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KRAS Mutation Profile in Colorectal Carcinoma and Novel Mutation – Internal Tandem Duplication in *KRAS*

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Oncogenic KRAS mutations are associated with resistance to anti-EGFR therapy in colorectal carcinoma. Since anti-EGFR monoclonal antibodies are employed in clinical practice in advanced colorectal cancer, KRAS mutations have become an important predictor of therapy outcome. Mutational analysis of KRAS was performed on 163 adenocarcinoma samples. Exons 1-3 of KRAS were analyzed using SSCP and sequencing. Fifty seven (35%) carcinomas had missense point mutations in one of codons 12, 13, 59, 61, 117. In accordance with the published data, missense mutations in codons 12 (66%) and 13 (22%) were the most frequent. Mutations in codons 59 and 117 occurred with the same frequency as in codon 61. The only detected insertion occurred in exon 2. 15-bp insertion resulted in tandem duplication of codons 62-66. Presumably, 5 additional amino acids affected switch II conformation and sustained Ras activity due to decreased GTP hydrolysis. We report this unusual new type mutation.

Introduction

KRAS is involved in different malignancies and developmental disorders. Oncogenic mutations result in sustained Ras activity [1]. Point mutations occur predominantly, leading to amino acids substitution. In frame insertion mutations are seen in isolated cases. In colorectal carcinoma, as well as in other malignancies, codons 12, 13, 61 are most frequently mutated [2]. Codons 12-13 encode a part of the phosphate-binding loop (P-loop), whereas amino acid at position 61 is located in the switch II region. Due to predominance of mutations in these hot spots, many researchers limited their analysis to these three codons or even to codon 12 and 13 only.

KRAS mutations are associated with a worse prognosis [3] and resistance to anti-EGFR therapy [4]. Since anti-EGFR monoclonal antibodies are employed in clinical practice, mutational analysis is needed to exclude resistant patients. Methods should be preferred which are capable of detecting also less frequent mutations in such an analysis.

Here we present the results of SSCP analysis in sporadic colorectal adenocarcinomas and novel mutation – internal tandem duplication in exon 2 of *KRAS*.

Material and Methods

Samples

One hundred sixty tree unselected sporadic colorectal carcinoma cases were studied. All the patients (91 men and 72 women, aged 34–87 years) underwent surgery at the 1st Department of Surgery, Collegium Medicum, Jagiellonian University, Kraków, Poland. Carcinomas were histologically classified according to the TNM and Astler-Coller classifications. The tumor and normal mucosa samples were cut into small pieces, snap frozen and stored in -80°C for further investigation. DNA was extracted from fresh-frozen tumor and corresponding non-neoplastic tissues according to the manufacturer's instruction (QIAamp DNA Mini Kit, Qiagen GmbH, Hilden, Germany).

Analysis of KRAS mutations

Mutational analysis of *KRAS* was performed by SSCP and sequencing. PCR products were amplified with Red-Taq polymerase (Sigma-Aldrich Corp, St Louis, USA) using the primers: 5'-GACTGAATATAAACTTGTGG 5'-CTGTATCAAAGAATGGTCCT for *KRAS* exon 1 and 5'-GACTGTGTTTCTCCCTTCT 5'-TGGCAAATA-CACAAAGAAAG for *KRAS* exon 2 (annealing temp. 55°C)) [5]. For *KRAS* exon 3, two primer sets were designed: 5'-TGTTACTAATGACTGTGC 5'-TTCAGTGTTACT-TACCTG (216bp) and 5'-AAAGAGTTAAGGACTCTG 5'-TTCAGTGTTACTTACCTG (164bp) (annealing temp. 52°C). Electrophoresis was carried out in TBE buffer (MP Biomedicals Corp, USA) using MDE gels (Cambrex Bio Science, Rockland, USA) (0.6 MDE for *KRAS* exon 1; 0.8 MDE for *KRAS* exons 2 and 3). Thermostatically controlled circulating water was used to maintain a constant temperature of 8°C for *KRAS* exons 2 and 3 and 18°C for *KRAS* exon 1. Gels were run for 18h (*KRAS* exons 2 and 3) or 15h (*KRAS* exon 1) with constant 170V and developed by silver staining. Mutations were confirmed by direct sequencing. The sequence data were collected and analyzed using an ABI PRISM 310 Genetic Analyzer (Applera Corp, Foster City, CA).

