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研究テーマと研究計画

QUALITY ASSURANCE OF NEXT GENERATION SEQUENCING (NGS)-BASED CANCER GENE PANEL TESTS

はじめに

Next Generation Sequencing (NGS) is the technology that enables us to determine the sequence of DNA (deoxyribonucleic acid) with much longer footage in much shorter time than the conventional Sanger sequencing method. This technical breakthrough of NGS facilitates the application of the DNA sequence to various fields including the clinical applications of genome sequencing. People now have easier access to the clinical examination of genotyping of cancers, immune disorders, hereditary disorders, and so on, and this technology acquires more and more importance; the clinical use of NGS has been approved in the Japanese National Health Insurance. The accuracy of the assay in this application, however, is still yet to be evaluated before it gains the reliability by clinical practitioners and patients as much as the other standard laboratory tests have, and this is where we can contribute to the development of the technology.

The entire workflow of NGS comprises of a multistep and complex workflow starting from sample preparation, library preparation, sequencing, and bioinformatics analysis. Furthermore, different NGS technologies and platforms exist, each harboring with their own inherent characteristics, limitations, and advantages. Those would lead to have the potential risk of introducing error in the any process involved in the tests. To overcome these issues, the best approach to the quality assurance needs to be explored in responding to the technical advancement and clinical demands on the NGS tests.

External quality assessment (EQA) is one of the measures for the quality assurance to evaluate the performance of the laboratories and guarantee the validity of tests results. Since NGS tests are various in platforms, applications and target of the genes, any specific methods for EQA or proficiency testing (PT) have not been universally applied to all the laboratories which perform NGS tests.

In the first year of my research work in Japan under the fellowship from Nakatani foundation, I worked for the implementation of the EQA program for NGS oncology tests. Based on obtained results, we concluded that most of the failures of detection of variants occurred due to dry-lab processes and platform specific errors. Based on these conclusions, we extended our current study to investigate on-site evaluation as EQA in the accreditation program under ISO 15189 for laboratories which performed NGS oncology test.

In addition, we have attempted to evaluate NGS inherited mutation detection tests involving commercial laboratories as EQA.

2 年目の研究活動実績

Methods

We commissioned on-site evaluation of the EQA in NGS oncology testing to perform a real time assessment of procedures and provide information for internal process improvement. On-site evaluation was accomplished by auditor's visit to the participant laboratories and discussion with laboratory staffs about the self-assessment of NGS oncology test.

ISO 15189 standard was deployed to assess the competence of medical laboratories which performed NGS oncology tests. The auditor made an assessment report according to the requirements of the ISO 15189 standard and reported to Japan accreditation board which made final decision for accreditation.

Participants

In this study a total of eight medical laboratories participated. As a platform, 3 (laboratory A, B, E, F, H) and 3 (laboratory C, D, G) laboratories used amplicon-based and hybrid-capture sequencing methods, respectively.

EQA samples

We prepared and assigned the value of two types of EQA sample including genome-based (Thermo Fisher Scientific K.K) and DNA-based (Horizon Discovery Ltd.) samples. Even tough other types of EQA samples were shipped to the laboratories due to their testing features and sequencing requirements.

Sample shipment

The EQA samples were shipped to the laboratories with conditions as follows. Genome-based sample was kept at -20 degree and DNA-based sample and the cell mixture were kept at 2-8 degree, respectively. Instructions for handling, storage, test of the samples and test report were provided together with dedicated sheets.

