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Validation of CRC and NSCLC somatic mutations detected with NGS using ddPCR

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Abstract

Next Generation Sequencing (NGS) has become powerful tool in molecular oncology. It allows multiparallel targeted sequencing that enables comprehensive assessment of tumor heterogeneity. Detection of mutations in colorectal cancer (CRC) and non-small cell lung cancer (NSCLC) defines patients diagnosis, therapy and prognosis. Multiple genes, their somatic mutations to be precise, carry different degrees of importance for each of these aspects. Ion AmpliSeq™ Colon and Lung Cancer Research Panel v2, which was used in this study, allows detection of hotspot mutations in 22 genes in a single reaction. Droplet digital PCR (ddPCR) has a unique advantage in low frequency mutation detection and it has been used as a validation tool for mutations that were detected with NGS. It has high sensitivity and enables accurate detection of a mutant allele against a background of abundant wild type alleles. For this study 35 samples of CRC and NSCLC were sequenced and selected samples were analysed with ddPCR for KRAS, NRAS, EGFR and BRAF genes. All the processed samples were successfully sequenced and had average base coverage >500X. NGS sequencing proved itself to be cost effective, has shorter turnaround time and is highly sensitive. Out of 35 samples, 25 had genetic alterations, while 10 samples were reported as wild type but were still tested with ddPCR as controls. In three samples low frequency somatic mutations were detected with NGS and mutation frequencies were verified using ddPCR.

Introduction

In the past few years, next generation sequencing (NGS) played an important role in understanding altered molecular pathways in cancer. First of all, it is a massive parallel sequencing process that consists of simultaneous sequencing of multiple targets in the same run. Having said that, it is cost effective, it has reduced turnaround time and it requires low DNA input which makes the method perfect for low copy DNA samples (cfDNA) (Serrati et al., 2016). Sequencing by synthesis is performed in

microscopic wells on semiconductor chip. In this study, Ion AmpliSeq™ Colon and Lung Cancer Research Panel v2 was used to screen CRC and NSCLC cancers. NGS sensitivity is high (2-6%) and it allows quantitative evaluation of the mutated allele (Quail et al., 2012; Ross et al., 2013). Detection of gene alterations in solid tumors define patients diagnosis, treatment plan and prognosis. Droplet digital PCR offers a number of unique advantages, especially when it comes to rare mutation detection and precise quantification of DNA. Compared to real-time PCR, ddPCR is considerably more sensitive and reproducible (Huggett et al., 2015). DdPCR enables accurate detection of a mutant allele in a background of highly abundant wild type allele. Bio-Rad QX200 ddPCR system combines water-oil emulsion droplet technology. Droplet generator partitions samples into maximum of 20 000 droplets and PCR amplification is carried out inside each droplet. Fluorescence is detected in each droplet using QX200 droplet reader. Using a well designed experiment setup, ddPCR can detect 1 mutant in a 10 000 wild type alleles. That kind of sensitivity makes ddPCR perfect for validation of low frequent somatic mutations detected by NGS. Colorectal cancer (CRC) is the second most frequent cancer in Europe and is responsible for 12% of cancer deaths (Ferlay et al., 2013). KRAS is predictive biomarker for the efficiency of anti-EGFR therapy in colorectal cancer (CRC) because KRAS mutant CRC patients are resistant to treatment with EGFR inhibitors. NSCLC accounts for 80-85% cases of lung cancers. Clinical molecular diagnosis of lung cancer consists of identifying druggable alterations (EGFR, RAS, BRAF, MET, ERBB2, ALK etc) (Garinet et al., 2018). New NCCN (The National Comprehensive Cancer Network) Guidelines (Benson et al., 2018) for CRC strongly recommend broader molecular testing and identifying rare driver mutations for which effective treatments already exists. In this study we validated ddPCR as a confirmation method for somatic mutations detected by NGS.

Materials and methods

For this study 35 samples of CRC and NSCLC were obtained from different medical centers from Bosnia and Herzegovina. All of the analyzed samples were FFPE blocks previously reviewed by a pathologist. Pathologist determined type and stage of cancer, area with the highest neoplastic cell content was marked and percentage of neoplastic cells was determined.

