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Circulating tumor DNA-based early detection of precancerous colorectal lesions using QClamp XNA-mediated real-time PCR

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The mutation status of the gene involved in early colorectal carcinogenesis and micro-invasive liquid biopsy allows detection of mutated circulating tumor DNA (ctDNA). Early detection of precancerous lesions and CRC is vital in proper treatment and hence associated to patient survival. The frequency of APC, CTNNB1, KRAS, and BRAF mutations in circulating free DNA (cfDNA) were analyzed to evaluate the performance of the mutated ctDNA in detecting polyp and adenoma using highly sensitive QClamp XNA based PCR. A total of 71 patients with low-risk adenoma or polyps were screened and there was no significant difference in the distribution of mutations gender, age, long-term medication, smoking history, alcohol drinking, and fecal occult blood. The positive predictive value (PPV) of the four genes' panel to detect low-risk adenoma and polyps was 39.44% (n = 28/71). Specifically, of all the 71 cases studied, there were 20 cases (28.2%) with APC mutations, 7 cases (9.9%) with CTNNB1 mutations, 4 cases (5.6%) with KRAS mutations, and 2 cases (2.8%) with BRAF mutations. Most mutations occurred at APC876. The four genes' panel detected by XNA-based PCR technique might be suited to efficiently and micro-invasively detect genetic alterations in cfDNA of patients with precancerous colorectal lesions.

1. Introduction

Colorectal cancer (CRC) is one of the most common cancer and the fourth leading cause of the cancer death worldwide (Wong and Ma 2014). A number of genetic alterations accumulation lead to the malignant transformation of epithelial cells in the colon or rectum (Fearon and Vogelstein 1990;Vogelstein and Kinzler 1993). WNT signaling pathway plays key roles in the progression of CRC (Tran et al. 2020). An abnormal activation and hyperactivation of WNT signaling pathway has been observed to be essential for tumor growth in CRC (Jardé et al. 2013).

WNT/β-catenin signaling is frequently activated in CRC due to the mutation of adenomatous polyposis coli (APC) which identified as the cause of hereditary colon cancer syndrome (Groden et al. 1991). Mutation of APC is a crucial initiating step in carcinogenesis which promotes the growth and progression of adenomas by activation of WNT signaling. APC mutations have been observed in the earliest CRC precursor including adenomas and aberrant crypt foci (Giaretti et al. 2004;Smith et al. 1994;Vogelstein et al. 1988). And β-catenin, encoded by CTNNB1, is an intracellular signal transducer in Wnt/β-catenin signaling pathway and it plays a critical role in tumorigenesis. APC and CTNNB1 mutations have been reported in precancerous colorectal lesions and are mutually exclusive in CRC (Kuipers et al. 2015;Shitoh et al. 2001). It is known that mutations in KRAS and BRAF would promote the development of carcinomas, and accumulated mutations in them would accelerate tumor metastases (Fearon 2011;Schepers and Clevers 2012;Vogelstein et al. 1988;Walther et al. 2009). BRAF encodes a serine/threonine protein kinase that functions downstream of RAS in the mitogen activated protein kinase (MAPK) pathway, which is important in regulating cellular responses to extracellular signals (Ciardiello and Tortora 2008). BRAF mutations occur in up to 10% of CRC patients and are mostly reported in 60% to 70% of serrated polyps (Normanno et al. 2009;Yang et al. 2015). BRAF V600E mutation accounts for 95% of all reported BRAF mutations (Oikonomou et al. 2014). Mutation of codon 600 in BRAF shows constitutive activation of the MAPK pathway. KRAS is an important and frequently mutated gene during colorectal carcinogenesis. KRAS mutations are found in 35–42% of CRCs and advanced adenomas (AA), and in 10% of non-advanced adenoma (NAA) (Jass et al. 2006;Pretlow and Pretlow 2005;Worthley and Leggett 2010;Zauber et al. 2013).