Evaluation of the performance

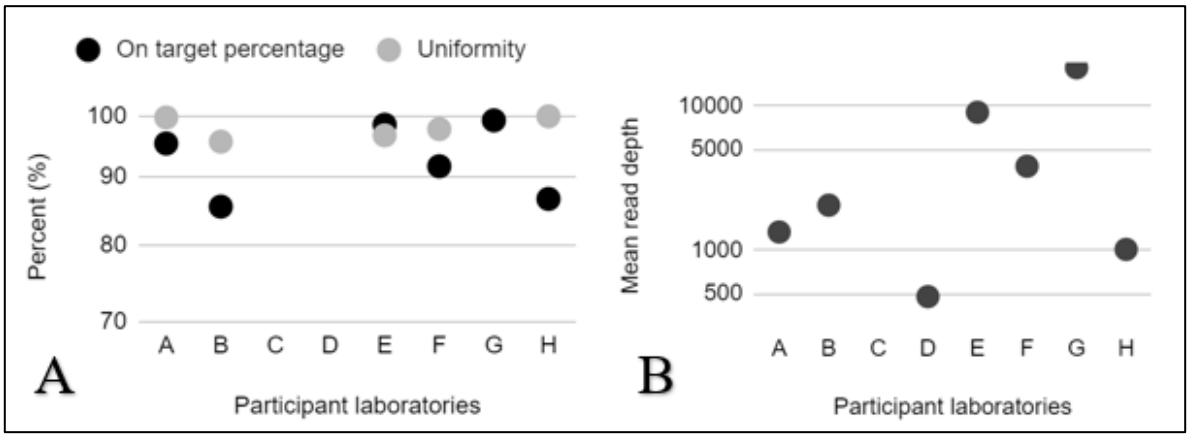
All the laboratories were required to perform the NGS analysis on the received samples using their routine procedure by their own panel and sequencing systems. The reporting sheet contained several questions including library quality indicators, sequence system, reagents used for DNA library preparation and sequencing, sequence analysis software, reference sequence data and quality metrics such as Q score, mapping qualities and variant allele frequency (VAF). Binary Aligned/Mapped (BAM) file was submitted to Tokai University as well. After receiving NGS results from the laboratories, we evaluated NGS performance, by above-mentioned indicators in the reports. The results were returned to laboratories by auditors through the onsite evaluation.

Comparison between laboratories were studied based on manufacturer`s reference data and results of our laboratory runs. BAM files were reviewed for false positive, false negative and low allele frequencies.

Results

Quality indices in the NGS oncology testing results from each of the participating laboratories were found to be acceptable (Figure 1). The mean library read length were between 100-400 bps. The mean read depth of the libraries from the participating laboratories were over 300, which was considered sufficient to assure the result of VAFs over 5%.

Figure 1. Quality metrics of the EQA samples generated by the participant laboratories. A. On-target percentage and uniformity. B. Mean read depth.

