectively, which was similar to other commercially available genetic tests. The amplification of MYC and KRAS was also detected. The analysis cost for each sample was JPY 94,000 (USD 850). These results demonstrate the potential of the TANRE-G panel as an effective tool for examining genetic alterations in gynecological cancers.

Keywords: genetic test, endometrial carcinoma, cervical carcinoma, ovarian carcinoma, gene panel

Introduction

Recent advances in next-generation sequencing (NGS) have enabled comprehensive genetic variants to be analyzed in a clinical setting as precision medicine, particularly for cancer [1]. A precision approach leads cancer patients to targeted therapies directed against specific genomic driver alterations in tumors identified with NGS-based testing [2]. Testing contents have recently involved more comprehensive gene panels instead of disease-focused gene panels [3]. In Japan, several types of NGS-based genetic tests are currently available; most use panels containing 100~200 comprehensive genes, and cost between 60,000 and 100,000 JPY (5,000~8,000 USD), which is not covered by medical insurance.

Due to prevailing precision approaches [4], we have encountered a number of "actionable" alterations, defined as alterations potentially targetable with established or investigational therapeutics directly or indirectly [5]. However, there are currently only a few FDA approved drugs, i.e., those targeting angiogenesis, homologous recombination deficiencies, and microsatellite instabilities [4].

While precision approaches offer useful diagnostic strategies for cancer patients, the following issues remain unsolved. Since there are many targeting genes on popular NGS cancer panels, including unfamiliar variants, such as variants of uncertain significances (VUSs), difficulties are often associated with interpreting the data obtained [6]. The handling of these "incidental" findings creates ethical dilemmas. Therefore, multiple and comprehensive cancer panels may currently result in many useless variants for patients, which inevitably causes confusion. Furthermore, although the cost of sequencing has gradually been decreasing [7], the cancer panels used in clinical sequencing are still too expensive for many terminal cancer patients and their families to request. However, the number of studies on the costs of clinical sequencing for advanced cancer patients is currently limited, and we often encounter difficulties understanding real transactions in clinical settings.

In addition, although multiple clinical studies evaluating precision medicine revealed that between 30 and 50% of patients had actionable mutations, only between 3 and 13% received treatments that had been adapted to individual druggable mutations [8]. However, even if druggable mutations are detected in the cancer panels of patients, it may be impossible to take targeted drugs covered by health insurance in many cases, or only a very small number of these patients may discover and join clinical trials offering suitable therapies. If some cases, these patients may eventually select a drug recommended by an expert meeting as off-label use at their expense, which often has been priced at astronomical levels, i.e., USD 70,000 to 130,000 for a course of treatment [9].

To address these issues, we designed a genetic testing approach focused exclusively on 28 actionable and druggable genes in gynecological cancers named Targeted variants ANalysis RElated to Gynecological cancers (TANRE-G), and retrospectively performed clinical sequencing as an experimental trial. This exploratory

research aimed to evaluate the cost-effectiveness and feasibility of clinical sequencing by the TANRE-G panel and show the prospects of this gynecologically specific panel.

Materials and Methods

Design

TANRE-G is based on the Ion AmpliSeq DNA assay workflow (Thermo Fisher Scientific, Wilmington, DE, USA). Amplicons (n=1471) were designed with Ion AmpliSeq DNA Designer. The 28 target genes of TANRE-G comprised actionable and potentially actionable genes, harboring frequent variants of oncogenic driver and tumor suppressor genes reported in gynecological cancers, and were selected based on the following databases: CanDL; https://candl.osu.edu/, CANCER GENOME INTERPRETER; https://www.cancergenomeinterpreter.org/home, CIViC; https://civicdb.org/home, OncoKB; http://oncokb.org/#/, and ClinicalTrials.gov; https://clinicaltrials.gov/ (Table 1). The TP53 gene was omitted from this panel because p53 variants did not have any impact on further treatment.

Patient and Samples

This study was a single-center case series with retrospective data collection conducted with the approval of the Ethics Committee of Shinshu University (approval No.591). Subjects were ensured of their right to opt out prior to the start. Eleven refractory or relapsed gynecological cancer cases (4 endometrial, 4 ovarian, and 3 cervical cancers) were selected from patients who underwent surgery at Shinshu University Hospital between 2016 and 2017. Median age was 57 years (44-78), 6 out of 11 cases were stage III-IV, and only 2 cases received neoadjuvant chemotherapy. Formalin-fixed paraffin-embedded (FFPE) tissues removed surgically and fixed within 48 hours were used in the following working flow.

