Original Article
KRAS gene mutations are more common in colorectal villous adenomas and *in situ* carcinomas than in carcinomas

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Abstract: We have evaluated the frequency of *KRAS* gene mutations during the critical transition from villous adenoma to colorectal carcinoma to assess whether the adenomas contain a *KRAS* mutation more frequently than carcinomas. We analyzed sporadic villous and tubulovillous adenomas, *in situ* carcinomas, and primary colorectal carcinomas from multiple patients. The cancers were further evaluated for mucinous status and microsatellite instability. Standard PCR molecular techniques were used for *KRAS* and microsatellite analyses. A *KRAS* mutation was found in 61.9% of 134 adenomas, 67.8% of 84 *in situ* carcinomas, and just 31.6% of 171 carcinomas. Our study clearly demonstrates that tubulovillous and villous adenomas, as well as both the benign and malignant parts of *in situ* carcinomas, are statistically more likely to contain a somatic *KRAS* gene mutation than colorectal carcinomas. This difference is confined to the non-mucinous and the microsatellite stable tumors. Our data support the possibility that non-mucinous and microsatellite stable carcinomas with wild-type *KRAS* gene may have had a mutation in the *KRAS* gene during their earlier stages, with the mutation lost during further growth.

Introduction

Colorectal cancer cells demonstrate a number of genetic changes, and it is now accepted that the accumulation of these changes, rather than a specific sequence, determines the development of the malignancy. Some of these genetic changes can be detected within colorectal adenomas, which are the precursor lesions for many colorectal cancers [1]. Current theories regarding tumor progression suggest that there is a process of clonal evolution involving progressively more biologically aggressive subpopulations, with the sequential accumulation of mutations in a number of genes [2]. The retention of all new mutations from one subpopulation to the next is implied in this concept. Therefore, it is assumed that once a molecular genetic change has occurred, it is retained throughout the progression of the benign adenoma into a colorectal cancer.

Somatic *KRAS* gene mutations are important in the carcinogenesis process [3], and they occur in approximately one-third of colorectal cancers [4]. Our previous studies [5], as well as the studies of others, have demonstrated the presence of *KRAS* mutations in adenomas, and have suggested, interestingly, that there may be a higher frequency of this mutation in villous adenomas than in carcinomas. Were this observation found to be consistent within a larger study group, the finding would provide a further clue concerning the molecular biology of colorectal cancer [6]. To clarify this issue, we have evaluated the frequency of *KRAS* mutations during the critical transition from villous adenoma to colorectal carcinoma by analyzing villous and tubulovillous adenomas, *in situ* carcinomas, and primary colorectal carcinomas from multiple patients. We also assessed the mucinous status and microsatellite instability for each
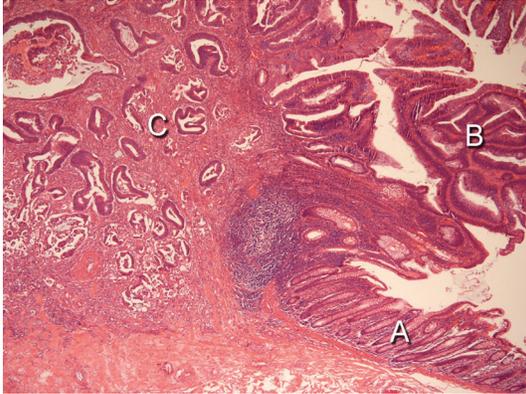
Figure 1. An *in situ* carcinoma. Hematoxylin and Eosin stain, 400X power. A: normal colonic mucosa. B: residual villous adenoma. C: *in situ* carcinoma.

carcinoma and its relationship to *KRAS* mutation.