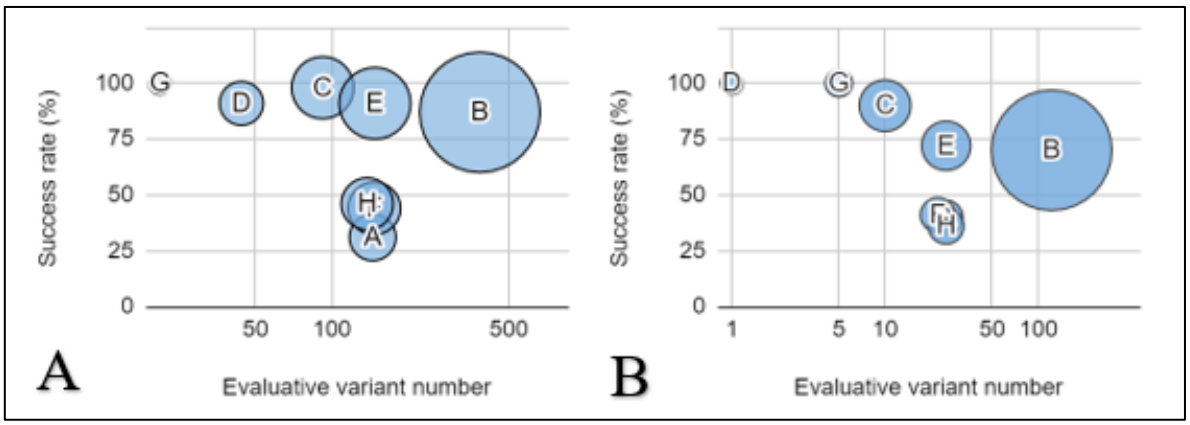


SNPs, MNP and INDELs of the genome-based and DNA based samples were determined by our laboratory using Ion Ampliseq Cancer Hotspot Panel on the Ion PGM system at our laboratory. The results were confirmed within reference ranges provided by manufacturer. VAFs of the genome-based and DNA-based samples were 5.3-25.9% and 0.4-32.8%, respectively. The detection results obtained by our laboratory was limited due to its reportable range of target variants. In addition, some VAFs of the DNA-based sample were detected as a false negative result due to its low allele frequency (1-2%). Criteria for low variant allele frequency (VAF) was defined by 5% difference from the reference range provided by manufacturer.

The participant laboratories detected 21 to 530 variants in the genome-based sample and 4 to 510 variants in the DNA-based sample by their own panel and sequencing systems. The success rate was

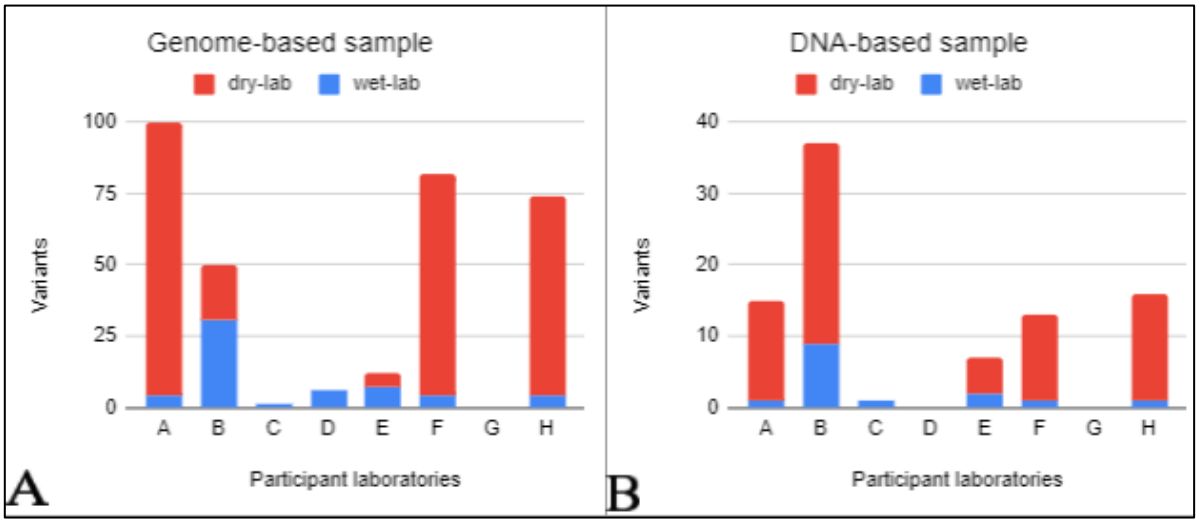
various among participant laboratories due to their reportable range and availability of the BAM file. The sequencing results of the participant laboratories are summarized on Figure 2.

Figure 2. Success rate of the participant laboratories. A. Genome-based sample, B. DNA-based sample. Bubble size represents total reported variants of each participant laboratories.



Lower-VAF and false negative results were regarded as failure to detection of the variants. The participant laboratories failed to detect 1 to 100 variants in the genome-based sample and 1 to 37 variants in the DNA-based sample. Most of the failures occurred due to dry-lab procedures such as software filtering process at variant calling and annotation (Figure 3).

Figure 3. Causes of failures (FN and Lower VAFs) to detection of the variants. A. Genome-based sample, DNA-based sample. Wet-lab process (sample processing steps), dry-lab process (bio-informatics steps)



Conclusion and future perspective

Based on obtained results we concluded following:

- Development of an EQA for NGS oncology tests has been challenging due to variation on platforms and complexity of applications and data analysis.
- There are several EQA methods available such as PT, rechecking/retesting, on-site evaluation, and inter-laboratory comparison methods. Advantage of our EQA scheme based on on-site evaluation was supportive for participant laboratories through discussion with auditors for the improvement of the internal process.
- Well-characterized genomic material or synthetic spike-in controls are important to calibrate NGS measurements and evaluate diagnostic performance.
- Accurate variant calling in NGS data is a critical step upon which virtually all downstream analysis and interpretation processes rely on it.

