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ORIGINAL ARTICLE

Droplet digital PCR revealed high concordance between primary tumors and lymph node metastases in multiplex screening of *KRAS* mutations in colorectal cancer

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Abstract

The proto-oncogene *KRAS* belongs among the most frequently mutated genes in all types of cancer and is also very important oncogene related to colorectal tumors. The detection of mutations in this gene in primary tumor is a predictive biomarker for the anti-EGFR therapy in metastatic CRC (mCRC); however, the patients with wild-type *KRAS* can also show resistance to the personalized medicine. The droplet-based digital PCR technology has improved the analytical sensitivity of the mutations detection, which led us to the idea about the optimization of this approach for *KRAS* testing. In this study, we report the application of ddPCR technology in order to analyze the presence of *KRAS* mutations in primary tumor and matched metastasis in lymph nodes (LNs) from patients with mCRC and address the question, whether the improvement in the detection method can lower the discrepancies of *KRAS* mutations detection between the primary tumor and regional LNs. Genomic DNA with wt*KRAS* and commercial DNA with mt*KRAS* (G12D) were used to set up the ddPCR reaction. Formalin-fixed paraffin-embedded tissues from primary tumor and positive lymph node from 31 patients with mCRC were analyzed using ddPCR and Sanger sequencing. *KRAS* status of primary tumors was known; however, the mutation status of lymph nodes was not detected previously. From 31 samples of primary tumors, our results corresponded to results from IVD kit in 30 cases. For one patient, ddPCR detected *KRAS* mutation in comparison with negative result of the IVD kit. In the samples of metastatic infiltrated LNs, ddPCR detected 16 samples as a WT *KRAS* and 15 lymph nodes showed positivity for *KRAS* mutation, whereby Sanger sequencing found *KRAS* mutations in 8 cases only. We also found two cases where genetic conditions of *KRAS* gene differed between primary tumor and infiltrated lymph node, both "low-grade" adenocarcinoma. Our study approved that ddPCR method is adequate technique with high sensitivity and in the future may be used as a diagnostic tool for evaluation of *KRAS* mutations, especially in infiltrated LNs of patients with mCRC.

Keywords Colorectal cancer · *KRAS* mutation testing · Droplet digital PCR · Lymph nodes

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Introduction

Colorectal carcinoma (CRC) represents the third most common cancer in men and the second in women population worldwide. CRC evolves in steps of successive accumulation of mutations in tumor suppressor genes and oncogenes. The mutations in *KRAS* gene and activation of the RAS/RAF/MAPK pathway belong to the early events in colorectal carcinogenesis [1], and together with mutations in the other oncogenes such as *BRAF*, *PIK3CA* and/or inactivation of *PTEN* tumor suppressor, play a role in the prediction of the EGFR-targeted therapies [2–4]. The diagnosis is based on clinical examinations using mainly colonoscopy with subsequent histopathological assessment. TNM staging recommended by ACJJ/UICC is the most common system for classification and prognostication of CRC and involves the extent of primary tumor, nodal status and assessment of distant metastases [5, 6]. However, approximately 25% of patients in stage I/II have experienced rapid tumor progression and vice versa some advanced-stage patients are long-term stable. In clinical trials, a new prognostic factor representing the infiltration of the tumor with immune cells has been identified [7–9]. Despite these new developments in staging systems, the assessment of lymph node infiltration remains very important parameter in the patient prognostication, and according to current guidelines, at least 12 LNs have to be assessed in order to accurate identification of stage II in CRC [10].