Materials and methods

Our research has involved a series of studies evaluating particular groups of patients with colorectal neoplasms with respect to molecular genetic abnormalities, both germ line and somatic. Each individual study was approved by the hospital Institutional Review Board, as was this analysis. All samples were archived material from the Department of Pathology. Clinical material primarily reflects a suburban community of middle economic level, with substantial representations from various minority groups (Asian, African-American) of both middle and low economic status. Histological slides stained with hematoxylin and eosin (H&E) and paraffin blocks were available for all cases. One clinical pathologist reviewed all histological slides and indicated the areas for molecular study. Criteria for differentiation of adenomas followed the World Health Organization criteria with respect to villous component: tubular adenomas, <20%; tubulovillous adenomas, 20 – 80%; and villous adenomas, >80%. Mucinous carcinoma was diagnosed when at least 50% of the tumor contained secretory mucin. Right side colonic segments were defined as cecum, ascending, hepatic flexure, transverse; and the left side was considered the splenic flexure, descending, sigmoid and rectum. We defined *in situ* carcinomas (ISCs) as tumors in which both a benign component and a malignant component were present (**Figure 1**). We limited our study to *in situ* cancers with villous or tubulovillous
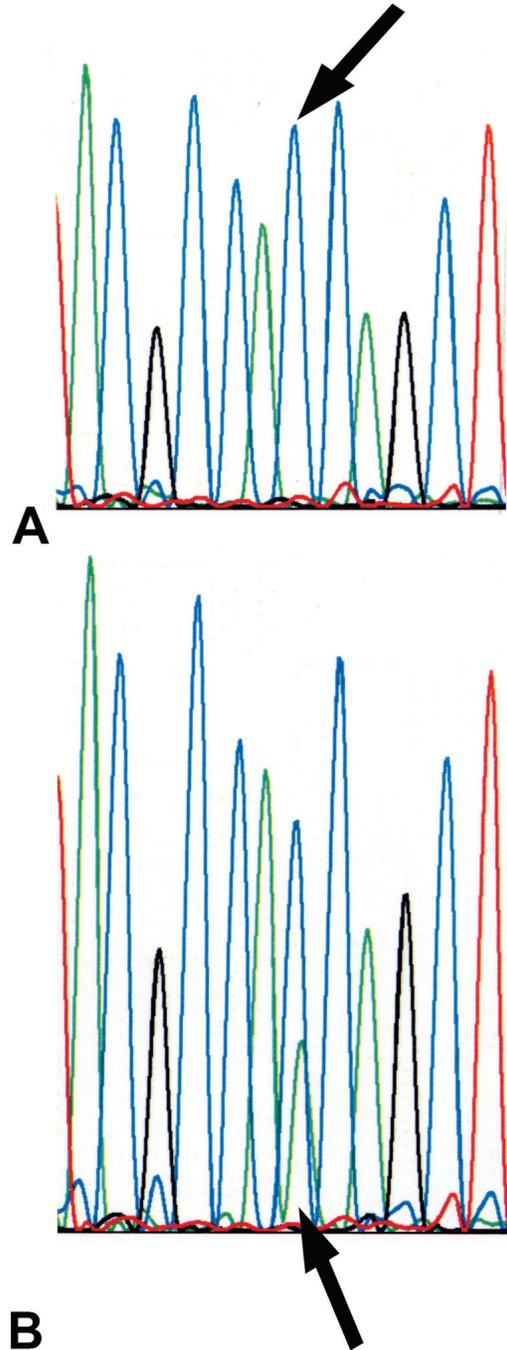

Figure 2. KRAS DNA sequence analysis. A: DNA from normal tissue. The arrow indicates the wild-type pattern with a single peak at the second nucleotide position of codon 12. B: DNA from an ascending colon carcinoma. The arrow indicates a heterozygous mutant peak under the wild-type peak.

benign areas and did not include the uncommon ISCs with only benign tubular areas.

Our series of studies contained patients who had numerous tumors removed over intervals

spanning as much as 20 years. Other patients were determined to have germ line mutations found most frequently in Ashkenazi Jews, including the *APC*I1307K, MSH21906G>C**, and *BLM*[Ash]. In this current analysis, we excluded patients with known germ line mutations and patients with multiple tumors, on the assumption that the development of mutations in tumors from these individuals might occur through mechanisms unrelated to the process occurring in isolated sporadic cases. Patients were included only if they were found, over time, to have the following: a maximum of only one or two other adenomas for the tubulovillous and villous group, and a maximum of only one other adenoma for the *in situ* carcinoma and the carcinoma groups.

