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Original Article

Major challenges related to tumor biological characteristics in accurate mutation detection of colorectal cancer by next-generation sequencing

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ABSTRACT

Next-generation sequencing (NGS) has been used in mutation detection of colorectal cancer (CRC). We here interrogated 747 CRC samples to detect mutations in 22 cancer-related genes by using NGS, and to explore some key challenges related to tumor biology. *RAS* mutations (*KRAS* or *NRAS* mutations), *RAS/BRAF/PIK3CA* mutations (mutations in *KRAS*, *NRAS*, *BRAF* or *PIK3CA*) and mutation burden (mutations in any of the 22 detected genes) were observed in 53.0% (396/747), 57.1% (431/747) and 84.2% (629/747) of specimens, respectively. Higher mutation frequencies were observed in biopsy specimens with ≥20% tumor cellularity than those with <20% tumor cellularity, but these differences were not observed in resection samples. Intratumor mutational heterogeneity was estimated by mutant allele frequency and tumor cellularity, and more likely to occur in *PIK3CA* mutant tumors. No significant differences of mutation frequencies were detected between primary and metastatic tumors. Additionally, specimens after chemotherapy showed lower mutation frequencies compared with specimens without chemotherapy. Together, our findings demonstrate that poor tumor cellularity, tumor heterogeneity and adjuvant therapy may confound the molecular diagnosis of CRC, and should be highlighted with prospective quality assessment during tissue process.

Introduction

Colorectal cancer (CRC) is one of the most common malignancies in the world [1]. Despite cancer diagnosis and therapy have greatly progressed, more than 50% of cases will ultimately develop metastatic CRC (mCRC), leading to poor prognosis [2].

Targeted therapy has been introduced into clinical practice in recent years. Anti-EGFR monoclonal antibodies, such as cetuximab and panitumumab, have been proven to be effective in mCRC treatment [3,4]. However, mutations in genes downstream of EGFR may cause resistance to anti-EGFR monoclonal antibodies [5,6]. *KRAS* and *NRAS* mutations have been confirmed as predictive markers of resistance to anti-EGFR therapy [7]. In these years, increased studies find that *BRAF* and *PIK3CA* mutations may also contribute to the lack of benefit from anti-EGFR therapy [8,9]. In addition to *KRAS*, *NRAS*, *BRAF* and *PIK3CA* mutations, mutations in other CRC-related genes may also affect the response to anti-EGFR targeted therapy and act as important prognostic markers [10–12]. Therefore, accurate detection of mutations in CRC is critical, since clinical molecular testing can provide a useful guide for treatment selection and outcome prediction.

Next-generation sequencing (NGS) has been developed in these years. Compared with traditional mutation testing, NGS can detect multiple gene mutations with higher sensitivity, using fewer amounts of input DNA, and at relatively lower cost. Thus, implementation of NGS platform for molecular diagnosis in cancer has become increasingly common in clinical practice [13]. However, this is challenging for molecular laboratories, since increasing number of biomarkers are mandatory to be detected using a single formalin-fixed paraffin-embedded (FFPE) sample [14,15]. In addition to identifying tumor histology, pathologists also need to choose the correct specimens and select the appropriate tissue blocks for further molecular diagnosis. Thus, the challenges faced in clinical practice for NGS testing of CRC are needed to be discussed and highlighted.

In this retrospective study, we interrogated 747 CRC samples using a validated clinical NGS assay to detect somatic mutations of

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22 cancer-related genes in an ISO15189-certified laboratory, and identified major tumor biological challenges to accurately detect mutations of CRC performed by NGS.

Patients and methods

Patient specimens

Between June 2014 and April 2017, a total of 756 specimens submitted for routine mutation testing at the Department of Pathology, Cancer Hospital, Chinese Academy of Medical Sciences (CAMS), were retrospectively collected in this study. The study has been approved by the Institute Review Board of the Cancer Hospital, CAMS. The methods were carried out in accordance with the approved guidelines. The informed consents were obtained from all patients. Nine specimens (9/756, 1.2%) were excluded from the analysis, because of scant tumor cells (specimens with tumor cellularity less than 10%) or insufficient amount/poor quality of DNA. NGS was successfully performed in 747 specimens from 739 tumors of 728 patients. The 747 specimens included 709 single specimens, 22 specimens from 11 paired primary and metastatic tumors, 10 specimens from 5 tumors (two different blocks from the same tumor) and 6 specimens from 3 paired pre-chemotherapy and post-chemotherapy tumors (Supplementary Fig. 1). All 728 cases were histologically examined by the pathologists before NGS testing, and were diagnosed as adenocarcinoma. Patient characteristics were listed in Supplementary Table 1.