The detection of *KRAS* mutations in primary tumor is a predictive factor for the EGFR-targeted therapy in metastatic CRC (mCRC); however, the patients with wild-type *KRAS* can show resistance to the personalized treatment, which can be due to the presence of alterations in the other EGFR signaling pathways mentioned above. The other reason of the non-responding cases can be the discordance between the *KRAS* mutations status of the primary tumor and matched metastases as the consequence of the tumor heterogeneity or the presence of mutated subclones selected during the therapy, which were not detected by the applied method [11, 12]. The meta-analysis that has been focused on the primary tumors and the matched metastases reported pooled concordance rate as 92% for *KRAS*, whereby mostly distant metastases were analyzed and different methods were compared [13]. Moreover, Baldus et al. [14] found that a discordance rate was 31%, when the primary tumor and lymph node metastasis were compared, an observation not described in distant metastases. Nowadays, commercially available certified kits with high analytical sensitivity based on allele-specific real-time PCR or high-resolution melting curve analysis are used for the *KRAS* mutations detection in clinical settings [15, 16]. Eventually, the improvement in diagnostic methods supports the findings that there are minimal differences between the primary tumor and distant metastases [17]. The Sanger dideoxysequencing is often used for confirmation of uncertain results and remains the easiest accessible validation method despite the limited sensitivity [18, 19]. The droplet-based digital PCR technology, that has improved the analytical sensitivity of the mutations detection, is based on amplification reactions, which take place in ten thousands up to millions of microscopic droplets on a nanoliter scale, and PCR amplification of the template molecules occurs in each individual droplet [20, 21]. Using very sensitive picodroplet digital PCR, Laurent-Puig et al. [22] showed that patients with mCRC and less than 1% of *KRAS*-mutated fraction may benefit from the anti-EGFR therapy.

Here, we report the original application of droplet digital PCR in order to analyze the presence of *KRAS* mutations in primary tumors of patients with CRC and corresponding infiltrated LNs. Furthermore, we would like to address the question, whether the improvement in the detection method can lower the discrepancies of *KRAS* mutations detection between the primary tumor and regional LNs and can help to find tumors showing intratumoral or intertumoral heterogeneity manifested by this discrepancy (Table 1).

Table 1 The histopathological characteristics of patients' cohort

Gender/mean age	
Female	14/66.4
Male	17/65.8
Histological typing	
Adenocarcinoma	25
"Signet ring cell" carcinoma	3
Mucinous adenocarcinoma	3
Histological grading	
G1	9
G2	15
G3	6
G4	1
Sideness	
Right colon	22
Left colon	7
Rectum	2
Lymph nodes stage	
N1a	4
N1b	10
N2a	8
N2b	8

Materials and methods

Patients and tumor characteristics

Formalin-fixed paraffin-embedded (FFPE) tissue sections from primary tumor and matched LN metastases were obtained from 31 patients with mCRC. This study was approved by the Ethics Review Board of the Jessenius Faculty of Medicine. The histopathological diagnosis was conducted by the experienced pathologists. Histological typing, grading, localization and staging of tumors were determined using the recommendation according to WHO and IUCC [6]. The main criterion of patients included in the study was the presence of at least one metastatic infiltrated LN. The LNs were identified using Carnoy's solution, and its positive histological status was assessed via hematoxylin and eosin (HE) staining. For FFPE samples of primary tumors, the mutation status of *KRAS* gene was identified with CE-IVD kit on COBAS 4800 [16]. The mutation status of metastatic infiltrated LNs was not known.

DNA isolation

Genomic DNA from FFPE tissue was isolated with the commercial kit blackPREP FFPE DNA Kit (Analytik Jena, Germany). Briefly, FFPE sections were lysed and shacked 1 h at 65 °C and 1 h at 90 °C, respectively. DNA was bound to the spin filter, washed and eluted to 40 µl of elution solution. The concentration of DNA was measured by Qubit Fluorometer 2.0 (Invitrogene, California, USA) with Qubit DNA BR Assay Kit (Life Technologies, California, USA).