DNA extraction and purification

All tissue specimens were formalin-fixed and paraffin-embedded. Histological slides stained with H&E were examined and the area of relevant tissue was identified and marked, as was an area of normal tissue. Consecutive unstained slides from the paraffin block were then prepared and the corresponding areas were isolated under a dissecting microscope by manual dissection. The paraffin wax was removed by xylene and ethanol washes. The cellular material was lysed in a proteinase potassium buffer solution. DNA was isolated and purified using the Qiagen QIAmp Tissue Kit (Qiagen Inc., Valencia, CA).

Sequence analysis of the KRAS gene

The codon 12/13 region of exon 1 in the *KRAS* gene was amplified using the primer set 5'-AAGGCCTGCTGAAAATGACTG-3' sense and 5-GGTCCTGCACCAGTAATATGCA-3'. Hot-start PCR was performed in 50 μl volumes with AmpliTAq Gold polymerase and ABI reagents (Applied Biosystems, Foster City, CA) using 100 ng of DNA, 50 pmols of primer, and 2.0 mM MgCl$_2$ on a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA). PCR consisted of an initial 8 minutes of denaturation at 94°C, followed by a total of 40 cycles of a 30 second denaturation at 94°C, a 30 second annealing, and a one minute elongation at 72°C, with a final 30 minute extension at 72°C. The annealing temperature was stepped down at 62°C, 60°C, and 58°C for 5, 20, and 15 cycles respectively. The post-PCR products were qual-

ity checked by agarose gel and then purified using the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA) prior to sequencing. The sequencing reactions were done in 20 μl volumes using 0.5X BigDye Terminator Cycle Sequencing Reagents (Applied Biosystems, Foster City, CA), 5.0 pmol of the reverse *Ki-ras* primer, and 1.0 μl of the purified PCR reaction. Reactions are run on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) for 25 cycles using 2 minutes of extension time. The sequencing reaction fragments were cleaned by isopropanol precipitation. Sequencing products were separated by capillary electrophoresis with an ABI 3130 Genetic Analyzer and the data was processed with Sequencing Analysis (Applied Biosystems, Foster City, CA) software (**Figure 2**).

Microsatellite instability (MSI) analysis

MSI was detected using the Bethesda panel of markers that include two mononucleotide markers Bat25 and Bat26, and three dinucleotide markers D2S123, D5S346, and D17S250. In all primer sets the forward primer contains a 5' fluorescent label while the reverse primer contains a 5'- GTGTCTT tail. PCR was performed in 30 μl volumes with AmpliTaq polymerase and ABI reagents (Applied Biosystems, Foster City, CA) using 100 ng of DNA, 8 pmols of primer, and 1.5 mM MgCl$_2$ on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). PCR consisted of an initial denaturation at 94°C, followed by 35 cycles of a 30 second denaturation at 94°C, a 30 second annealing at 55°C, and a 60 second elongation at 72°C, with a final 30 minute extension at 72°C. PCR products were separated by capillary electrophoresis with an ABI 3130 Genetic Analyzer and the data was processed with GeneMapper (Applied Biosystems, Foster City, CA) software. A tumor was defined as MSI-high if two or more of the five markers had a changed allele pattern, and is referred to as "MSI".

Statistical analysis

The main analysis focuses on whether the frequency of *KRAS* gene mutation differs for adenomas, *in situ* carcinomas, and carcinomas, controlling for mucin status and microsatellite instability for carcinomas. The *t*-test was used for two group comparisons, and analysis of variance was used for three group comparisons

Table 1. Total, excluded, and study groups of tumors. Percentages with a *KRAS* gene mutation indicated for the tumor types of the excluded group

Group	Total # studied	TV/Vil* (% mutated)	ISC† (% mutated)	Cancer
Total	914	241	205	468
Excluded	525	107 (49.5)	121 (50.4)	297 (31.0)
Study	389	134	84	171

*TV/vil = tubulovillous and villous adenomas. †ISC = *in situ* carcinoma.

