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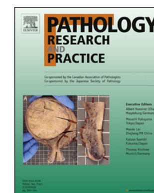

Multi-laboratory proficiency testing of clinical cancer genomic profiling by next-generation sequencing

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ABSTRACT

Next-generation sequencing (NGS) enables parallel analysis of multiple genomic targets. The increasing demand for NGS-based multiplexed molecular diagnostics requires standardized protocols and recommendations to ensure reproducibility and accuracy of test results for routine clinical decision making. However, the lack of clinical NGS data from multi-laboratory tests and the absence of inter-laboratory comparisons have hampered the establishment of instructive clinical NGS standards. To fill the gap, we set up Proficiency Testing (PT) for inter-laboratory comparison, in which formalin-fixed paraffin-embedded specimens from eight lung and eight colon cancers were analyzed by 15 European molecular diagnostic laboratories on three different platforms using multiple target enrichment systems. We first performed platform, test, and informatics pipeline validation and conducted sensitivity and specificity analysis by random *in silico* down-sampling. We then implemented a multi-level filtering strategy based on performance tests of base substitution, replicate runs, and Sanger sequencing verified variants. We finally applied the filter criteria to the NGS data from the respective PT participants and obtained high inter-laboratory agreement. We demonstrated accuracy, scalability, and robustness of NGS by means of PT, serving as a benchmark for detecting clinically actionable molecular alterations in research and diagnostic laboratories. In conclusion, this study strongly highlights the importance of establishing standards for NGS-based testing, particularly when the test results impact on clinical decisions, and systematically provides data sets from multiple different labs to infer such standards.

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1. Introduction

The concept of personalized medicine has already become a daily practice with the selection of specific drugs and therapies according to somatic genetic alterations [1,2]. Comprehensive characterization of mutations in clinically actionable genes and key cancer pathways proved beneficial in prognostic prediction and for guiding the selection of therapy in patients with advanced lung and colon cancer [3–6].

Next-generation sequencing (NGS) has become a high-throughput clinical routine and proven to be a cost effective approach to identify treatable alterations in multiple genes for optimal personalized treatment [7]. Although various large NGS trials have demonstrated the reliability of the technique [8], some clinical laboratories still verify NGS results with independent methods. Despite the efforts of international working groups such as the European Molecular Genetics Quality Network (EMQN) and the Association of Molecular Pathology/College of American Pathologists, standards for NGS quality assessment have not yet been properly defined [9,10].

In a plethora of publications regarding the establishment of customized or commercially available NGS assays on different platforms, molecular pathologists have compared their methods with the gold-standard techniques such as Sanger sequencing, digital droplet polymerase chain reaction (ddPCR) and fluorescence in-situ hybridization (FISH) [8,11–14], but the quality of those results were limited by inter-laboratory variability and diverse quality metrics. These problems emphasize the need for active participation in proficiency testing (PT) [12,15], which will improve clinical NGS performance and attain workflow-independent quality standards. Consequently, we initiated a PT for 15 clinical laboratories using formalin-fixed paraffin-embedded (FFPE) samples. Here, we demonstrate with our PT a high inter-laboratory agreement in calling clinical relevant mutations, which underlines the accuracy and robustness of current NGS platforms in clinical laboratories.

2. Results

2.1. Platform, test, and informatics pipeline validation

To assure the analytical validity of NGS in a clinical context and to assess PT performance, standardization of our NGS-based tests was performed according to previously published guidelines, including platform-, test-, and informatics pipeline-specific validation [15]. We first provided evidence that our platform (Ion Torrent PGM) could deliver reliable and reproducible sequencing results using commercially available control DNA that contains a distinct set of single and multiple nucleotide variants (SNVs, MNVs), insertions and deletions (InDels), and complex variants with defined frequencies (AcroMetrix Oncology Hotspot Control, Supplementary Table 1). Based on the results of two technical replicates derived from two independent NGS library preparations, we showed that the linear correlation coefficient for mutant allele frequencies (MAF) was $R = 0.9415$ ($P < 0.0001$, Fig. 1A). Moreover, we demonstrated that for the CHP2, we could reproduce MAFs from two technical replicates of clinical FFPE material of eight lung and eight colon carcinoma specimens with an average correlation coefficient of 0.90 (Supplementary Fig. 1). Second, we confirmed that our system correctly detected all disease associated variants in the 16 specimens that were previously assessed by Sanger sequencing with no additional mutations found in the targeted regions, reaching 100% sensitivity and specificity (Fig. 1B). Last, we validated three different informatics pipelines (CLC bio, GATK [16], and IR) based on the data from an independent EMQN PT with annotations of Sanger verified variants as reference. The highest concordance was obtained by the IR for CHP2 (Fig. 1C), i.e., platform-dependent algorithms for mapping and variant calling attained the best performance.