Droplet digital PCR for KRAS mutations screening

ddPCR™ KRAS Screening Multiplex Kit (Bio-Rad Laboratories, Hercules, California, USA) for 7 *KRAS* mutations (G12A, G12C, G12D, G12 V, G12R, G12S, G13D), which contains 20× concentrated assay mix and two tubes of 2x concentrated ddPCR supermix for probes, was used. The total volume of 20 µl ddPCR Master mix was prepared with 10 µl of 2× supermix for probes, 2 µl of assay mix, ultrapure water and 100 ng of DNA in variable volume. Each PCR reaction mix was loaded into the sample well of DG8™ cartridge (Bio-Rad Laboratories, Hercules, California, USA) and 70 µl of Droplet Generation Oil for Probes (Bio-Rad Laboratories, Hercules, California, USA) was added into each oil well of cartridge. The full cartridge was placed into QX200™ Droplet Generator (Bio-Rad Laboratories, Hercules, California, USA), which separated PCR mix into ~20,000 droplets in water-in-oil emulsion. Emulsified PCR mixes were then transferred to the 96-well plate by multichannel pipette, the plate was heat sealed with foil in PX1 Plate Sealer (Bio-Rad Laboratories, Hercules, California, USA) and placed into the T100™ Thermal Cycler (Bio-Rad Laboratories, Hercules, California, USA). The thermal cycling conditions of PCR reactions with ramp set up as ~2 °C/s were 10 min at 95 °C and 40 cycles with 30 s denaturation at 94 °C and 1 min annealing extension at 55 °C. The enzyme inactivation was run 10 min at 98 °C. The samples were held at 4 °C with cooling ramp set up as ~1 °C/s.

After amplification, the 96-well plate was loaded to the QX200™ Droplet Reader (Bio-Rad Laboratories, Hercules, California, USA) and droplets from each well were analyzed using QuantaSoft software (Bio-Rad Laboratories, Hercules, California, USA). Four main populations of droplets were shown by the analysis of emission in HEX or FAM wavelenghts—empty droplets without DNA, droplets containing only wild-type DNA with high HEX amplitude, droplets containing only mutant DNA with high FAM amplitude and droplets with both type of DNA with high HEX and also FAM amplitude (Fig. 1).

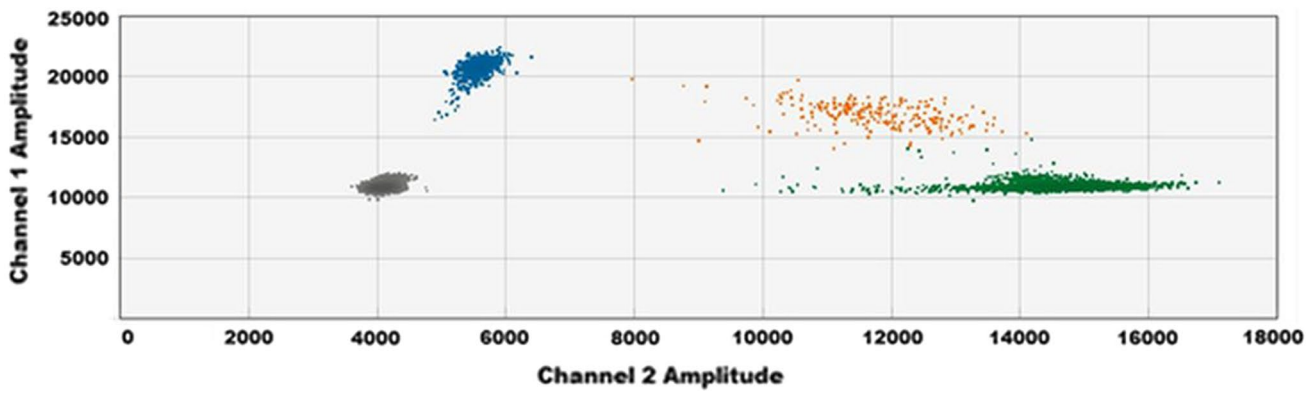

Fig. 1 The output data from ddPCR in the form of 2D histogram

Data analysis

Data obtained from QX200™ Droplet Reader were processed and analyzed by commercial QuantaSoft v.1.7 Software (Bio-Rad Laboratories, Hercules, California, USA). The threshold was set up using normal genomic DNA with wt*KRAS* and the commercial DNA from LS174T with mutated *KRAS* (G12D) and the cutoff for fractional abundance (FA) was calculated. The samples with FA ≥ 0.6 were assessed as positive, and samples with FA < 0.6 were assessed as negative.