Recommendations for molecular genotyping were made based on pathohistological report because of the targeted therapy prospects. Samples were transported with on standard room temperature. The manufacturer's protocols were followed without modification. Genomic DNA was extracted from all clinical tissue samples (FFPE) using QIAamp® DNA FFPE Tissue Kit (Qiagen, 2012) according to the manufacturer's protocol followed by quantification using Qubit 3 Fluorometer® and Qubit® dsDNA HS assay kit (Life Technologies, 2014). NGS libraries were prepared applying Ion AmpliSeq™ Library Kit 2.0 and Ion AmpliSeq™ Colon and Lung Cancer Research Panel v2 primers according to the manufacturer's instructions (Life Technologies, 2019). This is a hotspot panel designed to target clinically important regions of 22 genes covering 504 mutational hotspot regions of: AKT1 (NM_05163), ALK (NM_004304), BRAF (NM_004333), CTNNB1 (NM_001904), DDR2 NM_006182), EGFR (NM_005228), ERBB2 (NM_004448), ERBB4 (NM_005235), FBWX7 (NM_033632), FGFR1 (NM_000604), FGFR2 (NM_000141.2), FGFR3 (NM_000142), KRAS (NM_004985), MAP2K1 (NM_002755), MET (NM_000245), NOTCH1 (NM_017617.2), NRAS (NM_002524), PIK3CA (NM_006218.1), PTEN (NM_000314.4), SMAD4 (NM_005359.3), STK11 (NM_000455) and TP53 (NM_000546). Libraries are quantified with Ion Library TaqMan™ Quantitation Kit (Life Technologies, 2019).

Each library was diluted to 100 pM before final library mixing for chip templating on Ion Chef™ Instrument using Ion 510 & Ion 520 & Ion 530 Chef Kit. NGS sequencing was performed on Ion GeneStudio™ S5 instrument. Raw data was analysed using Torrent Suite Software 5.8.0 where the sequences were aligned to the h19 human reference genome. Expected coverage of 500X for each sample was acquired before further analysis of sequencing data. Mutations were analyzed using Variant Caller plug-in version v5.8.0.21. Parameter settings that were used are Generic-S5/S5XL

(510/520/530)- Somatic- Low Stringency. All detected variants were manually reviewed using ClinVar as a referent database. All 35 samples were also tested with ddPCR for specific KRAS, BRAF, NRAS and EGFR gene mutations. ddPCR™ KRAS G12/G13, NRAS Q61, BRAF V600, EGFR T790M, EGFR L861Q, EGFR L858R and EGFR del19 Screening Kits were used for detection of mutations. All of wild type controls were analyzed with ddPCR for each mutation. The multiplex assay was 20X concentrated and ddPCR supermix for probes was 2X concentrated. Samples were placed into a QX200 droplet generator which uses mirofluidics to portion each sample into 20 000 droplets. Formed droplets are then transferred to a 96-well plate and placed into Bio-Rad T100 thermal cycler. Following DNA amplification, the plate containing the droplets was placed in QX200 droplet reader which analyzes each droplet individually using two color system (FAM and HEX/VIC) (Bio-Rad, 2017). Chi-squared test was used to determine correlation between detected mutation frequencies acquired by NGS and ddPCR. For expected values, we used mutation frequencies detected by NGS and as observed values, we used mutation frequencies aquired by ddPCR. For chi-squared test 25 mutations were analyzed.

