Comparison Test of Sensitivity Between Next Generation Sequencing (NGS) Hotspot Panel and Droplet Digital PCR of KRAS G12 / G13 Mutation

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Orginal research

Abstract: Cancer is an abnormal proliferation of cells that is characterized by the presence of genomic alterations including DNA mutations, deletions, insertions, translocations, inversions, and others. *KRAS* gene is one of the most mutated genes across different cancer types. The most common mutations in *KRAS* are found in codons 12 and 13 of KRAS protein, which are associated with a lack of response to anti- epidermal growth factor receptor (EGFR) antibody therapy. This study assessed and compared the performance between two diagnostic methods: droplet digital PCR (ddPCR) and next generation sequencing (NGS). The main goal was to determine *KRAS* G12 / G13 mutant allele fraction using NGS and to compare the accuracy toddPCR. A total of 28 samples of non – small cell lung cancer (NSCLC) and colorectal cancer (CRC) were analyzed using ddPCR and NGS methods. Our results show that even though both methods exhibited high rate of concordance and correlation, the study proved that ddPCR is more superior when it comes to detecting low frequency mutations. Even though strong correlation was observed, based on the values obtained, we concluded that ddPCR is more accurate, reliable, and sensitive in comparison with NGS.

Keywords: ddPCR, KRAS mutation, NGS.

1. Introduction

It has been determined that *RAS* mutations are the most prevalent oncogenic alteration in human cancers. *KRAS*, followed by *NRAS,* is the member of *RAS* family that is most often mutated. Cancers of urogenital system, colorectal system, lung and pancreas all exhibit the *KRAS* mutation. The enormous heterogeneity that primary tumors with the *KRAS* mutation express has a significant effect on the frequency of the variant allele. This could lead to the metastases' erratic branching progression [1]. *KRAS*gene is recognized as the most frequently mutated oncogene in humanswith the majority of these mutations affecting codons 12 and 13 [2].

KRAS is a significant predictor of anti-epidermal growth factor receptor therapy response, and determining *KRAS* mutational status is critical for successful adenocarcinoma monitoring and treatment. Due to the advances made in field of regenerative medicine, personalized and target therapy it is of vital essence to accurately detect the presence of *KRAS* mutation as precise as possible [3].
The detection of *KRAS* mutation is mostly performed on archived surgical tumor tissue samples or tumor biopsy samples using traditional point mutation detection methods (quantitative PCR and amplification refractory mutation system) or sequencing technique (Sanger sequencing) [4 – 6].
In the past few years however, an increasing number of techniques and detection kits are being developed to detect *KRAS* mutations [7].

The water – in – oil emulsion of the PCR reaction mixture is achieved using droplet digital PCR (ddPCR) technology. This makes it possible to massively sub – partition the reaction into hundreds to millions of separate reactions, creating a synthetic enrichment effect that dramaticallyimproves the ability to detect uncommon mutations present in the sample at very low levels[8].
NGS, on the other hand, offers a low-cost, high-sensitivity, high-throughput platform for investigating genetic abnormalities in the clinical setting [9]. Given the inefficiency of traditional methods in terms of sample, cost, and time, the NGS platform has significant clinical potential for performing such multiplex genetic testing in FFPE CRC specimens [10].

In this study, we wanted to compare the *KRAS*G12/G13 mutation status using tissue and plasma samples of NSCLC and CRC in order to compare the efficiency of ddPCR with that produced by next – generation sequencing.

2. Materials and Methods

Patient samples

A total of 28 samples were included in this study, where 10 samples were from patients with colorectal cancer, 15 samples were from patients with non-small cell lung cancer, and 3 samples were plasma samples from patients with NSCLC. All samples and diagnostic procedures were carried out at Alea Genetic Center, Sarajevo, Bosnia and Herzegovina. The Ethics Committee decision was obtained from International Burch University, Number 04 – 117/21. The study was carried out according to the Declaration of Helsinki.