Library preparation, emulsion PCR, and enrichment

Tumor tissues (as test samples) and normal myometrial tissues (as normal references) were collected by needle microdissection from several 10-μm-thick FFPE tissue sections. The ratio of tumor cells was confirmed to be between 60 and 80% across all samples. The genomic DNA of each sample was extracted using the MagMAX FFPE DNA/RNA Ultra Kit (Thermo Fisher Scientific, Wilmington, DE) according to the manufacturer's instructions. The concentration and quality of extracted DNAs were checked using the Qubit dsDNA HS Assay Kit with the Qubit 3.0 Fluorometer (Thermo Fisher Scientific) and quantitative PCR (qPCR) with the TaqMan FFPE DNA QC Assay (Thermo Fisher Scientific), respectively. Libraries were generated from 10 ng of DNA per

sample using the Ion AmpliSeq Library Kit 2.0 (Thermo Fisher Scientific), quantified by applying a qPCR analysis with the Ion Library TaqMan Quantitation Kit (Thermo Fisher Scientific), and pooled to an equimolar concentration of 20 pM for template preparation. Template preparation, which comprised emulsion PCR, the enrichment of beads containing the template, and chip loading, was performed using the Ion Chef system and Ion PGM Hi-Q View Chef Kit following the manual (Thermo Fisher Scientific).

Sequencing and data analysis

The sequencing of multiplexed templates was performed using the Ion PGM system on Ion 318 v2 chips with the Ion PGM Hi-Q View Chef Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. A primary data analysis was performed by Torrent Suite Software v5.8 (Thermo Fisher Scientific) with default settings using hg19 as reference genome data. The data of normal DNA was only used individually for the subtraction of germline alterations, such as SNPs. Variant calling was executed using the Torrent Variant Caller plugin (5.8.0.19) in the Torrent Server and visualized using Integrative Genomics Viewer Version 5.01 (0) (Broad Institute). After the sequence was aligned and filtered on a tumor-normal pipeline, a variant analysis and annotations were performed by IonReporter software 5.6 with default settings. Candidate variants selected by these processes were filtered based on the predicted impact on protein function by SIFT (Sorting Intolerant From Tolerant, http://provean.jcvi.org/index.php) and Polyphen-2 (Polymorphism Phenotyping version 2, http://genetics.bwh.harvard.edu/pph2/). These functionally affected variants were finally defined as 'Pathogenic', 'Likely Pathogenic', or others by ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/). Furthermore, if the candidate variant was a frameshift or nonsense mutation affecting known functional domains of a tumor suppressor gene, it was also regarded as 'Pathogenic' regardless of the registration in ClinVar.

Prediction of copy number alterations

Copy number alterations (CNAs) were detected using an Ion Reporter Software Copy Number Variation Analysis (Thermo Fisher Scientific) based on a hidden Markov model [10]. CNAs with precision scores ≥10 were included in the analysis according to the manufacturer's instructions. The copy number of the tumor cells was calculated by correcting with the ratio of tumor cells in the tissue section. According to previous studies, copy numbers of ≥6 were defined as high-level amplification, while copy numbers of 3-5 were regarded as moderate amplification [11]. In the present study, high-level and moderate amplifications of oncogenic driver genes were defined as "Likely pathogenic" variations.

Confirmation of Genetic Alterations

Each variant sequence detected by the above workflow was confirmed by Sanger sequencing. Immunohistochemistry (IHC) was performed to confirm CNAs. Primers for Sanger sequencing were designed using Primer Designer Tool online (Thermo Fisher Scientific) or Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). IHC was performed using 3-μm-thick FFPE tissue sections as described previously [12]. Antibodies for MYC (9E10, 1:200 dilution, Novus Biologicals, Littleton) and KRAS (12063-1-AP, 1:300 dilution, ProteinTech) were used as the primary antibody.

Statistical Analysis

The significance of differences between the TANRE-G panel and NCC Oncopanel was evaluated with a two-tailed Fisher's exact test. A P value of less than 0.05 was considered to be significant. All statistical analyses were performed using R software (https://www.r-project.org/).