Results and Discussion

Out of 35 analysed samples 18 were from patients diagnosed with CRC and 17 with NSCLC. All 35 samples were successfully sequenced and had average base coverage of more than 500X. Number of samples which have one of analyzed mutations was 25, while 10 tested samples were wild type. Wild type samples were further tested with ddPCR to verify precision of NGS somatic mutation detection. Regarding sequencing performance, average number of reads for 35 samples was 490580. In CRC samples, none of the EGFR mutations were detected by NGS or ddPCR. BRAF V600 mutation was detected in 3/13 patients (23,07%), KRAS G12/13 mutation was detected in 8/13 patients (61,53%) and NRAS Q61 mutation was detected in 2/13 patients (15,38%). NRAS G12/13 mutation was not detected in the CRC patients. The similar result was reported for Belgian CRC patients where the highest mutation frequency was for KRAS mutation (46%) and the lowest (0.3%) for EGFR mutations (D'Haene et al., 2018).In NSCLC, none of the NRAS mutations were detected by NGS or ddPCR. EGFR L861Q mutation was detected in 1/12 NSCLC patient (8,33%), EGFR L858R mutation was detected in 2/12 patients (16,66%), EGFR T790M mutation was detected in 3/12 patients (25%), EGFR del19 mutation was detected in 2/12 (8,33%), BRAF V600 mutation was detected in 2/12 patients (16,66%) and KRAS G12/13 was detected in 4/12 patients (33,33%) (Table 1.). The prevalence of *EGFR* mutations in NSCLC patients ranges from 40% in Asian patients to 11–17% in Caucasian patients (Garinet et al., 2018).

Table 1. Percentage of specific mutations found in CRC and NSCLC

Mutation	CRC (%)	NSCLC (%)	Total (%)
EGFR L861Q	0	8,33	4
EGFR L858R	0	16,66	8
EGFR T790M	0	25	12
EGFR del19	0	16,66	8
BRAF V600	23,07	16,66	20
KRAS, G12/13	61,53	33,33	48
NRAS, G12/13	0	0	0
NRAS Q61	15,38	0	8

In our study, the most frequent mutation in both CRC and NSCLC was KRAS G12/G13 mutation. In three samples low frequency somatic mutations were detected by NGS and verified using ddPCR. In sample number 3, KRAS Gly13Asp mutation was detected by NGS with frequency of 3,5%; sample 3 was also tested with ddPCR and it showed the same mutation with frequency of 2,6% (Figure 1. and Figure 2.). Sample number 15 showed EGFR L861Q mutation with frequency of 4,3% on NGS and with ddPCR the same mutation was detected with the frequency of 4,1%. Sample number 21 had KRAS Gly12Val mutation with frequency of 3,9% with NGS and with ddPCR the same mutation was detected with the frequency of 3,1%. Our study is in correlation with other studies that suggest that the most frequent mutations in CRC are in KRAS gene (Dinu et al., 2014; D'Haene et al., 2018).

Value of $\chi 2$ for 27 observed mutations was 30,474 which is lower than critical value of 38,885. p-value was lower than 5% which proves that the results are

statistically significant and there is no difference between observed NGS and expected ddPCR frequencies. A major advantage of NGS is its ability to detect multiple mutations on multiple targets simultaneously. NGS sequencing has the advantage over other conventional methods in terms of cost, sensitivity and turnaround time which is of high importance for oncological patients. Earlier reports (D'Haene et al., 2015) validated NGS for surgical resection, biopsies and cell blocks on 90 CRC and NSCLC patients using Colon and Lung Cancer panel. For our laboratory, NGS is found to be cheaper and faster when multiple alterations must be screened. The number of genetic alterations associated with targeted treatment is increasing, so genetic profiling that includes more than few genes is imperative (Tsoulos et al., 2017). In the present study, targeted NGS panel was used for simultaneous analysis of alteration in 22 genes that are commonly mutated in CRC and NSCLC. This panel is cost effective, has good sequencing performance, high sensitivity and is compatible with low amounts of DNA which are expected from FFPE samples.