Sanger sequencing

The DNA isolated from the FFPE samples of infiltrated LNs were analyzed also by Sanger sequencing method. Primer pair 5′-GGTACTGGTGGAGTATTTGATAGTG-3′ and 3′-CAAGATTTACCTCTATTGTTGGATCA-5′ designed by us were used for the examination of *KRAS* mutations within exon 2. The PCR conditions were: 10 min. denaturation at 95 °C followed by 35 cycles of 30 s denaturation at 95 °C, 30 s annealing at 64 °C and 30 s extension at 72 °C. The last step was 10 min final extension at 72 °C. The products of reaction were purified by NucleoSpin Gel and PCR Clean-up (Machery-Nagel GmbH & Co. KG, Germany) and sequenced with BigDye Terminator version 1.1 cycle sequencing kit (Applied Biosystems, California, USA) according to the manufacturer's protocol. The analysis was performed by ABI 3500 sequencer (Applied Biosystems, California, USA), and results were resolved by Chromas DNA Sequencing Software (Technelysium Pty Ltd., Australia).

Results

Detection of KRAS mutations by ddPCR™ KRAS screening multiplex kit

Using ddPCR™ KRAS Screening Multiplex Kit, we were able to detect seven most frequent *KRAS* mutations in exon 2 (codon 12 and 13). We first determined thresholds for our assay. We used commercial DNA isolated from the cell line LS174T bearing a heterozygous *KRAS* mutation G12D as a positive control and DNA isolated from blood of healthy patient as a negative control. After we set up threshold as a 8800 for channel 1 and 5000 for channel 2, respectively, we applied these values to our samples and according to that, fractional abundance was calculated for each sample of primary tumor and metastatic infiltrated lymph node using QuantaSoft software.

According to our data, we assessed samples with FA ≥ 0.6 as a mutant positive and samples with FA < 0.6 as a mutant negative. From 31 samples of primary tumors, ddPCR detected mt*KRAS* in 17 cases and 14 samples were diagnosed with WT *KRAS*. Our results corresponded to results from IVD kit, used for diagnosis of primary tumors, in 30 cases. For one patient, ddPCR detected *KRAS* mutation, whereas IVD kit assessed this sample as a WT *KRAS* (Table 2 left). When we compared the primary tumor samples with corresponding metastatic infiltrated LN, ddPCR detected 16 samples as a WT *KRAS* and 15 LNs showed positivity for *KRAS* mutation. When we compared results obtained with the IVD kit in the primary tumor and ddPCR in matched LNs, one patient showed discordant mutation status and was positive in primary tumor and negative in LNs. When we compared the results obtained with ddPCR (primary tumor and LNs), two patients have distinct genotypes regarding the *KRAS* mutations status and were mutated in primary tumor and negative in LNs (Table 2). We analyzed mutation status of 31 metastatic infiltrated lymph nodes also by Sanger sequencing and found *KRAS* mutations in 7 cases only (Table 2).

Identification of intra- and intertumoral heterogeneity

We found two cases where genetic conditions of *KRAS* gene differed between primary tumor and infiltrated LN. In one patient, primary tumor showed mutation in exon 2 of *KRAS*, while histologically positive lymph node was diagnosed as WT for *KRAS* mutation. This patient was diagnosed with "low-grade" adenocarcinoma in *colon ascendens* and classified as a pT4apN1b by TNM system.

The second case was a patient with two tumors, one "high-grade" in *colon sigmoideum* that was positive by COBAS assay in primary tumor and LN metastasis as well (data not shown) and TNM was pT2pN2a, the second primary tumor localized in rectum was tested positive by ddPCR and negative by COBAS assay and diagnosed as "low-grade" adenocarcinoma pT3bpN2a. LN of this tumor was confirmed as WT *KRAS* by ddPCR and also by Sanger sequencing (Table 3).

Table 2 The detection of *KRAS* mutations in patient's samples using two different methods

	Primary tumor		Lymph node	
	IVD kit	ddPCR	Sanger seq.	ddPCR
mt*KRAS* ex.2, cod.12/13	16	17	7	15
WT *KRAS* ex.2, cod. 12/13	15	14	24	16

Table 3 The discordant mutation status of *KRAS* between primary tumor and tested lymph nodes

	Primary tumor		Lymph node	
	IVD kit	ddPCR	Sanger seq.	ddPCR
1.	WT *KRAS*	ex.2; cod. 12/13	WT *KRAS*	WT *KRAS*
2.	ex.2; cod. 12/13	ex.2; cod. 12/13	WT *KRAS*	WT *KRAS*