KRAS mutation detection

DNA isolation from FFPE and plasma samples was carried out using standardized protocols given by the manufacturers. Briefly, deparaffinization of the FFPE samples was done in xylol for 30 minutes, followed by alcohol washing and drying.QIAamp DNA FFPE Tissue Kit was subsequently used for DNA isolation [11]. For the isolation of DNA from plasma samples, QIAamp Circulating Nucleic Acid Kit [11] was used on 1.5 – 2 ml of plasma. After the isolation, the quantification of the isolates was done using QubitFluorometric Quantification under the category of high double – stranded DNA [12]. All samples were stored at -20°C until the further analysis.

Next – Generation Sequencing

All samples were selected based on the presence of *KRAS* mutations in codons 12 and 13, with variant allele frequency ranging 0.6% up to 50.8%. *KRAS* mutational status was determined using Colon/Lung hotspot panel on Ion GeneStudio™S5 instrument. The primary source was analyzed by Torrent Suite Software 5.8.0. The sequences were paired in accordance to the h19 human reference genome. The variants detected were analyzed using ClinVar platform as a referent database. 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 guidelines [13]. This is a hotspot panel intended to identify statistically significant regions of genes, including *KRAS* mutational hotspot regions [13].

Droplet Digital PCR

KRAS tissue genotype analysis was performed using ddPCR *KRAS* G12 / G13 Screening Kit assay which covers codon 12 and 13.

The reaction mixture required for ddPCR contained the ddPCR*KRAS* G12 / G13 screening kit assay which included: Bio – Rad ddPCR™Supermix for Probes and Bio – Rad ddPCR™*KRAS* G12 / G13 Screening Assay. The final volume of 20 µL consisted of extracted DNA, SuperMix for probes (10 µL), *KRAS* screening assay (1 µL) and distilled water which was added in accordance with the amount of DNA sample included in the reaction. The droplet generator partitioned each sample in the well into nearly 20, 000 nanoliter – sized droplets, which were further amplified in a conventional 96 – well PCR plate.

The reaction conditions were set as following: enzyme activation (95°C for 10 minutes and 1 cycle), denaturation (94°C for 30 seconds and 40 cycles), annealing / extension (55°C for 1 minute and also 40 cycles), enzyme deactivation (98°C for 10 minutes and 1 cycle) and hold as an optional step at 4°C. After that, the droplets were transferred to the Bio – Rad QX200™ Droplet Reader.

The QuantaSoft Software was used to analyze the generated droplets using a two – color detection system FAM and HEX / VIC [14]. The software is designed to classify the droplets into three categories: wild type, mutants and empty droplets which, based on the level of fluorescence threshold, exhibit no target.

Statistical Analysis

The two-sample t-test was used to compare mean mutation frequency values between NGS and ddPCR obtained results.

3. Results

Clinical characteristics

The clinical features of patients diagnosed with colorectal cancer and non – small cell lung cancer, the type of *KRAS* mutation, and mutant allele fraction as determined by NGS and ddPCR platforms are shown in Table 1. Briefly, the study included 16 (57 %) male and 12 (43 %) female patients. The patients' average age was 66 (67 for male patients and 66 for female patients).

Table1. Clinical features of patients diagnosed with cancer and *KRAS* sensitivity results.

Sample Number	Age	Gender	Sample Type	Analysis Type	*KRAS* Mutation	NGS Frequency	ddPCR Frequency
1	1949	F	FFPE tissue	NSCLC	GLY12ALA	30%	42%
2	1950	M	FFPE tissue	NSCLC	GLY12ALA	3.2 %	0.72 %
3	1962	M	FFPE tissue	NSCLC	GLY12CYS	13 %	21.3 %
4	1950	F	FFPE tissue	NSCLC	GLY12CYS	10 %	10.6 %
5	1951	F	FFPE tissue	NSCLC	GLY12ASP	37 %	60.4 %
6	1958	F	FFPE tissue	NSCLC	GLY12VAL	4.1 %	3.3 %
7	1967	F	FFPE tissue	NSCLC	GLY12VAL	19.1 %	22.1 %
8	1983	M	FFPE tissue	NSCLC	GLY12ASP	37.2 %	42.4 %
9	1951	M	FFPE tissue	CRC	GLY12ALA	14.7 %	28 %
10	1959	M	FFPE tissue	NSCLC	GLY12CYS	29.5 %	66 %
11	1963	F	FFPE tissue	NSCLC	GLY12CYS	29.5 %	34 %
12	1956	F	FFPE tissue	CRC	GLY13ASP	33.6 %	44 %
13	1954	M	FFPE tissue	CRC	GLY12VAL	0.60 %	0.62 %
14	1945	M	FFPE tissue	NSCLC	/	0%	0.41 %
15	1944	F	FFPE tissue	CRC	/	23 %	25 %
16	1949	M	FFPE tissue	CRC	/	25 %	32.1 %
17	1958	M	FFPE tissue	CRC	/	20 %	23.2 %
18	1958	F	FFPE tissue	NSCLC	GLY12ALA	10.2 %	0 %
19	1952	F	FFPE tissue	CRC	GLY12VAL	36 %	38 %
20	1950	M	FFPE tissue	CRC	GLY12ASP	18 %	21.5 %