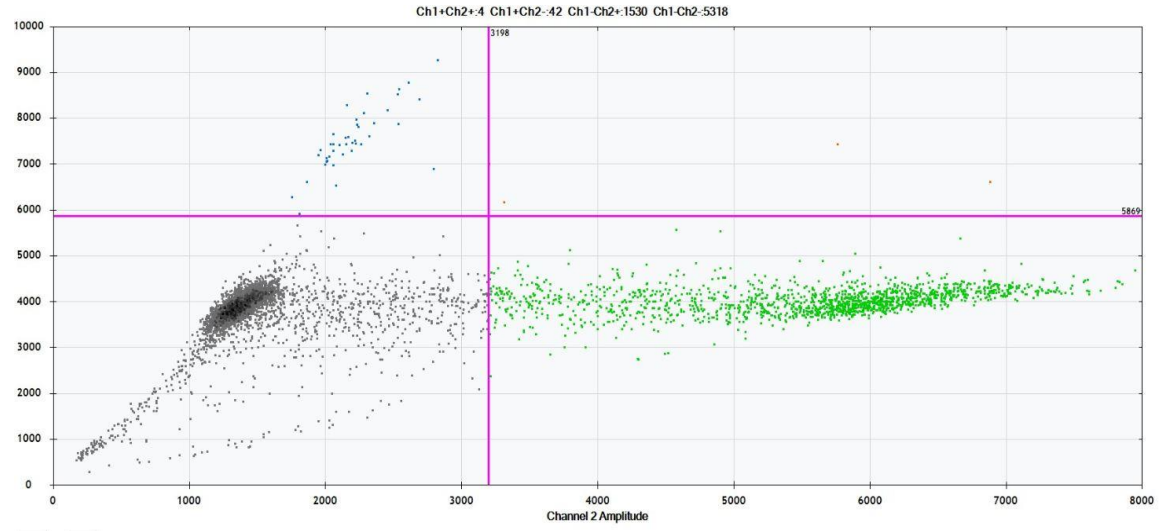

Figure 1. Generated droplets for KRAS G13 mutation in sample 3

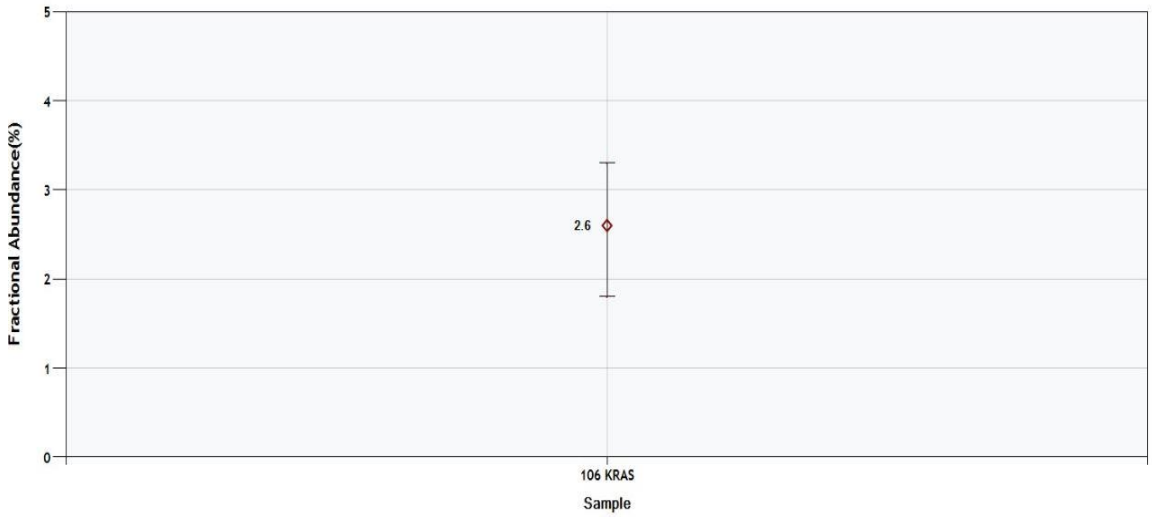

Figure 2. KRAS G13 mutation with the frequency of 2,6% detected by ddPCR in sample 3

Conclusions

Targeted sequencing by NGS is a method of choice for CRC and NSCLC molecular profiling due to the capability of providing necessary informations which guides physicians to an informative decision regarding targeted therapy. Ion AmpliSeq™ Colon and Lung Cancer Research Panel v2, used on a Ion S5 platform, proved itself as highly informative and sensitive, and henceforth valuable in clinical practice. This study showed that the most frequent mutation in CRC and NSCLC is KRAS G12/G13 mutation. The study showed that there is no difference between observed genotypes obtained with NGS or ddPCR (Figure 3.). ddPCR can be used as a validation tool for mutations detected by NGS.