Currently, colonoscopy is the gold standard screening method for the diagnosis of CRC. However, colonoscopy is invasive and accompanied by dietary restriction requirement, which decreases patient's compliance (Kuipers et al. 2013). Thus, effective non-invasive methods detecting early stage of CRC and precursors are highly desirable. The "liquid biopsy" method has been developed over the past few years and is currently used clinically for therapeutic guidance. Compared with a classic biopsy, liquid biopsies are more convenient for patients (Alix-Panabières and Pantel 2016;Diaz and Bardelli 2014;Merker et al. 2018). And ctDNA tests have shown promising results for early detection of cancer, MRD detection, and prediction of disease recurrence (Abbosh et al. 2017; Chaudhuri et al. 2017; Cohen et al. 2018; Tie et al. 2016). Indeed, several mutation and aberrant methylation of genes have been reported to be associated with the tumorigenesis of CRC (Durso et al. 2017; Fearon and Vogelstein 1990;Heiss and Brenner 2017;Raut et al. 2019;Song and Li 2015;Vogelstein and Kinzler 1993). Approval of Cologuard (multi-target stool DNA test that examines KRAS mutation, NDRG4 and BMP3 methylations, β-actin, plus a hemoglobin immunoassay) (Imperiale et al. 2014) and Epi proColon (blood-based test that examines SEPT9 methylation) (Potter et al. 2014; Schmidt and Diehl 2007; Song and Li 2015) by the Food and Drug Administration have further confirmed that DNA mutations and methylation can serve as applicable biomarkers for non/micro-invasive CRC screening. However, the sensitivity of early detection is limited by low concentration of ctDNA released from pre-cancerous lesions and early stage of CRC.

Researchers have sought for strategies to improve the sensitivity, such as COLD-PCR, ARMS, droplet-digital PCR (ddPCR) (Bando et al. 2011; Didelot et al. 2012; Hindson et al. 2013;Milbury et al. 2011;Taly et al. 2013) and blocking oligonucleotides employed in PCRs such as peptide nucleic acid (PNA) and locked nucleic acid (LNA) (Ishige et al. 2018; Kyger et al. 1998; Saarbach et al. 2019). As expected, BRAF V600E detection in melanoma is highly improved by COLD-PCR (Milbury et al. 2011). Allele-specific real-time PCR ARMS-PCR is a highly sensitive and specific mutation detection method for JAK2 V617F in Formalin-fixed and paraffin-embedded (FFPE) samples from CRC patients (Jarry et al. 2004). And ddPCR is one of techniques to detect ctDNA at early cancer stages and to be used as a blood screening test with its high sensitivity (0.1%). In addition, the development of next generation sequencing (NGS) has offered unprecedented progress in detection of somatic mutations with outstanding accuracy, sensitivity and high throughput. NGS has been proved to successfully detect mutations in cfDNA from the peripheral blood of GIST, colorectal cancer, and lung cancer patients (Couraud et al. 2014; Olmedillas-López et al. 2018; Wada et al. 2016). However, the current strategies and methods still vary in their sensitivity, assay complexity and costs.

In the present study, we applied molecular QClamp technology (Powell and Zhang 2014). It is a real-time PCR technology that utilizes a sequence specific oligonucleotide blocker (XNA) with a modified backbone chemistry. The use of a modified backbone increases the binding affinity and hence the melting temperature (Tm) of the blocking oligomer leads to more efficient clamping of WT alleles. It can detect low frequency genetic mutations (<0.1%) in DNA samples obtained from patient tumor biopsy or whole blood samples (Myers et al. 2016). The objectives of this study were to evaluate the value of early detection of ctDNA in blood of precancerous colorectal lesions using a highly sensitive QClamp XNA based PCR technique. A panel of target genes APC, KRAS, BRAF, CTNNB1 was selected to analyze the frequency of mutations in precancerous lesions using QClamp XNA based PCR (Schneider et al. 2010; Scholtka et al. 2009). This research will further advance study on the use of ctDNA in detection of neoplastic lesion, which could also be applicable in many other cancers.