Discussion

In colorectal cancer treatment, we recognize three groups of drugs directed against specific molecules—drugs targeting vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR) and kinase inhibitors [23]. Unfortunately, because of the resistance to therapeutics, high percentage of mCRC patients still do not benefit from this type of therapy. The most important role in the effectiveness of anti-EGFR therapies plays *KRAS* gene and its mutation state. The investigation of *KRAS* mutations is required before treatment and is performed on primary tumor [3], therefore the mutation status in metastases is not known before therapy. The recently published meta-analysis has been focusing mainly on distant metastases, where the concordance rates between primary tumor and corresponding metastases are above 90% [13]. However, when concentrating on LN metastases, conflicting results have been reported with high discordance rates about 31% [14]. When more sensitive methods were applied, higher concordance was revealed [24]. We have firstly used ddPCR to analyze infiltrated LNs of patients with mCRC and identified high concordance in *KRAS* mutation status between primary tumors and matched infiltrated LNs about 93.54%. The discordant mutation status was determined in two cases of low-grade adenocarcinoma. In one case, the patient suffered from two tumors in different localizations. One tumor was localized in *colon sigmoideum* and both, the tumor sample and the corresponding metastasis have been exhibiting *KRAS* mutation, which was detected with the COBAS 4800 platform (data not shown). The second tumor was localized in rectum, was negative by qPCR and positive using droplet digital PCR, although the corresponding metastasis was negative. Even though this result has no impact on the indication of the anti-EGFR therapy, because the first tumor showed *KRAS* mutation, it seems that there is a distinct driver mutation in this metastasis as in the primary tumor. There is widespread opinion that molecular heterogeneity regarding the *KRAS* is mostly present in early stage of the diseases [25]. Our two discordant cases are about low-grade adenocarcinoma, which should be deeper characterized regarding the tumor heterogeneity and other driver mutations.

The development of new molecular technologies that show high accuracy in the mutation detection, allows to select patients with CRC in more precise way [26]. Droplet digital PCR represents one of these methods with the high potential for future diagnostic purpose [22, 27, 28]. The main reason why we decided to optimize ddPCR for *KRAS* testing is the sensitivity of ddPCR in comparison with Sanger sequencing and next-generation sequencing. We addressed the question whether the improvement in the detection method can lower the discrepancies of *KRAS* mutation detection between the primary tumor and regional LNs and showed that the concordance rate really increased on 93.54%. This approach has revealed cases of tumor heterogeneity. Tumor heterogeneity could be important in therapeutic decision making [29].

In regard to 31 samples of histologically positive LNs, the mutation state was unknown. When we applied Sanger sequencing, the results were in concordance with the primary tumor in 8 cases only. The Sanger sequencing was chosen because it is still considered to be the gold standard of molecular diagnostic, but the sensitivity of that technique is significantly lower than for other novel methods [19, 30], which was also proved by our results. According to that and despite the important role of Sanger sequencing in molecular biology, the testing of patients for targeted therapy design should be done with diagnostic procedures, which showed higher sensitivity than direct sequencing [26].

Conclusion

We have shown that there is high concordance between the presence of *KRAS* mutations in primary tumors and corresponding LN metastases when very sensitive method is applied. We obtained discordant results only in cases of low-grade adenocarcinoma and believe, similar to the others, that molecular heterogeneity of *KRAS* does not include advanced cases and is not very relevant for predictive testing. Despite this, it was concluded by us and others that normally the primary tumor and when possible the metastasis too, should be tested for the presence of *KRAS* mutations [17]. Moreover, our findings represent an evidence for the necessity of using more sensitive and effective methods in predictive testing when metastases are tested. In specific cases, the negative results of tested metastases by so sensitive methods can be a hint for of the presence of another high frequent driver mutation. The detection of subclonal populations of cells in primary tumor is a big challenge and it needs complex analyses along with the right interpretation methods for attained results. The approaches such as "liquid biopsies" using droplet digital PCR methods [31] are new facilities to study tumor heterogeneity in a noninvasive manner.

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Compliance with ethical standards

Conflict of interest 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 ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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