21	1952	M	FFPE tissue	NSCLC	GLY12VAL	24 %	28 %
22	1953	M	FFPE tissue	NSCLC	GLY12ASP	50.8 %	51 %
23	1952	F	FFPE tissue	NSCLC	GLY12ASP	21.6 %	13 %
24	1959	M	FFPE tissue	CRC	GLY12CYS	15 %	16 %
25	1953	M	FFPE tissue	CRC	GLN61HIS	8.1 %	/
26	1975	F	Plasma	NSCLC	GLY12CYS	4.1 %	3.2 %
27	1950	M	Plasma	NSCLC	GLY12ASP	9.4 %	10 %
28	1953	M	Plasma	NSCLC	/	ND	0.6 %

Mutational status

Out of a total of 28 samples, one mutation was detected in codon 13, Gly13Asp, six patients were characterized with Gly12Cys mutation, six patients exhibited mutation Gly12Asp, five of them had Gly12Val mutation and four of them had presence of Gly12Ala mutation. Based on the numerical values provided in the table, ddPCR exhibited higher sensitivity rate in majority of cases, which can also be observed in a Fig. 1 shown below.

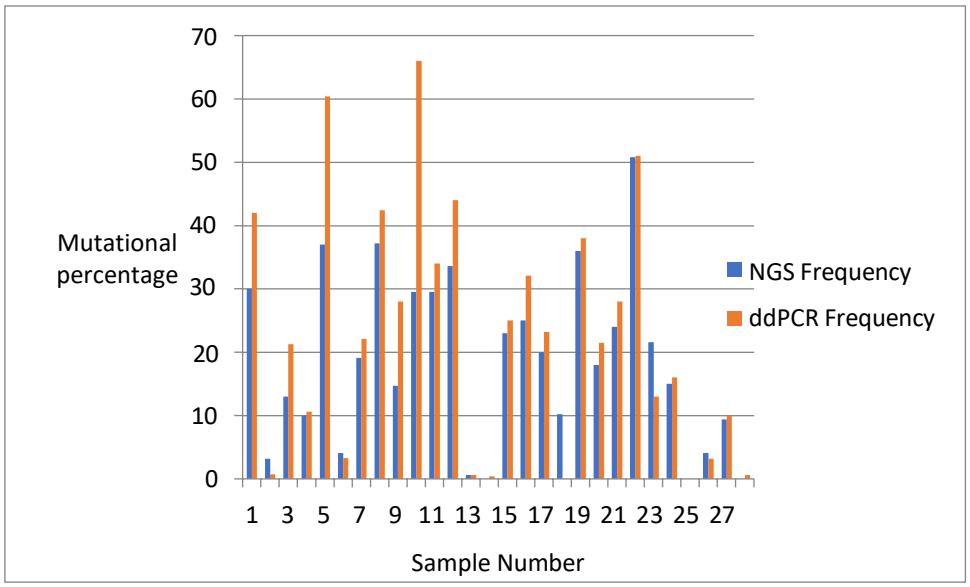

Figure 1. Mutation frequencies detected by ddPCR and NGS.