Assessment of tumor cellularity

All specimens were fixed in 10% neutral buffered formalin for 16–48 h, and then were embedded in paraffin. Tumor cellularity of each sample was assessed using the corresponding HE slide. Tissue blocks with ≥10% tumor cellularity were selected for further analysis. The selected blocks were sectioned to collect enough tumor tissues for DNA extraction. Next, one additional 3 μm section was stained with HE, and the percentage of tumor cells were estimated to further correct the tumor cellularity. Tumor cellularity was assessed by two pathologists (Dr. W. L and Dr. T. Q), independently. The percentage of tumor cells was estimated with 5% increments, and the final tumor cellularity was calculated as (tumor cellularity from Dr. W. L+ tumor cellularity from Dr. T. Q)/2. When more than 10% of difference in tumor cellularity was assessed between the two pathologists, or the tumors were mixed with dense lymphocytic infiltrates or mucin, the percentage of tumor cells was further estimated by a third pathologist (Dr. J. Y). Final tumor cellularity was identified as (tumor cellularity from Dr. W. L+ tumor cellularity from Dr. T. Q+ tumor cellularity from Dr. J. Y)/3. The flow chart was listed in Supplementary Fig. 2. Macrodissection was performed when necessary. When macrodissection is used, an area with high and homogeneous tumor cellularity was marked by the pathologists and dissected to remove necrosis, mucin lakes, or prominent lymphocytic infiltrates. Estimation of tumor cellularity was performed on this marked area.

Isolation of genomic DNA

DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) tissues, using QIAamp DNA FFPE Tissue Kits (Qiagen, Germany), according to the manufacturer's instructions. NanoDrop ND-1000 Spectrophotometer (NanoDrop, USA) was used to detect the absorbance ratios of 260/280 nm to evaluate DNA quality. Quantus™ Fluorometer was used to determine DNA quantity.

DNA library construction and sequencing

Ten ng of genomic DNA was used to prepare amplicon libraries, with the Ion Ampliseq Colon and Lung Cancer Panel. There were 22 cancer-related genes in the panel, including *KRAS*, *NRAS*, *BRAF*, *PIK3CA*, *EGFR*, *AKT1*, *ERBB2*, *PTEN*, *STK11*, *MAP2K1*, *ALK*, *DDR2*, *CTNNB1*, *MET*, *TP53*, *SMAD4*, *FBXW7*, *FGFR3*, *NOTCH1*, *ERBB4*, *FGFR1* and *FGFR2*. The amplicon libraries were conducted with Ion AmpliSeq Library Kit 2.0 (Thermo Fisher, MA, USA). After PCR amplification and adapter ligation according to the manufacturer's protocol, the amplicon libraries were quantified using Ion Library Quantification Kit (Thermo Fisher, MA, USA). Next, each library was diluted to a concentration of 100 pM, and pooled in equal volumes. Emulsion PCR and template preparation (the template-positive Ion Sphere Particles, ISPs) were performed using Ion OneTouch Template Kit and Ion OneTouch system (Thermo Fisher). Finally, ISPs were sequenced on Ion PGM with the Ion 316 Chip or Ion 318 Chip, following the manufacturer's instructions.

Data analysis and validation

Successful sequencing was identified when the total reads >300 000 and AQ20 > 98% (1 misaligned base per 100 bases). Mutations were annotated through Torrent Variant Caller and viewed with Integrative Genomics Viewer. Variants with >1000 × coverage and ≥5% mutant allele frequency were considered true.