MSI analysis

PCR, using a panel of 5 microsatellite markers located near APC, p53, BATRII, BAT26, and BAX, was performed according to the previously reported protocol [6]. All the cases that demonstrated MSI or LOH in at least 1 microsatellite marker were examined with a panel of 9 microsatellite markers (D2S123, D5S346, D3S1611, D18S35, NM-23, D7S501, D1S2883, TP53-pentanucleotide, TP53-dinucleotide) using a commercial kit (Applera Corp). The labeled products were separated on an ABI PRISM 310 Genetic Analyzer (Applera Corp). According to the MSI/LOH results, the cancers were classified into 3 groups: MSS (microsatellite stable cancers, without detectable instability), MSI high, and MSI low [7].

Results

Mutational analysis performed on 163 sporadic colorectal adenocarcinoma cases revealed *KRAS* mutations in 58 (35.6%) tumors. Fifty seven tumors had missense point mutations. Fifty one mutations were localized in exon 1: 12 cases G12V (GGT>GTT), 10 cases G12C (GGT>TGT), 9 cases G12D (GGT>GAT), 4 cases G12A (GGT>GCT), 3 cases G12S (GGT>AGT). 12 cases G13D (GGC>GAC), 1 case G13C (GGC>TGC). Four point mutations were detected in exon 2: 2 cases A59G (GCA>GGA), 2 cases Q61H (CAA>CAT; CAA>CAC). Only 2 mutations were found in exon 3, both in the same codon: K117N (AAA>AAT; AAA>AAC).

The only detected heterozygous insertion occurred in exon 2. The mutated allele contained 15-bp insertion, which is tandem duplication of sequence corresponding to codons 62-66 (c.198_199insGAGGAGTACAGTGCA) or sequence including a part of codons 61 and 66 (c.197_198insAGAGGAGTACAGTGC). This insertion resulted in tandem repeat of codons 62-66 in coding sequence and introduced pentapeptide repeat (p.A66_M67insEEYSA). Figure 1 presents the result of sequencing analysis.

A lesion with tandem duplication mutation was localized in the splenic flexure of a 64-year old woman. Histological examination classified it as a well-differentiated (G1), partially mucinous adenocarcinoma, with T2N0M0

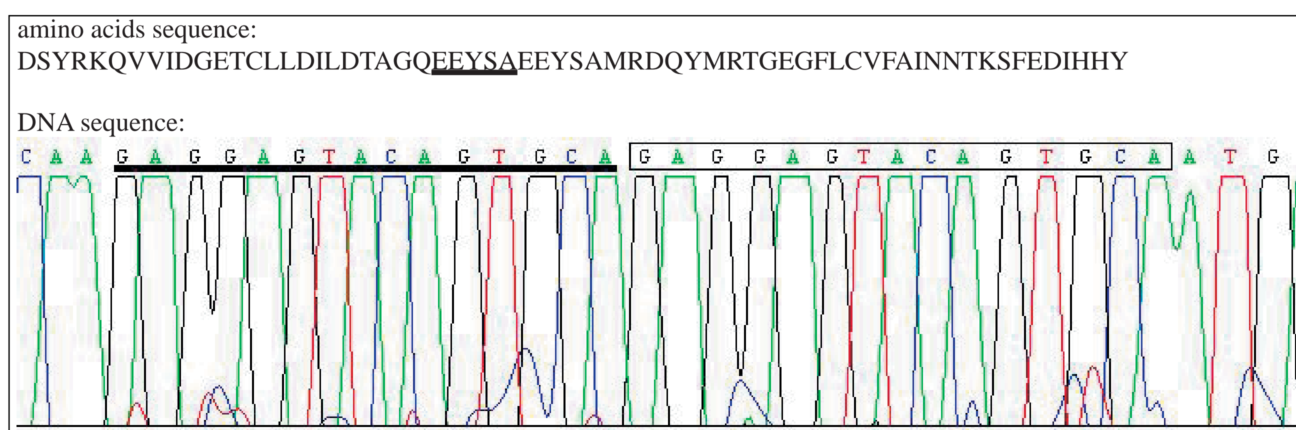

Fig. 1. Mutated allele with internal tandem duplication in exon 2. Codons 62-66 are underlined. 15-bp insertion resulted in tandem repeat of codons 62-66 (in frame) and amino acids sequence EEYSA repeat in protein.