Table 2. Clinical characteristics of study patients and their tumors

	Adenomas No. (%)	Cancer part of ISCs No. (%)	Cancer No. (%)
Characteristic			
Number	134	84	171
Age (mean)	66.9	66.9	69.2
S.D	11.5	12.6	12.5
Gender			
Male	59 (44)	38 (45.2)	75 (44)
Female	75 (56)	46 (54.8)	96 (56)
Location			
Right	66 (49.3)	61 (72.6)	93 (54.4)
Left	68 (50.7)	23 (27.4)	78 (45.6)
Microsatellite status			
Stable		73 (92.4)	141 (84.4)
Unstable		6 (7.6)	26 (15.6)
Mucin status			
Mucinous		21 (25)	31 (18.4)
Non-mucinous		63 (75)	137 (81.6)

potentially resulting in a greater likelihood of a cancer. The incidence of a *KRAS* mutation was statistically more common in the excluded adenomas than the excluded carcinomas ([odds ratio] OR = 2.19, [confidence interval] CI = 1.39-3.44, $p </= 0.0006$), and also more common in the carcinomatous portion of the ISCs than in the carcinomas (OR = 2.26, CI = 1.47-3.49, $p </= 0.0002$) (**Table 1**). For all excluded tumors, 80.6% of the *KRAS* mutations were in codon 12, 17.0% were in codon 13 and 2.4% were other. Of the 166 mutations in codon 12, 143 (86.1%) were in the second position and 23 (13.9%) were in the first position. Of the 35 mutations in codon 13, only one was in the first position, all others were in the second position. With our exclusion criteria, the final study group consisted of a total of 389 tumors, with 134 (34.4%) tubulovillous and villous adenomas, 84 (21.6%) *in situ* carcinomas, and 171 (44%) carcinomas. Clinical characteristics of age, gender and location for these three study groups of tumors are shown in **Table 2**.

Adenomas

The adenoma study group contained 34 tubulovillous and 100 villous adenomas. A comparison between these two subtypes of adenomas showed no statistical difference in mean age of the patient when the tumor was removed (64.7 vs 67.6 years; *p*=0.20), percentage of patients of male gender (50% vs 58%; *p*=0.42), percentage of tumors from the left colon (64.7% vs 46%; *p*=0.06), or the percentage of tumors with a *KRAS* gene mutation (67.6% vs 60.0% *p*=0.43). Therefore, these two groups were combined for further analysis.

A *KRAS* mutation was detected in 83 (61.9%) of the 134 adenomas. One adenoma had two

regarding mean age by group. The chi-square test was used to assess differences in proportions for characteristics by tumor group. We derived odds ratios for *KRAS* status for each group in a case-case analysis of adenoma or *in-situ* cancer compared to cancer. These case-case odds ratios were also derived controlling for mucinous status and microsatellite instability using the Mantel-Haenszel procedure with test for heterogeneity. Ninety-five percent confidence intervals were derived for the odds ratios [7].

Results

Selection of study cases

Our series of studies included a total of 914 tumors, and the excluded group consisted of 525 tumors from 406 patients (**Table 1**). A higher percentage of carcinomas, rather than adenomas or *in situ* cancers, were excluded, reflecting that excluded patients had germ line mutations or multiple tumors, both features

Table 3. Numbers of tumors by colon segment and percentage with a *KRAS* gene mutation

Tumor type	Cecum # (%)	Ascend # (%)	Transv # (%)	Right[*] # (%)	Descend # (%)	Sigmoid # (%)	Rect # (%)	Left[*] # (%)
Adenoma	15 (87)	22 (68)	22 (55)	7 (86)	12 (75)	43 (53)	12 (33)	1 (100)
In situ cancer	28 (71)	17 (82)	6 (50)	10 (80)	2 (50)	17 (47)	3 (100)	1 (0)
Cancer	39 (38)	30 (37)	22 (23)	2 (0)	7 (14)	63 (29)	4 (75)	4 (25)

[*]Tumor labeled only as right or left colon.