Based on our experience of the EQA for NGS oncology test, we attempted to evaluate NGS tests for inherited diseases. For this pilot study, we sent genome-based sample as a EQA sample, which contains the variants responsible for following case scenario.

Case scenario: The participant laboratories were provided the case scenario of megalocephaly, hemangiomas, and papilloma like rashes, and requested to choose target genes based on the case scenario. According to the case scenario most probable diagnosis were PTEN hamartoma tumor syndrome, Cowden syndrome (*PTEN* gene), Proteus syndrome (*AKT1* gene), Tuberous sclerosis complex (*TSC1/TSC2* genes) and PIK3CA-Related Overgrowth Spectrum (*PIK3CA* gene). These disorders are closely linked in phenotype because of mutations that occur predominantly within the phosphoinositide 3– kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) regulatory pathway. The genes involved in these syndromes are *AKT1, PIK3CA, PTEN, TSC1* and *TSC2* genes (Table 1).

Table 1. Types of abnormal growth observed with mosaic mutations of the PI3K/PTEN/AKT/TSC/ mTORC1 signaling pathway

Gene	***PIK3CA***	***PTEN***	***AKT1***	***TSC1/TSC2***
Syndrome	PIK3CA-Related Overgrowth Spectrum	PTEN Hamartoma Tumour Syndrome	Proteus Syndrome	Tuberous Sclerosis Complex
Segmental overgrowth	+++	+	+++	+
Hamartomas	+++	+++	+++	+++
Malignant tumours	+	+++	+	+

Nathan N et al. Mosaic Disorders of the PI3K/PTEN/AKT/TSC/mTORC1 Signaling Pathway. *Dermatol Clin*. 2017;35(1):51-60.

Participant laboratories and EQA sample

In the inherited mutation detection study, a total of three medical laboratories participated. The laboratories were named as X, Y and Z. The genome-based sample contains 27 pathogenic variants on the *AKT1* (1), *PIK3CA* (5), *PTEN* (20) and *TSC2* (1) genes which prove the clinical symptoms.

VAFs of the pathogenic variants were between 5-35% and represent mosaicism except the variant on the *TSC2* gene.

The performance was evaluated in a manner for NGS oncology tests study and result reports were sent back to the participant laboratories for discussion and process improvement.

Quality indices in the NGS inherited mutation detection results from each of the participating laboratories were found to be acceptable except laboratory Z. The mean library read length were between 150-200 bps. The mean read depth of the libraries from the participating laboratories were over 250, which was considered sufficient to assure the mosaicism over 5% of VAF.

PTEN gene was chosen as a target of interest by all participant laboratories. Laboratory X failed to choose other three genes, laboratory Y failed to choose *AKT1* and *PIKCA* genes and laboratory Z failed to choose *AKT1* and *TSC2* genes, respectively.

Comparing the variants (mosaicism) in the sample to the variants which were found in BAM file generated by the participant laboratories, 27 variants were regarded as evaluative variants. The success rate was various among participant laboratories due to their targeted genes and VAF criteria of a lower VAF to report.

In conclusion, selecting target genes based on clinical symptoms is crucial to NGS testing for inherited mutation detection. Also, variant filtering and annotation processes could lead to false negative results and clinicians should be aware of lower-VAFs in the samples with mosaicism.

Well characterized and validated EQA sample is important for establishing EQA scheme for detection of inherited germline variants. Therefore, our further studies will be focused on the generation of EQA samples and quality issue on detection performance of inherited germline variants.

留学先での生活・交流の様子

During my last 2 years, I have learned how to work in research lab and basic techniques to handle with various types of samples and working on NGS testing and analyzing the test results. Also, I have learned to write drafts and prepare for submission of scientific articles to have them published in international journals.

【謝辞】

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