stage according to the TNM scale. In addition to *KRAS* mutation, low-level microsatellite instability was detected (MSI-L). The patient underwent surgery and after 43 months was lost to follow-up. There was no recurrence during the follow-up period.

Discussion

Missense mutations in codons 12, 13, 59, 61, 117 are described in the literature. As expected, missense mutations in codons 12 (66%) and 13 (22%) were the most frequent. Mutations in codons 59 and 117 occurred with the same frequency as in codon 61.

Oncogenic insertions in *KRAS* are very rarely reported. There are only 6 known cases with insertion in exon 1, found in human malignancies (leukemia and rectal carcinoma). All of them were 3-bp in frame insertions. Three cases had insertion c.36_37insGGT, which is codon 12 duplication [8, 9]. In 2 cases, codon 10 was duplicated (c.30_31insGGA) [10]. 3-bp insertions in *KRAS* were usually tandem duplications. The only exception was insertion after codon 9 of sequence GTA (c.27_28insGTA), which corresponds to codon 8, but in protein this insertion also results in repeat of the preceding amino acid (p.V9_G10insV) [2, 11]. Longer insertions in exon 1 were observed in rat renal mesenchymal tumors induced by injection of nitrosamine. 9-bp (codons 10-12) and 12-bp (codons 10-13) fragments were tandemly duplicated, leading to tandem repeat of amino acids in the P-loop [12].

Insertion mutations in exon 1 of *KRAS* involved the phosphate-binding loop (codons 10-16). Mutant accumulated in cells in the active GTP-state. Biochemical analysis confirmed that the higher Ras-GTP levels corresponded to a dramatic decrease in intrinsic GTP hydrolysis, as well as resistance to GTPase-activating proteins [10]. Similar results were obtained with insertions in HRAS gene. Mutations of HRAS with three amino acids tandem duplications within the phosphate-binding loop have been investigated both in vitro and in vivo. The mutants bound preferentially GTP with an attenuated binding affinity for GDP. Furthermore, both the intrinsic, as well as the GAP-stimulated GTP hydrolysis were drastically decreased [13].

This is the first report on internal tandem duplication longer than 1 codon in the *KRAS* gene and also the first insertion detected in exon 2 in humans. This insertion resulted in tandem repeat of codons 62-66 that encode a part of the switch II region (codons 59-67). Switch II functions as an element of conformational mechanism allowing a release of the gamma-phosphate after GTP hydrolysis, and regulates binding to Ras regulators and effectors. Guanine nucleotide-exchange factors (GEFs) highly accelerate the exchange of GDP to GTP and activate Ras. After GEF binding, lysine 16 (a residue of the P-loop) is reoriented toward carboxylate from the switch II region, the highly conserved glutamic acid residue (Glu62). In GTP-bound conformation, there is hydrogenbond from gamma-phosphate oxygen to the main chain NH group of the glycine 60 residue [14]. Glutamine 61 is essential for Ras GTP hydrolysis, missense mutations at this position block this enzymatic reaction [15]. Oncogenic missense mutations in switch II have been reported for codons 59-62, with codon 61 as the most frequent. Tandem duplications in exon 2 have been detected so far in chemically induced mouse tumors. One lung adenoma contained a repetition of codons 52-60 and two liver adenomas had duplicated codons 64-73 and 66-75. These tandem repeats also involved the switch II region [16].

The novel tandem duplication mutation has not been functionally characterized. Presumably, 5 additional amino acids caused a conformational change in switch II, which attenuated GTP hydrolysis and stabilized Ras protein in the active state. Notably, low-level microsatellite instability has been detected in this carcinoma, what may have connection with tandem duplication.

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