Table 4. *KRAS* gene findings in 84 *in situ* carcinomas by benign and malignant portions

Benign / Malignant	Number (%)
Wild / wild	24 (28.6)
Same mutation	43 (51.2)
Different mutations	4 (4.8)
Mutated / wild	3 (3.6)
Wild / mutated	10 (11.9)

separate *KRAS* mutations for a total of 84 mutations. Of the 69 tumors with mutations in codon 12, there were 12 mutations in the first position (c.34G>A = 8, c.34G>C = 1, and c.34G>T = 3); and 57 mutations in the second position (c.35G>A = 23, c.35G>C = 1, c.35G>T = 33). One of the 15 mutations in codon 13 was in the first position (c.37G>T), and 14 were in the second position, (c.38G>A). For the adenomas, there was no difference between tumors with or without a *KRAS* mutation by gender (for females, *p* = 0.84); or by age (67.8 vs 65.4, *p* = 0.24). A total of 66 adenomas (49.3%) were from the right colon and 68 (50.7%) were from the left colon. A *KRAS* mutation was less likely to be found in an adenoma from the left colon (54.4%) than from the right colon (69.7%), but this was not statistically different (*p* = 0.07). The distribution of the adenomas and percentage with a *KRAS* mutation is shown in **Table 3**. Of the 134 adenomas, 86 (64.2%) were tested for MSI. Only one (1.2%) adenoma demonstrated microsatellite instability and it contained only wild type *KRAS* gene.

In situ carcinomas

A *KRAS* mutation was detected in the benign portions of *in situ* tumors (ISCs) in 50 of 84 (59.5%) ISCs, while the malignant portion contained a *KRAS* mutation in 57 of 84 ISC (67.8%) (p = 0.052). There were 61 (72.6%) ISCs found in the right colon and 23 (27.4%) from the left colon. The distribution of the ISCs with percentages of *KRAS* mutations is shown in **Table 3**.

Both the benign and malignant parts of 24 of 84 (28.6%) ISCs contained only wild type *KRAS* genes. The mutational changes in the two parts of the other 60 ISCs are shown in **Table 4**.

Four ISCs demonstrated a mutation in the first codon position of codon 12: c.34G>T = 3, c.34G>A = 1. The second position of the codon 12 contained a mutation in 39 lesions: c.35G>A = 21, c.35G >T = 11; and codon 13: c.38G>A = 7. There was no difference between the ISCs with or without a *KRAS* gene mutation in the carcinomatous portion when controlling for age (68.0 vs 64.4, *p* = 0.22); or gender (56.1% for females with a mutated ISC, *p* = 0.71). A *KRAS* mutation was less likely to be found in the carcinomatous portion of an ISC from the left colon (52.2%) than from the right colon (73.8%), but this was not statistically different, (*p* = 0.06). A total of 79 (94%) ISCs were assayed for MSI within the malignant part of the tumor, and microsatellite instability was detected in six (7.6%). In 5 of these 6 tumors the *KRAS* gene was wild type, and one demonstrated a *KRAS* mutation. All 84 ISCs were evaluated for mucin within the carcinomatous area and 21 (25%) were mucinous. Eleven of the mucinous ISCs contained a *KRAS* mutation (52.4%), and 46 of the 63 non-mucinous ISCs (73%) contained a *KRAS* mutation. This difference did not reach statistical significance, (*p* = 0.08).

Carcinomas

A *KRAS* gene mutation was found in 54 (31.6%) of 171 carcinomas. There were 93 (54.4%) carcinomas from the right side and 78 (45.6%) from the left side. The distribution of the cancers and percentage with a *KRAS* mutation is shown in **Table 3**. There were 44 mutations in codon 12, 8 were in the first position (c.34G>A = 3, c.34G>C = 1, c.34G>T = 4), and 36 mutations in the second position (c.35G>A = 14, c.35G>C = 5, c.35G>T = 17). There were 9 mutations in codon 13, all in the second posi-

Table 5. *KRAS* gene mutation for each tumor type, with or without mucin or MSI

	Tumor Type						Odds Ratio In Situ versus Cancer	95% Confi-dence Interval	*P*-value	Interaction effect
	Adenomas[*]		In situ cancer[†]		Cancer[‡]					
Ki-ras	No.	Mutated n (%)	No.	Mutated n (%)	No.	Mutated n (%)				
Total	134	83 (61.9)	84	57 (67.8)	171	54 (31.6)	4.6	2.6-8.0	<0.0001[§]	
Mucinous status										
Non-mucinous	-	-	63	46 (73.0)	137	40 (29.2)	6.6	3.4-12.9	<0.0001[§]	0.01
Mucinous	-	-	21	11 (52.4)	31	14 (45.2)	1.3	0.4-4.1	0.6	
Microsatellite status										
Microsatellite stable	-	-	73	52 (71.2)	141	50 (35.5)	4.5	2.4-8.3	<0.0001[§]	0.4
Microsatellite unstable	-	-	6	1 (16.7)	26	3 (11.5)	1.5	0.1-18.0	0.7	

[*]Adenomas vs. Cancers: O.R. = 3.5, C.I. = 2.2 – 5.7 *p* = <0.0001. [†]5 *in situ* cancers not assayed for microsatellite instability. [‡]3 cancers not assayed for mucin. [§]denotes statistical significance.

tion c.38G>A. One carcinoma contained a mutation in codon 19, c.57G>T.