Table 1: Pathological characteristics of the Low-risk adenoma and polyp cases in the present study

Characteristics	Variable	No.
Gender	Female	22
	Male	49
Age	<65	44
	≥65	27
Long-term medication	No	55
	Yes	16
Smoking history	Current/former	14
	Never	57
Alcohol drinking	Current/former	16
	Never	55
Fecal occult blood	No	66
	Yes	5

2. Investigations and results

2.1. Clinical characteristics of samples

A total of 71 patients with low-risk adenoma or polyps were screened for inclusion in present study. Clinical characteristics are shown in Table 1, including gender, age, long-term medication, smoking history, alcohol drinking, and fecal occult blood.

2.2. Mutation characteristics of patients' samples

The mutation status of target genes APC, KRAS, BRAF, and CTNNB1 were analyzed among the 71 patients with low-risk adenoma or polyps using QClamp XNA based-real time PCR. The correlation between mutation status and clinical characteristics was analyzed. There was no significant difference in the distribution of mutation status by gender, age, long-term medication, smoking history, alcohol drinking, and fecal occult blood (Table 2).

2.3. Mutation of circulating tumor DNA in colorectal lesions

We evaluated 71 low-risk adenoma or polyps for missense mutations in APC (codon 86, 1309, 1367, and 1450), CTNNB1 (codon 41 and 45), BRAF (codon 600) and KRAS (codons 12 and 13). The incidence of all four genes analyzed and revealed that 28 out of 71 patients exhibited mutations (39.44%), at least one of four genes altered (Fig. a). Specifically, 20 patients had a APC mutation (28.2%), 7 patients had a CTNNB1 mutation (9.9%), 4 patients had a KRAS mutation (5.6%), and 2 patients had a BRAF mutation (2.8%).4 patients had both APC and CTNNB1 mutations and 1 patients had both APC and KRAS mutations. All mutation sites could lead to missense or frameshift of AA. Mutations of APC and CTNNB1 were more frequent in low-risk adenoma and polyps. From a diagnostic point of view, the sensitivity of the four genes panel to detect low-risk adenoma and polyps was 39.44% (n = 28/71). No patients had both KRAS and BRAF mutations.

Table 2: Characteristics of low-risk adenoma and polyp patients harboring mutations

Variable		Mutation*	Wild type	$\chi 2$	P value
Gender	Female	8 (28.6%)	14 (32.6%)	0.0085	0.9263
	Male	20 (71.4%)	29 (67.4%)		
Age	<65	17 (60.7%)	27 (62.8%)	0.0055	0.941
	≥65	11 (39.3%)	16 (37.2%)		
Long-term medication	No	21 (75.0%)	34 (79.1%)	0.0122	0.912
	Yes	7 (25.0%)	9 (20.9%)		
Smoking history	Current/former	7 (25.0%)	7 (16.3%)	0.3569	0.5502
	Never	21 (75.0%)	36 (83.7%)		
Alcohol drinking	Current/former	8 (28.6%)	8 (18.6%)	0.4785	0.4891
	Never	20 (71.4%)	35 (81.4%)		
Fecal occult blood	No	26 (92.9%)	40 (93.0%)	0.2005	0.6543
	Yes	2 (7.1%)	3 (7.0%)		

