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Advances in Brief

Type and Number of Ki-*ras* Point Mutations Relate to Stage of Human Colorectal Cancer[1]

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Abstract

Point mutations in the Ki-*ras* gene belong to the genetic key events in tumorigenesis of colorectal cancer. The type and number of point mutations were detected in specimens from patients with colorectal carcinomas staged as Dukes B and C using single-stranded conformational polymorphism analysis and sequencing. G-A transitions in codon 12 were exclusively found in Dukes B tumors, G-T transversions mainly in Dukes C, and G-C transversions only in Dukes C tumors. Apparently, the G-T and G-C transversions are associated with metastatic behavior of colorectal carcinomas, while G-A transitions are not. In several samples, multiple point mutations could be detected in codon 12, the frequency of multiple mutations increasing with the stage of the tumor.

Introduction

A model for a cascade of genetic alterations in colorectal cancer has been described by Vogelstein *et al.* (1). In this model, Ki-*ras* point mutations, in particular in codon 12 and to a lesser extent in codons 13 and 61, are considered to be a rather early event. Various mutations in codon 12 have been reported: G-A transitions in the first (Gly-Ser) and the second position (Gly-Asp) (2–4); and G-T transversions in the first (Gly-Cys) and second base (Gly-Val) are most frequently found (5, 6). Also, G-C changes in the first (Gly-Arg) and in the second (Gly-Ala) position are reported to occur (3, 7), although the frequency is lower. These different types of Ki-*ras* point mutations have been attributed to different mutagenic dietary components or to ethnic variation (6). It has been suggested that G-A transitions could occur as spontaneous mutations due to errors in DNA replication (8), whereas both G-A transitions and G-T transversions could be the result of mutagenic action (2). The variance in the spectra of mutations in different stages of tumor progression as mentioned in the cited reports (5, 6, 9) can be explained by various methodological differences as used in Ki-*ras* point mutation detection or by the selection of the investigated cancer samples.

Against the background of the potentially different mechanisms underlying the type of Ki-*ras* point mutations, we studied a series of nonmetastasized Dukes B tumors and metastasized Dukes C tumors with respect to type and frequency of point mutations in the first exon of the Ki-*ras* gene using analysis with the SSCP[4] technique and sequencing.

Materials and Methods

DNA Extraction and Amplification. Formalin-fixed, paraffin-embedded tissue samples of 31 Dukes B and 42 Dukes C colorectal tumors were retrieved from the pathological archives of various Dutch hospitals. Patients were surgically treated between 1974 and 1982. From these samples, 10-μm sections were cut; the microtome and blade were thoroughly cleaned with xylene between the specimens to prevent cross-contamination. Tumor tissue was scraped off, and after deparaffination, the tissue was incubated with proteinase-K. A 178-bp fragment of the first exon of the Ki-*ras* gene was amplified using PCR in a Hybaid Thermal Reactor (Westburg). The sense primer was 5′ CTG TAT CAA AGA ATG GTC CTG CAC 3′; the antisense primer was 5′ AGG CCT GCT GAA AAT GAC TGA ATA 3′. After denaturation for 3 min at 94°C, 500 ng DNA was allowed to run for 30 s at 92°C, 90 s at 60°C, and 150 s at 72°C for 40 cycles in 10 mM Tris (pH 8.3), 50 mM KCl, and 2 mM $MgCl_2$.

SSCP and Sequence Analysis. For SSCP analysis, 0.1 μl of these DNA fragments was reamplified with a set of nested primers: 5′ AAA ATG ACT GAA TAT AAA CTT GTG G 3′ and 5′ CTC TAT TGT TGG ATC ATA TTC GTC 3′. After denaturation for 1 min at 92°C, reamplification was done in 20 cycles of 30 s at 92°C, 30 s at 60°C, and 30 s at 72°C, followed by 20 cycles of 30 s at 92°C, 60 s at 60°C, and 30 s at 72°C.

The PCR products of 114 bp were analyzed by electrophoresis on a 12% nondenaturing polyacrylamide gel containing 10% glycerol at 25 W for 18 h at room temperature (10).