There was no difference between the carcinomas with or without a *KRAS* gene mutation when controlling for gender (53.7% for females with a mutated cancer, *p* = 0.66), or location (29.5% of left side carcinomas had a mutation and 33.3% of the carcinomas from the right side, *p* = 0.59); or age (68.4 vs 69.6, *p* = 0.58). There were only 5 carcinomas occurring in patients less than 45 years of age at the time of diagnosis, and two contained a *KRAS* mutation while three did not. There was no difference in the percentages of cancers with a *KRAS* mutation across three age distributions of </= 59 years (35.1%), 60-74 years (30.1%), and >74 years (30%) (*p* = 0.84).

A total of 167 cancers were assessed for microsatellite instability, and 141 (84.4%) cancers were microsatellite stable while 26 (15.6%) demonstrated MSI. A *KRAS* mutation was more frequently seen in cancers that were microsatellite stable (35.5%) than in those with MSI (11.5%), (*p*=0.02). A total of 168 cancers (98.2%) were assessed for the presence of mucin, and 31 cancers (18.4%) contained mucin while 137 (81.6%) did not. Of the 26 (15.6%) cancers with microsatellite instability, 25 were tested for mucin and 9 (36%) contained mucin and 16 (64%) did not. A higher percentage of cancers with MSI contained mucin (36%) than did microsatellite stable cancers (15%), (*p* = 0.01). Mucinous cancers were more likely to have a *KRAS* mutation (45.2%) than non-mucinous cancers (29.2%), but this

difference did not reach statistical significant (*p*=0.08).

Comparisons among tumor types

There was no difference in the percentage of tumors with a *KRAS* mutation between the adenomas (61.9%) and the ISCs (67.8%), (OR = 0.77, CI = 0.43-1.37, *p* = 0.37). However, a *KRAS* gene mutation was significantly less frequent in the carcinomas (31.6%) than in either the adenomas or in the *in situ* tumors (**Table 5**). The difference in frequency of *KRAS* mutation between adenomas plus ISCs compared to carcinomas is also statistically significant, (OR = 3.9, CI = 2.5-5.9, *p* < 0.0001).

A statistically significant difference was found between the frequency of a *KRAS* mutation in non-mucinous ISCs (73%) and non-mucinous carcinomas (29.2%), (OR = 6.6, CI = 3.4-12.8, *p* < 0.0001). There was no statistical difference between mucinous ISCs and mucinous carcinomas regarding the frequency of *KRAS* mutation (OR = 1.3, CI = 0.4-4.1, *p* = 0.6) (**Table 5**).

The percentage of microsatellite stable ISCs (carcinomatous portion) with a *KRAS* mutation (71.2%) is statistically greater than the percentage found in microsatellite stable carcinomas (35.5%) (OR = 4.5, CI = 2.4-8.3, *p* < 0.0001). The numbers of ISCs and carcinomas found to be microsatellite unstable was small, 6 and 26 respectively. There was a slightly greater percentage of unstable ISCs with *KRAS* mutation than unstable carcinomas, but this was not statistically significant (OR =1.5, CI =0.1-18.0, *p*

=0.7) (**Table 5**). Overall odds ratios are higher in the adenomas and the *in situ* carcinomas than in the carcinomas. However, this relationship is confined to the non-mucinous and the microsatellite stable tumors. In the mucinous and microsatellite unstable tumors, there is much less difference in the incidence of *KRAS* mutation between the tumor types, with test of heterogeneity 0.01 for mucin status and 0.4 for microsatellite status.