Results

We evaluated the performance of the TANRE-G panel in the retrospective exploratory analysis of 11 gynecological FFPE tumor-normal samples. All samples successfully underwent the targeted sequencing of genes on the TANRE-G panel. A TANRE-G assay generated approximately 5.5 million reads per specimen with the following characteristics for tumor and normal tissues: mean coverage depths in tumor and normal amplicons were approximately 3400× and 350×, respectively; mean coverage depths in 86% of all tumor amplicons and 82% of all normal tissue amplicons were >500× and >100×, respectively; and <1% of tumor amplicons and <3% of normal tissue amplicons were <20× in their mean coverage depths.

Somatic variants detected

Table 2 shows 32 candidate somatic variants in 8/11 (72%) cases from sequencing with the TANRE-G panel. Each number of variation type was as follows: missense 22 (69%); nonsense 6 (19%); amplification 3 (9%); frameshift-deletion 1 (3%). Regarding 'Pathogenic' or 'Likely pathogenic' somatic alterations as actionable somatic alterations, 24/32 (75%) actionable variants were detected. The gene having the most frequent variations was PIK3CA with 7 variants, followed by PTEN with 5 variants. Most of these gene alterations, especially those defined as 'Pathogenic' were consistently with previous reports in each primary cancer, and were shown in pBioPortal (http://www.cbioportal.org/) and/or COSMIC (https://cancer.sanger.ac.uk/cosmic) (Supplementary Table 1). Twenty-four candidate somatic variants were extracted in 4/4 (100%) cases of endometrial cancer.

Eighteen (18/24; 75%) of these were actionable variants, most of which were related to an aberrant PI3K pathway by PIK3CA or PTEN mutations (4/4 cases; 100%) or an aberrant Receptor Tyrosine Kinase (RTK)/RAS signaling pathway by ERBB2 or KRAS variants (3/4 cases; 75%). In 3/4 (75%) of ovarian cancer cases, six candidate somatic variants were identified, and 3/6 (50%) were actionable variants; PIK3CA and ARID1A variants in one (25%) clear cell ovarian carcinoma case, and BRCA2 gene in one (25%) high-grade serous ovarian carcinoma case. In Case 7 with the BRCA2 variation, the tumor relapsed 4 times, however, she has been successfully treated with platinum-based chemotherapy followed by PARP inhibitors as maintenance therapy. Only one (33%) out of the three GAS cases had actionable variants in the KRAS gene. All actionable somatic mutations were validated by Sanger sequencing.

Although the target region of TANRE-G panel is not large enough (0.14Mb) to precisely calculate the tumor mutation burden (TMB, mutation frequency /Mb), that of case 1 and 4 were 62 and 82 /Mb, respectively. Therefore, TMB data obtained by TANRE-G panel may be applied to select the candidates for MSI testing.

CNAs was assessed in 5 cases (45%) under the detection algorithm. We detected 3 moderate amplifications as actionable variants in 2 driver oncogenes: One was MYC (copy number = 3 in case 6, and copy number = 5 in case 7), the other was KRAS (copy number = 7 in case 9). This KRAS amplification may explain the high variant allele frequency (VAF) of it (Table 2). These results were confirmed by IHC (Supplementary fig 1). The specimens from cases 8 and 11 were immunostained as a control for MYC and KRAS due to their normal copy number, respectively. The direct material costs for an analysis of one sample by TANRE-G, including chemicals, was approximately USD 850 (JPY 94,000), which is less expensive than other gene panels (e.g., FoundationOne CDx: USD 5800, MI Tomor Seek: USD 3500).

Comparison with another panel assay

In a comparison of the TANRE-G panel with the NCC Oncopanel for the first 131 cases [13], at least one pathogenic somatic variant was observed in 8/11 (73%) cases in the TANRE-G panel, whereas 104/131 (79%) were noted in the NCC Oncopanel (*P* value, 0.700). Furthermore, at least one actionable somatic variant was observed in 8/11 (73%) cases in the TANRE-G panel, whereas 59/131 (45%) were noted in the NCC Oncopanel (*P* value, 0.115). Neither panel showed a significant difference in detecting pathogenic or actionable somatic variants, which indicates that the TANRE-G panel is a similar or alternative test to the NCC Oncopanel, at least in gynecological clinical settings.