For sequencing, 50 μl of the 178-bp PCR product obtained with the first PCR reaction were purified by precipitation with isopropanol and 4 M ammonium acetate. After centrifugation at 12,000 rpm (4°C), 150–200 fmol DNA were sequenced according to a modification of the method of Sanger (11) with the dsDNA Cycle Sequencing System (Life Technologies, Inc.) following the supplier's instructions. In brief, 1 pmol of a primer (5′ CTC TAT TGT TGG ATC ATA TTC GTC 3′) was 5′-end-labeled with 10 μCi [γ-^{32}P]ATP (Amersham) and T4-polymerase and applied in the dideoxy chain termination reaction; the 178-bp DNA fragment was subjected to 20 cycles at 95°C for 30 s, at 58°C for 30 s, and at 70°C for 30 s, followed by 10 cycles at 95°C for 30 s and at 70°C for 60 s. After terminating the reaction, 2.5-μl volumes were applied to a pre-run (30′; 1000 V) 8% urea-polyacrylamide gel (acrylamide:*N,N′*-methylenebisacrylamide = 29:1, v/v) and separated at 1800 V during 2 h. The gel was autoradiographed overnight with Kodak X-Omat film.

Results

Fig. 1 shows representative results obtained with SSCP analysis. The reproducibility of the method was determined by reanalyzing, in an independent experiment, a representative selection of 12 paraffin sections from 6 Dukes B and 6 Dukes C samples, each containing a point mutation. In 11 of 12 samples, the same mutation was detected, indicating a good reproducibility of the SSCP analysis.

Results of the SSCP analysis are shown in Table 1. Of 31 Dukes B tumors, 17 (55%) showed mutations in the first exon of Ki-*ras*. Table 2 summarizes the type of mutations in codon 12; 12 of the 17 mutations appeared to be G-A transitions, and 5 mutations were G-T transversions. No G-C changes were observed in these tumors.

In 22 of 42 Dukes C samples (52%), one or more point mutations in codon 12 were detected by SSCP analysis. In these specimens a

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[4] The abbreviations used are: SSCP, single-stranded conformational polymorphism; PCR, polymerase chain reaction; bp, base pair(s).

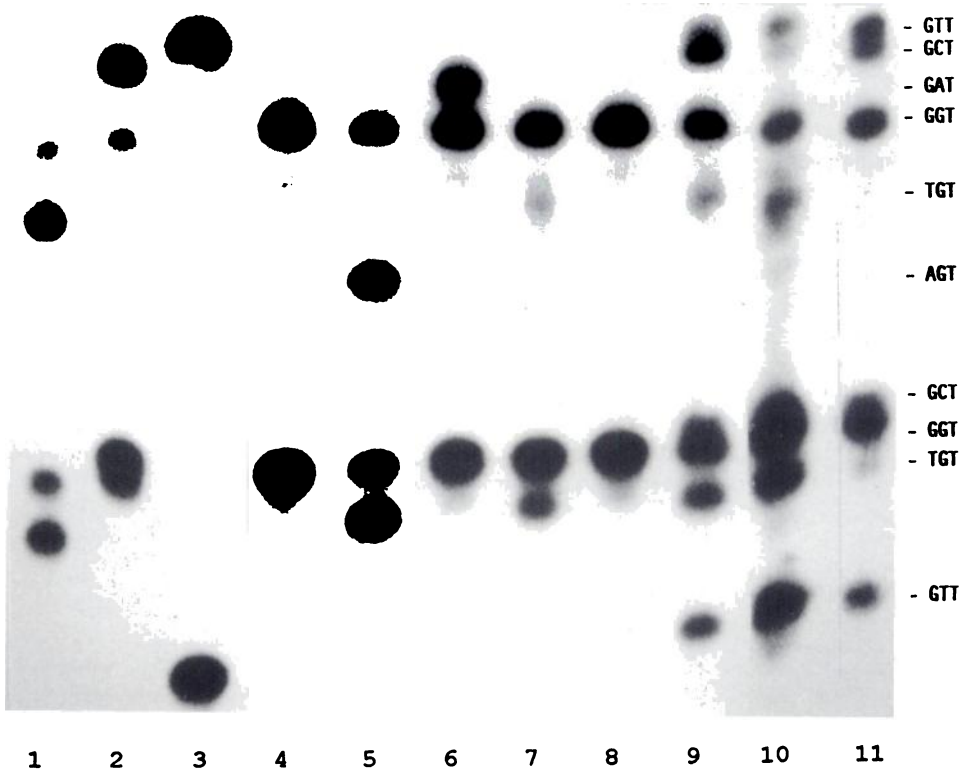

Fig. 1. Representative samples with different mutations in the first exon of Ki-*ras* that were determined by SSCP. *Lanes 1–6,* samples with respective sequences of TGT, GCT, GTT, GGT, AGT, and GAT in codon 12. *Lanes 7–11,* tumor samples. *Lane 7,* TGT; *Lane 8,* nonmutated GGT; *Lane 9,* TGT, GTT, and GCT; *Lane 10,* TGT, GTT, and GCT; *Lane 11,* GTT and GCT. Note that all tumor samples contain wild-type DNA.