Calculation of heterogeneity score (HS)

Heterogeneity score was calculated as described by Normanno et al. [16], with some modifications. Briefly, assuming that somatic mutations usually affect one allele, the HS was calculated as mutant allele frequency*2/tumor cellularity. Thus, HS represented the fraction of tumor cells with a specific mutation. HS = 1 suggested all tumor cells having the mutation; HS < 1 suggested only a fraction of tumor cells showing the mutation; HS > 1 indicated that copy-number variation may exist in the genes (gain of the mutant allele or loss of the wild-type allele).

Statistical analysis

Chi-square test or Fisher's exact test was performed to explore the relationship between tumor cellularity, tumor heterogeneity, adjuvant therapy and mutation frequencies. Student's t-test or nonparametric ANOVA was performed to compare the HS values of *KRAS*, *NRAS*, *BRAF* and *PIK3CA* mutations. Mutant allele frequencies in tumors with concomitant *RAS* and *PIK3CA* mutations were assessed by correlation analysis. All data were analyzed using the SPSS 18.0 Software. The 2-sided P values < 0.05 were considered statistically significant.

Results

Mutation profiling of CRC

NGS was successfully performed in 747 specimens from 739 tumors of 728 CRC patients. *RAS* mutations (*KRAS* or *NRAS* mutations) were observed in 396 of 747 (53.0%) specimens. *RAS/BRAF/PIK3CA* mutations (mutations in *KRAS*, *NRAS*, *BRAF* or *PIK3CA*) were observed in 431 of 747 (57.1%) specimens. Mutation burden (mutations in any of the 22 detected genes) was observed in 629 of 747 (84.2%) specimens. There was no statistical difference between the groups with or without mutation burden regarding tumor type, sampling site and sample type (Supplementary Table 2). Moreover, the most commonly mutated gene was *TP53* (56.2%), followed by *KRAS* (48.6%), *PIK3CA* (9.6%), *FXBW7* (5.1%), *NRAS* (4.7%), *SMAD4* (3.7%) and *BRAF* (3.5%). Two rare *KRAS* mutations, including a p.D47Y (c.139G > T) mutation and a p.Q61L (c.182_183AA > TT) mutation not reported in the COSMIC database, were detected in *KRAS* exon 3. The detailed information of the mutation profiling was described in Fig. 1.

Tumor cellularity and mutations

Tumor cellularity was assessed by the pathologists. NGS testing was not conducted in specimens that contained fewer than 10% tumor cells and could not be macrodissected (Supplementary Fig. 3A–3D). All samples were categorized into three groups based on the estimated percentage of tumor cells: Group 1: 10%–19% tumor cellularity, Group 2: 20%–30% tumor cellularity and Group 3: >30% tumor cellularity. There were 27 specimens in Group 1, 192 specimens in Group 2 and 528 specimens in Group 3. *RAS* mutations were more likely to be detected in Group 3 (293/528, 55.5%) as compared to Group 1 (9/27, 33.3%) (P = 0.024). *RAS/BRAF/PIK3CA* mutations were more frequently to be detected in Group 3 (319/528, 60.4%) as compared to Group 1 (9/27, 33.3%) (P = 0.005). Significant differences were also observed in the frequency of mutation burden when Group 1 was compared with Group 2 or Group 3 (Group 1 vs Group 2, 63.0% vs 83.9%, P = 0.016; Group 1 vs Group 3, 63.0% vs 84.8%, P = 0.006) (Fig. 2A). We further analyzed the mutation frequencies in biopsy and resection specimens, respectively. In biopsy specimens, lower frequencies of *RAS* mutations, *RAS/BRAF/PIK3CA* mutations and mutation burden were observed in Group 1 compared with Group 2 or Group 3 (*RAS* mutations, Group 1 vs Group 2, 11.1% vs 44.3%, P = 0.036; Group 1 vs Group 3, 11.1% vs 56.0%, P = 0.012; *RAS/BRAF/PIK3CA* mutations, Group 1 vs Group 2, 11.1% vs 49.5%, P = 0.017; Group 1 vs Group 3, 11.1% vs 62.5%, P = 0.003; mutation burden, Group 1 vs Group 2, 44.4% vs 86.6%, P = 0.001; Group 1 vs Group 3, 44.4% vs 85.3%, P = 0.001). However, no significant differences were observed between Group 2 and Group 3 (Fig. 2B). Moreover, there were no statistically differences among Group 1, 2 and 3 in resection specimens (Fig. 2C).