Conflict of interest

The authors have declared no conflicts of interest.

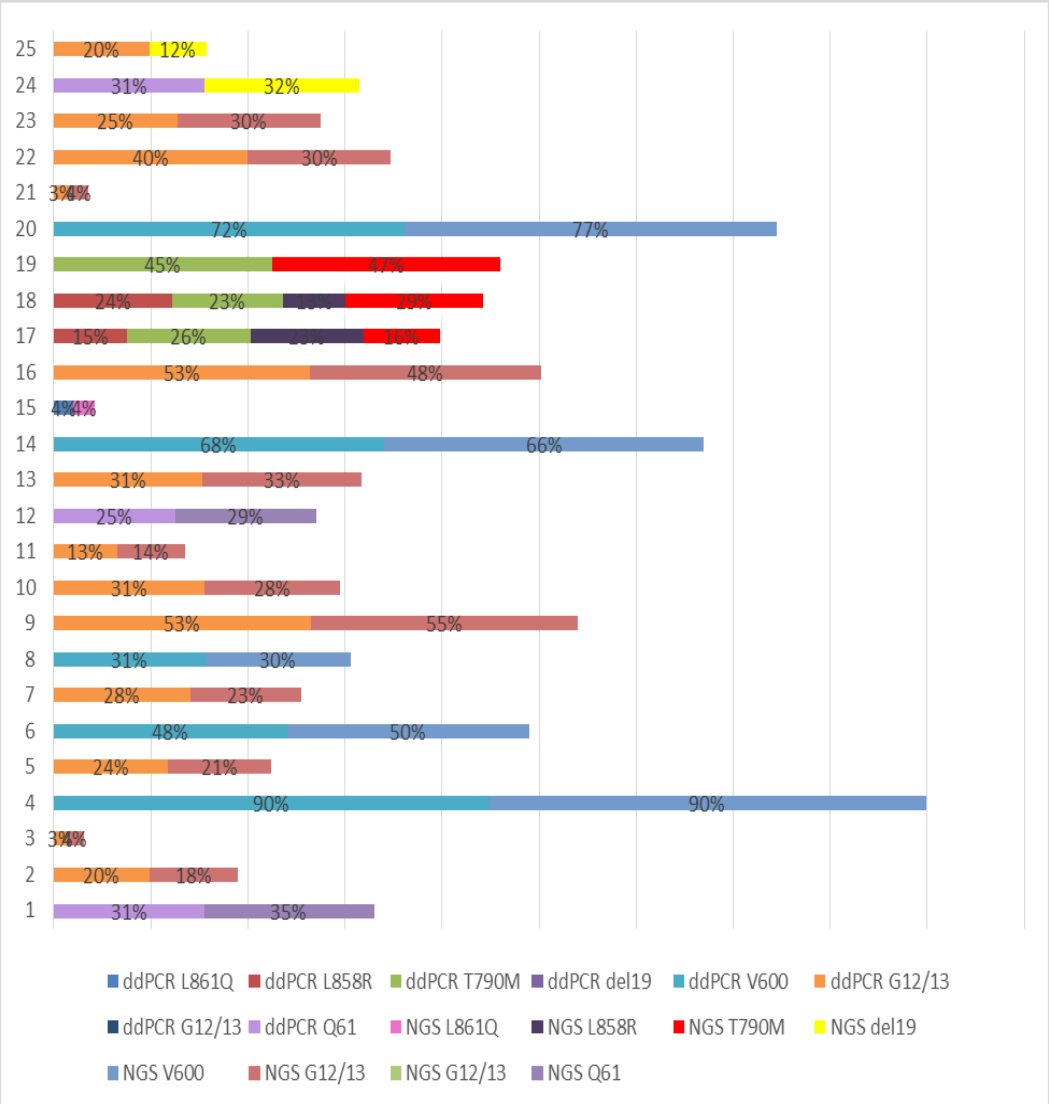

Figure 3. Frequency of mutations detected using ddPCR and NGS

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