2.2. Sensitivity and specificity analysis

To investigate which filter criterion could be applied to achieve highest sensitivity and PPV in variant calling across different NGS assays and platforms, we analyzed BAM files of the PT data. Due to the absence of reference specimens with all possible somatic mutations in all cancer-associated genes, we developed a validation strategy based on reference samples from AcroMetrix by CHP2 using PGM, which contains a wide range of known genomic alterations with defined MAFs. For validation of base substitution detection accuracy, we used a total of 377 variants across the targeted amplicon regions, representing allele frequencies from 5% to 35% with two major MAF categories of 5–15% and 15–35% predefined by AcroMetrix (Fig. 2A, Supplementary Table 1). For the sample mean amplicon coverage (MAC) of 2,252x, we obtained a sensitivity of 96.95% (254/262) for base substitutions at MAF $\leq$15%, and a sensitivity of 84.35% (97/115) at MAF > 15%, where low sensitivity at MAF > 15% could be explained by the smaller amount of true positive. High specificity was obtained with a PPV of 92.03% (254/276) with 22 false positive cases at MAF $\leq$15%, and a PPV of 94.17% (97/103) at MAF > 15%, when excluding genomic alterations (MAF > 35%, in the pool of AcroMetrix) from false positive called variants (Supplementary Table 2). Moreover, MAF measured in samples match the expected frequency (Fig. 2B).

Next, we evaluated the detection sensitivity as a function of varying sample MAC from 10% to 100% of the original coverage at 2,252x, using a strategy of *in silico* down-sampling by selecting subsets of reads at random [8]. The detection sensitivity at MAF > 15% dropped faster than MAF $\leq$15% as coverage decreased. At about 1,126x (50% of 2,252x), we obtained a base substitution sensitivity of 96.18% at MAF $\leq$15%, as were 84.35% at MAF > 15%, and a PPV of 90.97% at MAF $\leq$ 15% and of 94.17% at MAF > 15%, and an overall sensitivity of 97.66% and PPV of 95.67% (Fig. 2C and Supplementary Table 2).

2.3. Proficiency testing

We launched an NGS-based clinical PT for 15 molecular pathology laboratories (11 from Switzerland, two from Germany, and two from Austria). The laboratories were equipped with different NGS platforms (Ion Torrent PGM (n = 11), Ion Torrent Proton (n = 1), and Illumina MiSeq (n = 2) and the analyses were performed using multiple target enrichment systems (CHP2 and CLP2; TruSeq Amplicon-Cancer Panel (TruSeq panel); EGFR 18–21 MASTR (EGFRMM) and SOMATIC 1 MASTR (somaticMM); and custom HaloPlex panel (HaloPlex)). To exclude pre-processing parameters such as fixation time, tumor heterogeneity and DNA isolation methods, all participants received DNA isolates of eight lung and eight colorectal adenocarcinoma FFPE-tissue specimens. For DNA isolate no. 1, four participants were assigned to the CHP2, seven participants to the CLP2, and one participant to the EGFRMM and somaticMM panels. Three other hospitals received DNA isolate no. 2 for the CHP2, the CLP2, and the TruSeq Panel, respectively. For each panel assignment, the individual participant's BAM files were collected and the variants called by IR. The variants in the variant call format (VCF) files of each participant were compared with that of the USZ (Fig. 3). Finally, we applied multi-level filtering to the VCF files. One of the two exceptions were hospital H06 that used a MiSeq to analyze their data (MiSeq Reporter 2.4.60.8), and the other was hospital H10 that was excluded due to technical problems in adapting the HaloPlex enrichment system to the DNA extracted from FFPE samples (no data was received for analysis).

For multi-level filtering strategy, we first selected (Prefiltering) only SNVs, MNVs and InDels in coding exonic regions (Fig. 3). Subsequently, we filtered out (Filtering 1) duplicate entries of the same variant, synonymous variants, non-pathogenic variants according to ClinVar (version 1), and variants with more than 1% minor allele frequency according to dbSNP (version 138). Last, the minimum amount of MAF and mutant allele coverage was defined as 4% and 50 reads,

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