Clinica Chimica Acta 413 (2012) 1605–1611

Contents lists available at SciVerse ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Characteristics and prevalence of *KRAS*, *BRAF*, and *PIK3CA* mutations in colorectal cancer by high-resolution melting analysis in Taiwanese population

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ARTICLE INFO

Article history:
Received 17 April 2012
Received in revised form 27 April 2012
Accepted 28 April 2012
Available online 8 May 2012

Keywords:
Colorectal cancer
KRAS gene
BRAF gene
PIK3CA gene
Direct sequencing
High-resolution melting

ABSTRACT

Background: The identification of *KRAS*, *BRAF*, and *PIK3CA* mutations before the administration of anti-epidermal growth factor receptor therapy of colorectal cancer has become important. The aim of the present study was to investigate the occurrence of *KRAS*, *BRAF*, and *PIK3CA* mutations in the Taiwanese population with colorectal cancer. This study was undertaken to identify *BRAF* and *PIK3CA* mutations in patients with colorectal cancer by high-resolution melting (HRM) analysis. HRM analysis is a new gene scan tool that quickly performs the PCR and identifies sequence alterations without requiring post-PCR treatment.

Methods: In the present study, DNAs were extracted from 182 cases of formalin-fixed, paraffin-embedded (FFPE) colorectal cancer samples for clinical *KRAS* mutational analysis by direct sequencing. All the samples were also tested for mutations within *BRAF* V600E and *PIK3CA* (exons 9 and 20) by HRM analysis.

Results: The results were confirmed by direct sequencing. The frequency of *BRAF* and *PIK3CA* mutations is 1.1%, and 7.1%, respectively. Intriguingly, we found that nine patients (4.9%) with the *KRAS* mutation were coexistent with the *PIK3CA* mutation. Four patients (2.2%) without the *KRAS* mutation were existent with the *PIK3CA* mutation. Two patients (1.1%) without the *KRAS* mutation were existent with the *BRAF* mutation.

Conclusions: In the current study, we suppose that HRM analysis is rapid, feasible, and powerful diagnostic tool for the detection of *BRAF* and *PIK3CA* mutations in a clinical setting. Additionally, our results indicated the prevalence of *KRAS*, *BRAF*, and *PIK3CA* mutational status in the Taiwanese population.

1. Introduction

Colorectal cancer (CRC) is the third most frequent tumor worldwide, with > 70,000 new cases per year for both sexes in the United States [1]. Similarly, CRC is the third leading cause of cancer deaths in both sexes in the Taiwanese population [2]. Recently, significant improvements have been made in patient survival, after metastasis development, by improving new therapies. Anti-EGFR-targeted therapies with monoclonal antibodies such as cetuximab and panitumumab are a successful strategy for the treatment of metastatic CRC or after the failure of conventional chemotherapy. These agents bind the epidermal growth receptor (EGFR) on tumors cells and then block the downstream intracellular signaling pathways. One member of this pathway is *KRAS* and much evidence shows that the patients with *KRAS* mutations do not benefit from the addition of cetuximab or panitumumab to standard chemotherapy [3]. Therefore, *KRAS* mutation testing should be performed in all individuals with advanced CRC refractory to first-line regimens to identify which patient's tumors will not respond to the expensive monoclonal antibody inhibitors of EGFR.

KRAS encodes a membrane-associated GTPase that is an early player in many signal transduction pathways. KRAS acts as a molecular on/off switch that recruits and activates proteins that are necessary for the propagation of growth factor and other receptor signals, such as c-Raf and PI 3-kinase. When activated, KRAS is involved in the dephosphorylation of GTP to GDP, after which it is turned off. The rate of GTP to GDP conversion can be dramatically accelerated by an accessory protein of the guanine nucleotide activating protein (GAP) class, for example, RasGAP [4]. In CRCs, *KRAS* point mutations occur early in the adenoma–carcinoma sequence and are believed to contribute to the growth and increased atypia of adenomas [5]. Activating mutations of the *KRAS* gene have been widely studied as markers for cancer prognosis. These gene mutations, principally in codons 12 and 13, occur in approximately one-half of CRCs, and population-based studies have suggested that the mutations might be associated with some tumor phenotypes [6]. Recently,

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doi:10.1016/j.cca.2012.04.029

high-resolution melting (HRM) analysis has been applied for use in the screening for the *KRAS* mutation in CRC [7].