*carrying at least one of the four above-mentioned genes

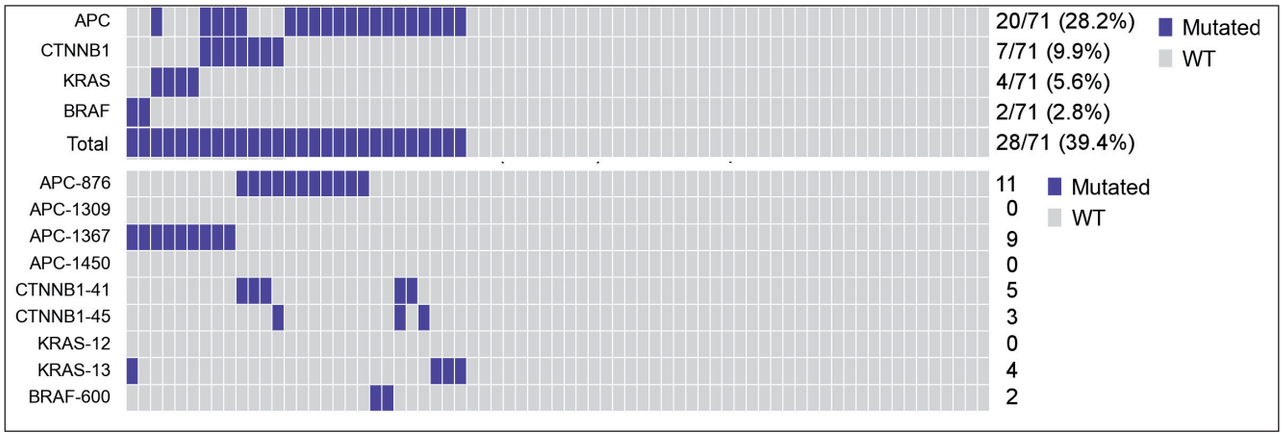

Fig.: Mutation status of APC, CTNNB1, KRAS, and BRAF genes was investigated using real-time polymerase chain reaction (PCR). a. Mutation frequency of APC, CTNNB1, KRAS, and BRAF genes was analyzed. b. Mutation frequency of amino acid sites in the four genes were listed.

Furthermore, mutation frequency of amino acid sites was calculated. 11 patients harbor an APC 876 mutation which was the most frequent mutation sites. Interestingly, 3 patients had both APC 876 and CTNNB1 41 mutations. 1 patient had both APC 876 and CTNNB1 45 mutations. 1 patient had both APC 1367 and KRAS 13 mutations. none of patients harbored mutation of APC 1309, 1450, or KRAS 12.

3. Discussion

In our present research, we investigated the mutations of ctDNA in blood specimens of 71 patients with precancerous colorectal lesions, using a highly sensitive QClamp XNA based PCR technique. Four target genes (APC, KRAS, BRAF, and CTNNB1) were selected for further analysis. Our results indicated that the PPV of the four gene panel for detecting precancerous lesions was 39.44%, and APC mutations occurred in 28.2% of the patients.

Firstly, we screened 71 patients with low-risk non-advanced adenomas or polyps for further analysis, and collected the clinical characteristics, including gender, age, long-term medication, smoking history, alcohol drinking, and fecal occult blood. Subsequently, four target genes APC, KRAS, BRAF, and CTNNB1 were selected to be detected, as their involvement in the WNT and the Ras-Raf-MEK-MAPK signaling pathway and crucial role in adenoma-carcinoma progression (Ciardiello and Tortora 2008; Jardé et al. 2013; Tran et al. 2020). We found that there was no significant correlation between mutation status and clinical characteristics. Moreover, missense mutations in the four genes were further detected. And the results revealed that APC, CTNNB1, KRAS, and BRAF mutations occurred in 20 (28.2%), 7 (9.9%), 4 (5.6%), and 2 (2.8%) patients, respectively. The sensitivity of the four gene panel to detect low-risk precancerous colorectal lesions was 39.44% (n = 28/71). Notably, most of the mutations were found in APC, and there were 4 patients with both APC and CTNNB1 mutations.

Numerous studies have evidenced the crucial role of some abnormal gene expressions in tumor progression (Huang et al. 2018). As one of the most common cancers, CRC usually shows no obvious symptoms at an early stage and has a metastasis with the tumor development, leading to a poor long-term prognoses of patients (Ladabaum et al. 2020; Simon 2016). Early screening of CRC will undoubtedly improve the prognosis of patients. The genetic instability of CRC were reported to be resulted from complicated mechanisms, including chromosomal instability (CIN) and microsatellite instability (MSI) (Muller et al. 2016). Furthermore, the inactivating mutations or losses in APC tumour suppressor gene has been demonstrated to related to CIN, as an early event in CRC (Muller et al. 2016). On this point, our findings were consistent with the previous study. There are APC mutations in most of adenomas and CRC, resulting in hyperactivation of the WNT pathway (Silva et al. 2014; Suehiro et al. 2008). Additionally, aberrant CTNNB1 signaling is also a key factor related to the pathogenesis and progression of CRC (Bienz and Clevers 2000), and there were 4 patients showed both APC and CTNNB1 mutations in our study. Either the mutations of CTNNB1 or the biallelic inactivation of APC could result in the deregulation of CTNNB1 signaling (Cancer Genome Atlas 2012). Moreover, KRAS and BRAF have been revealed to be correlated with the progression and metastasis of CRC (Afrasanie et al. 2019;Cicenas et al. 2017), but in our research, the mutation rates of KRAS and BRAF in low-risk non-advanced adenoma or polyp samples were relatively low.