Statistical analysis

Using χ2 test for the observed mutations, we obtained the statistically significant results, with p – value being less than 0.05% . The chi square values obtained were 6.11 in case of CRC samples and 17.51 in case of NSCLC samples. Mean mutation frequencies were 20.74 and 23.61 for NGS and ddPCR results, respectively. Although ddPCR appears to be more sensitive when compared to NGS, the obtained p value was 0.53, which means that this difference in mean values was not statistically significant. The mean mutation frequencies between the two methods were also compared with respect to mutation type, cancer type, sample type as well as sex, but all the obtained p values were above the critical 0.05 level.

4. Discussion

In sample number 14, the patient tested negative for *KRAS* mutation by NGS and positive by ddPCR (0.41%). Taking into consideration that droplet digital PCR also detected low frequency mutation, NGS could not trace the mutation because the sensitivity level it usually detects is set at 1%. For sample number 18, NGS detected the mutation at 10.2% but ddPCR did not. Sample number 25 exhibited Q61 mutation, which could not be detected by ddPCR as expected since it is designed to trace exclusively *KRAS* G12/G13 mutations. Patient under sample numbered by 28 is the one who has already gone through the treatment process after being diagnosed with NSCLC, so the droplet digital PCR in this case was done with the goal of monitoring the eventual progress of the disease.

The comparison of different molecular methods used in detection of *KRAS* G12 / G13 mutational status has shown that ddPCR is more sensitive NGS. Besides droplet digital PCR and next generation sequencing represented in this work, the methods used in analysis process might also include Sanger sequencing, peptide nucleic acid – clamping (PNA clamping assay), and quantitative PCR.

In a study published by Kyung Ha Lee and colleagues, the identification of *KRAS* G12 / G13 mutational status in CRC patients was accomplished using ddPCR and compared to the results of Sanger sequencing and the PNA clamping assay.Compared to Sanger sequencing method and PNA-clamping assays, their study found that ddPCR retained high sensitivity and specificity (the percentage was 100% in both cases).When compared to NGS panel sequencing, ddPCR and Sanger sequencing also showed higher sensitivity expressing values of 96.43 % and 100 % respectively. When it comes to specificity, the obtained results were

98.11 % in case of ddPCR and 92.45 % in case of Sanger sequencing. NGS panel sequencing, on the other hand, allowed for scanning of numerous genes, including KRAS G12/G13 status, but demonstrated low sensitivity level and greater computation value [3]. Thus, ddPCR, Sanger sequencing and PNA-clamping assay showed comparable outcomes for *KRAS* G12 / G13 mutations. The quantity of DNA required for ddPCR was 1 L, which was significantly less than the amount required for Sanger sequencing (20 ng) and the PNA-clamping assay (7 l). This practicalbenefit is useful when detecting *KRAS* G12 / G13 mutations in both, biopsied tissues and liquid biopsy samples.Moreover, the *KRAS* G12 / G13 multiplex kit was unable to differentiate the mutation codon site and did not cover the entire spectrum of KRAS mutation sites. The ddPCR platform is equipped with two fluorescence filters and can perform at least duplex reactions [3].

Janku F. and others presentedcomparable results. They demonstrated that the *KRAS* G12 / G13 mutations from patients with advanced malignancies can be detected using ddPCR on a small amount of plasma cfDNA that has not been amplified, with satisfactory concordance of 85 %, sensitivity of 84 % and specificity of 88 % [15].

Advantages of NGS include detection of different types of mutations as compared to ddPCR which cannot, as shown in the example of Patient 25. On the other hand, ddPCR is good for detection of a specific mutation at a low frequency. In sample 18, we could not detect the mutation by ddPCR. Our results show that NGS and ddPCR are equivalent methods for the detection of the percentage of *KRAS* mutations.

5. Conclusion

Even though CRC and NSCLC remain as a primary reason of death among all cancer types, utilization of novel therapies based on EGFR inhibition, showed significant results and increased the survival rate among the patients.

In this study we investigated the sensitivity of two sophisticated diagnostic methods: NGS and ddPCR. Even though both methods can provide meaningful and robust diagnostic information, when comparing the methods, we found that ddPCR is more sensitive and superior then NGS, primarily due to its capability of tracing the *KRAS* mutation with higher specificity and accuracy.

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