BRAF, a member of the *RAF* gene family (*BRAF*, *ARAF1*, and *RAF1*), encodes a serine–threonine protein kinase that is a downstream effector of activated KRAS. Mutated BRAF activates a signaling cascade involving proteins in the mitogen-activated protein kinase system, resulting in cell proliferation [8]. Approximately 15% of CRC have the *BRAF* mutation and this is relevant to poor prognosis [9]. The hotspot mutation is the T-to-A transversion at nucleotide 1796 causing V600E. This mutation is predisposed to the inhibition of apoptosis and also aids in increasing invasiveness [10]. Meanwhile, *KRAS* and *BRAF* mutations are mutually exclusive in CRC [11]. This suggests that they occur in different tumor types and might have different outcomes. On the other hand, studies showed that the *BRAF* V600E mutation confers resistance to EGFR monoclonal antibodies in patients with chemotherapy-refractory *KRAS*-wild-type metastatic CRC [12]. Moreover, a part of CRC patients without in *KRAS* and *BRAF* mutations fails to respond to anti-EGFR therapy, and this may be due to mutations in the *PIK3* gene.

The PIK3s are a family of lipid kinases that are grouped into classes with a different structure and substrate preference [13]. PIK3Ks are heterodimeric kinases that are involved in the control of cellular growth, transformation, adhesion, and also apoptosis [14]. Several studies showed that the p110α isoform which is encoded by *PIK3CA* is mutated in approximately 15–18% of CRCs [15,16]. In CRCs, more than 80% of *PIK3CA* mutations occur in either exon 9 or exon 20 [11].

HRM analysis is rapidly becoming the most important mutation-scanning methodology that allows mutation scanning and genotyping without the need for costly labeled oligonucleotides. It is a closed-tube method, indicating that PCR amplification and subsequent analysis are sequentially performed in the well, making it more convenient than other scanning methodologies. Recently, we have used this method of genotyping and mutation scanning [7,17,18]. The aim of this study was to understand the *KRAS*, *BRAF*, and *PIK3CA* gene status in a Taiwanese cohort of CRC patients using direct DNA sequencing and HRM analysis.

2. Materials and methods

2.1. Sample preparation and DNA extraction

The specimens consisted of 182 formalin-fixed, paraffin-embedded (FFPE) colorectal adenocarcinomas submitted for clinical *KRAS* mutational analysis. All samples were tested for *BRAF* V600E and *PIK3CA* mutations within exon 9 and exon 20. FFPE samples were deparaffinized and air dried, subsequently, DNA was isolated using the proteinase K and QIAamp® mico DNA extraction kit (QIAGEN) according to the manufacturer's protocol.

2.2. Design of primers for HRM assay

A good amplicon design is essential for obtaining robust and reproducible HRM analysis. The difference between wild-type and heterozygote curves becomes smaller and more difficult to differentiate when the product length increases. Besides, extra care is needed to design PCR reactions that avoid primer dimers and non-specific amplification in HRM analysis.

DNA samples were amplified for the *KRAS* regions, including codons 12 and 13, using primers according to the previously published works [7]. The 153 bp PCR products with a single band were resolved on 2% agarose gels and visualized after staining with ethidium bromide. The cycling conditions for *KRAS* codons 12 and 13 involved a 35-cycle PCR program (denaturation at 96 °C for 10 s; annealing at 50 °C for 5 s; and elongation at 60 °C for 4 min). Mutation status was determined by direct sequencing.