Table 1 *Mutations in the first exon of the Ki-ras gene in relation to tumor progression*

Sequence	Amino acid	Dukes B No. of tumors (%)	Dukes C No. of tumors (%)
GGT (wild type)	Gly	14 (45)	20 (48)
TGT	Cys	1 (3)	1 (2)
AGT	Ser	4 (13)	0 (0)
CGT	Arg	0 (0)	0 (0)
GTT	Val	2 (6)	8 (19)
GAT	Asp	8 (26)	0 (0)
GCT	Ala	0 (0)	1 (2)
GTT, GCT	Val, Ala	0 (0)	7 (17)
TGT, GTT	Cys, Val	1 (3)	1 (2)
TGT, GTT, GCT	Cys, Val, Ala	0 (0)	3 (7)
Codon 13:CGT	Arg	0 (0)	1 (2)
Codon 8:GTG		1 (3)	0 (0)
		$n = 31$	$n = 42$

Table 2 *Frequency of the base transitions and transversions in codon 12 of the Ki-ras gene*

Base substitution	Dukes B No. of mutations	Dukes C No. of mutations
G → A	12	0
G → T	5	24
G → C	0	11

total number of 35 mutations in codon 12 was found. In contrast with the samples of the Dukes B series, no G-A transition could be detected in Dukes C specimens. Twenty-four of these 35 mutations were found to be G-T transversions; the other mutations were found to be G-C transversions. Thus, the type of mutation appeared to differ significantly in different stages of tumor development; 82% (24 of 29; $P = 0.008$) of all G-T transversions in codon 12, as well as all 11 G-C transversions ($P = 0.009$), were observed in Dukes C tumors. In contrast, all detected G-A transitions ($P < 0.001$) were found in Dukes B tumors.

In nine samples, two mutations, and in three samples, three mutations of codon 12 were detected. The number of tumors with multiple mutations was significantly higher ($P = 0.003$) in Dukes C tumors (26%) than in Dukes B tumors (3%).

The only mutations in the first exon outside codon 12 appeared to be one in codon 13 and one in codon 8 (Table 1).

In order to validate the SSCP results, all samples were analyzed also by sequencing. In 77% of the mutated samples, the same mutation was detected with both methods. However, in 23% of the mutated tumors, the mutation that was detected by SSCP could not be confirmed by sequencing. Assuming that mutated cells are mixed with populations of nonmutated cells, such as stromal cells, this discrepancy can be explained by a different sensitivity of both methods. Experiments to establish the sensitivity of these methods demonstrated that SSCP could detect at least 5% mutated cells in a heterogenous cell population, whereas for sequencing analysis, at least 10% mutated cells were required for detection.[5]

Discussion

The results of our study indicate that Dukes B and Dukes C tumors show very significant differences in genetic makeup. This could imply that the concept that all Dukes C tumors eventually evolve from Dukes B tumors is too simplistic. Instead, it is conceivable that the metastatic potential of these tumors is related to the nature of genetic alterations in codon 12 of the Ki-*ras* oncogene, G-T and/or G-C transversions endowing the tumor cells with the capacity to metastasize. Consequently, the five Dukes B tumors with G-T transversions would progress toward the Dukes C stage, whereas tumors which only show a G-A transition do not progress into a higher stage due to the absence of (lymphogenous) metastatic potential. In our view, the functional impact of the ras protein resulting from different types of mutations could be important. In the literature thus far, data on this issue are scarce. One study demonstrated different transforming capacities, with G-T mutants being more potent than G-A mutants, as

[5] P. Moerkerk, J. W. Arends, M. van Driel, A. de Bruïne, A. de Goeij and J. ten Kate, manuscript in preparation.

determined by colony morphology in rat fibroblasts transfected with mutated H-*ras* genes (12). Another study showed that GAT-mutated colonic tumors did not give rise to recurrences (13).

The number of multiple mutations of the *ras* gene was significantly higher in Dukes C tumors (Table 1), which implies a heterogeneous population of tumor cells. This could reflect an increase of the genetic instability of tumors in advanced stages of tumor progression (14). This phenomenon was also observed in our recent model study in which a mutated H-*ras* gene, transfected into a colon tumor cell line SW480, led to increasing genetic instability (15).

In conclusion, the results of our study demonstrated a basically different genetic makeup of tumors in different stages of tumor extension. In addition, the occurrence of multiple point mutations in the Ki-*ras* gene in Dukes C as compared to Dukes B tumors indicates that colon tumor progression is accompanied by increased genetic instability. These results could imply that the type and number of Ki-*ras* point mutations fundamentally affect the biological behavior of the tumor.

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