Furthermore, we have used a highly sensitive QClamp XNA-PCR technology in this study (Powell and Zhang 2014), which is a kind of real-time PCR technology utilizing a sequence specific oligonucleotide blocker with a modified backbone chemistry (Myers et al. 2016). Based on this, binding affinity and the melting temperature (Tm) of the blocking oligomer can be increased, resulting in a more efficient clamping of WT alleles. It has been evidenced that low frequency genetic mutations (<0.1%) in DNA could be detected (Myers et al. 2016). Accordingly, the mutations of KRAS (5.6%) and BRAF (2.8%) were also detected in this study. Applying QClamp XNA-PCR to explore the target mutation rates in low-risk non-advanced adenoma or polyp samples, not only benefits for investigating early events in CRC but also is conducive to better application of ctDNA in early screening of CRC.

In conclusion, we detected the mutation rates in APC, CTNNB1, KRAS, and BRAF of 71 patients with precancerous colorectal lesions, using the QClamp XNA based PCR technology. The PPV of the four gene panel for detecting precancerous lesions was 39.44%, and APC mutations occurred in 28.2% in the patients. Our findings will provide more alternatives for early detection of CRC and contribute to the application of ctDNA in clinical strategies for various cancer treatments.

4. Experimental

4.1. Subjects population

Blood samples from patients were sourced from Tianjin People Hospital between September 2019 and September 2020. Individual age, gender, cancer history, colorectal polyp history, taking drugs in long-term, and feces occult blood were obtained from the pathology request form. 101 participants were diagnosed as colorectal disease or advanced adenoma and 30 participants were excluded because of withdraw consent or dropout. The sample set consisted of 71 low-risk non-advanced adenomas and polyps according to the 2020 US MULTI-SOCIETY TASK FORCE. low-risk non-advanced adenomas and polyp refers to having 1–2 tubular adenomas with low-grade dysplasia, each <10 mm in size or hyperplastic polyp.

4.2. DNA extraction

Blood samples were collected before the colonoscopy, stored at 4 °C and processed within 1–4 h. Blood samples were centrifuged at 3000 rpm for 10 min at 4 °C and

plasma samples were stored at -80 °C. Prior to DNA extraction, plasma was centrifuged at 13,200 rpm for 10 min at 4 °C. cfDNA was extracted from blood samples using QClamp Circulating Nucleic Acid Kit (55114, Qiagen) according to manuscript instruction.

4.3. Mutation analysis using QClamp XNA PCR

QClamp XNA PCR is a real-time PCR technology that utilizes a sequence specific oligonucleotide blocker that has a modified backbone chemistry. ColoScape™ kit is provided by DiaCarta, Inc. It is a gene panel including APC, CTNNB1, KRAS, and BRAF. The limit of detection (LOD) of the kit was 0.1% for the multiplex mutation assay. qRT-PCR was performed on LC480-II thermal cycler (Roche Diagnostics). The XNA based real-time PCR reaction conditions used in the detection of the mutations were 95°C for 2 min, followed by 50 cycles of 95°C for 20 s, 74°C for 40 s, 62 °C for 30 s and 72 °C for 30 s.

4.4. Statistical analysis

Fischer's exact test or Pearson's X^2 test was applied for comparison of the categorical variables. Two-tailed p-values of less than 0.05 were considered to be statistically significant. All statistical analyses and graphical data were performed using Python Software (python-3.9.2, Python). p values <0.05 were considered to be statistically significant.

4.5. Ethical considerations

The study was approved by the ethics committee of Tianjin People Hospital (No. 2021-B18). Patients signed informed consent forms approving the use of his/her biological samples for research purpose.

Availability of data and materials: The datasets used and analysed in the present research are available from the corresponding author on reasonable request.

Conflicts of interest: The authors declare no conflict of interest.

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