The set of primers for HRM, specific for *BRAF* V600E and *PIK3CA* exon 9 and exon 20, were designed while fulfilling the requirements of the LightCycler® 480 System Gene Scanning Assay. All the amplicons were designed to be smaller than 300 bp. In the present study, the three pairs of primers for HRM analysis were selected using Primer3 software (Table 1). Appropriate primers were named as *H1–H6* as shown in Table 1. All the primers synthesized were all of standard molecular biology quality (Protech Technology Enterprise Co., Ltd, Taiwan).

2.3. The HRM technique

PCR reactions were carried out in a 10 μl final volume using the LightCycler ® 480 High-Resolution Melting Master (Reference 04909631001, Roche Diagnostics) 1× buffer – containing Taq polymerase, nucleotides and the dye ResoLight – and 20 ng DNA. The primers and $MgCl_2$ were used at a concentration of 0.25 μM and 2.5 mM, respectively, for identifying the mutation status of *BRAF* V600E, *PIK3CA* exon 9, and exon 20.

The HRM assays were conducted using the LightCycler® 480 Instrument (Roche Diagnostics) provided with the software LightCycler® 480 Gene Scanning Software Version 1.5 (Roche Diagnostics).

The PCR program required a SYBR Green I filter (533 nm), and it consisted of an initial denaturation–activation step at 95 °C for 10 min, followed by a 45-cycle program (denaturation at 95 °C for 15 s, annealing at 60 °C or 62 °C (Table 1) for 15 s, and elongation at 72 °C for 15 s with reading of the fluorescence; acquisition mode: single). The melting program included three steps: denaturation at 95 °C for 1 min, renaturation at 40 °C for 1 min, and subsequent melting that consists of a continuous fluorescent reading of fluorescence from 60 to 90 °C at the rate of 25 acquisitions per °C.

2.4. Gene scanning

The melting curve analysis performed by the Gene Scanning Software consists of three steps: normalization of melting curves, which involves equaling to 100% of the initial fluorescence and to 0% of the fluorescence remnant after DNA dissociation: shifting of the temperature axis of the normalized melting curves to the point where the entire double-stranded DNA is completely denatured: and, finally, the generation of difference plots, allowing the capture of the differences in melting profile between the reference sample curves from the test samples. If the shape of the melting curves is not similar to each other, then we will confirm it by direct DNA sequencing to prevent a false negative result. Furthermore, an analysis of the melting curves with a high-sensitivity setting of 0.5 was carried out by Gene Scanning Software (the default sensitivity setting of the Gene Scanning Software is 0.3.).

2.5. Direct sequencing

To confirm the results of HRM analysis, sequencing analysis was also performed in all samples. After HRM analysis, the samples were purified using a PCR-M™ clean-up system (VIOGEN, Sunnyvale CA 94086, U.S.A.). The PCR products generated after HRM were directly sequenced. The sequence reaction was performed in a final volume of 10 μl, including 1 μl of the purified PCR product, 2.5 μM of one of the PCR primers, 2 μl of the ABI PRISM terminator cycle sequencing kit v3.1 (Applied Biosystems)

Table 1
Primers use for HRM analysis of *BRAF* and *PIK3CA* gene mutation.

Detection for	Sequence (5′ to 3′)	Annealing temp. (°C)
PIK3CA Exon 9	H1 5′-GCCTGCTGAAAATGACTGAA-3′ (forward) H2 5′-CATTTTAGCACTTACCTGTGACTCCA-3′ (reverse)	58
PIK3CA Exon 20	H3 5′-TGAGCAAGAGGCTTTGGAGT-3′ (forward) H4 5′-TCATTTTCTCAGTTATCTTTTCAGTTCAAT-3′ (reverse)	58
BRAF	H5 5′-CATAATGCTTGCTCTGATAGGAAA-3′ (forward) H6 5′-TCAGCACATCTCAGGGCCAAA-3′ (reverse)	58