Discussion

NGS has enabled us to perform personalized oncologic strategies for targeted therapy. We herein conducted exploratory research on a customized, cost-effective NGS panel “TANRE-G” for the identification of relevant somatic alterations specifically in gynecological cancers. We performed a retrospective analysis of archival tumor-normal FFPE samples in recurrence or refractory cases. We effectively identified mutations through deep targeted sequencing using the Ion Torrent platform and its useful annotation software Ion Reporter [14]. The prevalence of detecting actionable or potentially actionable mutations in TANRE-G was similar to that in existing panels. For example, actionable mutations were detected in 45% in the NCC Oncopanel [13], 47% in Foundation One, and 36% in MSK-IMPACT [15].

Endometrial cancer is expected to have the most actionable mutations in TANRE-G. Likewise, a recent study revealed that the prevalence of actionable mutations or CNAs in all cancers was the highest (98%) in endometrial cancer, resulting in the cost of finding at least one actionable mutation for this cancer to be the second lowest among all cancers (USD 5,897) [16].

Drugs for potentially actionable mutations may be developed in rapid succession. The use of a comprehensive panel may be advantageous for unintentionally identifying new actionable drugs already approved for other diseases with the same mutations. However, basket trials on one drug for a single mutation in a number of tumors have only recently started [4]; therefore, further time is needed to obtain information, which results in delays in the treatment of patients using these drugs. Therefore, we consider TANRE-G to be a beneficial and cost-effective choice, particularly for gynecological patients.

One issue needs to be addressed; when a higher number of genes is adopted in one panel, more VUSs are also found, which results in difficulties judging pathogenicity in clinical settings. We selected candidate genes that did not cause increases in germline or somatic VUSs because there are many functionally unknown variants that may be pathogenetic [17]. In general, the fewer genes mounted in gene panels, the fewer VUSs detected. Based on our results in TANRE-G, we only found one VUS in case 1 (Table 2), thereby confirming the pathogenicity of VUSs.

Following the recommendations of the American College of Medical Genetics and Genomics (ACMG), we do not necessarily inform patients of all results of VUSs. However, we need to consider that changes in VUSs to actionable ones in the near future may be detected by upcoming new methods, such as whole genome clinical sequencing, and this information may be provided to patients.

Thus, TANRE-G is a simple gene panel test. By using this panel, we may examine the minimum necessary genes, judge whether they are actionable, and consider the next treatment policy. Furthermore, CNV and TMB may be used as references. The discovery rate of actionable genes is expected to be high from the

present results, suggesting lower costs. Therefore, this may become an excellent option, particularly for gynecological cancer patients in the local area.

In conclusion, TANRE-G proved to be a clinical model that is a reliable, fast, and cost-effective targeted NGS panel. Many selected genes are useful for finding new candidate variants to target or new functional therapies, but this may potentially be very rare. In contrast, many VUSs are identified that provide patients with helpful or sometimes harmful information. We currently cannot offer patients sufficient consultations on the genes listed in ACMG secondary findings [18] because of the lack of genetic counselors, particularly in Japan (approximately 200 counselors). However, as discussed above, our exploratory TANRE-G panel may become a shortcut to resolving these issues by merely defining gynecological target genes. We consider the effectiveness of our panel to support its clinical application to advanced gynecological cancer patients in near future.

Conflict of Interest disclosure statement

The authors declare that they have no conflict of interest.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the Ethics Committee of Shinshu University (approval No.591) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Blanket consents had been obtained from all individual participants included in this study for using their resected tissue-samples to any studies with anonymization. It was accepted in the Ethics Committee that the additional consent was unnecessary from any participants because of analyzing only somatic alterations of the genes on the TANRE-G panel. All individual participants were ensured of their right to opt out prior to the start.

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Supplementary materials

Supplementary fig 1: Hematoxylin and eosin (H&E) and immunohistochemistry (IHC) staining of cases with the MYC or KRAS amplification. a Case 6 of low-grade serous ovarian carcinoma showed the MYC amplification as a copy number (CN) = 3. b Case 7 of high-grade serous ovarian carcinoma showed the MYC amplification as a CN = 4. c Case 8 of high-grade serous ovarian carcinoma showed the normal CN = 2 in MYC as a control. d Case 9 of mucinous carcinoma, gastric type showed the KRAS amplification as a CN =5. e Case 11 of mucinous carcinoma, gastric type showed the normal CN =2 in KRAS as a control. Each group (a–e) of images showed H&E (left, ×40) and IHC (right, ×40).