Discussion

Numerous studies have reported the frequency of *KRAS* gene mutations in colorectal cancer. The largest series was the "*RASCAL*" study of 2721 colorectal cancer cases reporting an incidence of 37.7% [4]. Similar frequencies were reported subsequently [8-10]. Information on the frequency of *KRAS* gene mutation in adenomas is more limited, particularly with respect to adenomas with villous features. The largest study reported a *KRAS* mutation in 78 (32.8%) of 238 tubulovillous and villous adenomas. This study, however, included only 30 villous adenomas, with 12 (40%) having a mutation [11]. A smaller study reported a *KRAS* gene mutation in 25 of 49 (51%) adenomas with moderate dysplasia [12], while another small study found a *KRAS* mutation rate of 15.9% (7 of 44) in a mixture of tubulovillous and villous adenomas [13]. None of these three articles included comparable data on carcinomas. We previously reported a frequency of 52.5% (21 of 40) for *KRAS* gene mutations in tubulovillous and villous adenomas, and we suggested the possibility of a higher incidence of *KRAS* mutation in villous adenomas than in colorectal carcinomas [5]. By comparison, the frequency of *KRAS* mutations in tubular adenomas is quite low [1]. We previously reported a frequency of only 9.2% in 553 tubular adenomas [14]; consequently, we have not included data on tubular adenomas in this report.

The frequency of *KRAS* gene mutation in carcinomas was the same for both our excluded group and our study group. However, the frequency of *KRAS* mutations was borderline lower for adenomas (p </= 0.054) and lower for ISCs (p </= 0.013) in the excluded group than for the study group, possibly suggesting that other molecular mechanisms may be more critical for tumorigenesis in the excluded adenomas and ISCs.

KRAS mutations in carcinomas with mucinous features

In the *RASCAL* study, mucinous status was reported for 790 (60%) of the accumulated cases, and mucin was present in 252 (31.9%) cancers [4]. A *KRAS* mutation was present in 44% of the mucinous cancers and in 35.1% of the non-mucinous cancers. This is statistically significant, with *p* =0.0161, (O.R. = 1.45, C.I. = 1.07-1.97). Similar results have been reported in other, smaller studies [15], indicating that *KRAS* gene mutations are more common in mucinous cancers than in cancers without mucin. Our data on carcinomas are similar, but did not reach statistical significance. In contrast, our mucinous ISCs had a lower incidence of *KRAS* mutation than the non-mucinous ISCs, but this also did not reach statistical significance.

KRAS gene mutations in cancers with microsatellite instability

Microsatellite instability (MSI-H) is detected in 10% to 15% of sporadic colorectal cancers [16], and studies have shown a lower frequency of *KRAS* gene mutation in colon carcinomas with microsatellite instability than in microsatellite stable cancers [17]. Our data support this finding. There have been several reports showing that microsatellite unstable carcinomas are frequently mucinous [18, 19]. As a *KRAS* mutation is more common with mucinous carcinomas, and mucin is more common with MSI-H tumors, one would anticipate a higher incidence of *KRAS* mutation in MSI-H tumors, but this is not the usual finding. It is paradoxical that *KRAS* mutation is actually more common with microsatellite stable tumors. Perhaps the reports detailing carcinomas with MSI and mucin included patients for whom MSI developed through several different mechanisms – germ line as well as somatic – and that the correlation between MSI and mucin is not the same for all mechanisms. It is also possible that other unknown, or additional molecular genetic changes in the tumors may overwhelm the relationship between mucinous feature and *KRAS* mutation.

Our study clearly demonstrates that tubulovillous and villous adenomas, as well as both the benign and malignant parts of *in situ* carcinomas, are statistically more likely to contain a

somatic *KRAS* gene mutation than colorectal carcinomas. However, approximately 70% of colorectal carcinomas lack a mutation in the *KRAS* gene. It is possible that carcinomas with wild-type *KRAS* develop through a process devoid of *KRAS* mutations; for example, the serrated adenoma pathway involving *BRAF* gene mutations and MSI. However, MSI is detected in just 15% of all colorectal carcinomas and this pathway does not explain all *KRAS* wild-type colorectal cancer. Furthermore, the frequency of *KRAS* mutations in our microsatellite stable carcinomas was half the frequency found in adenomas or microsatellite stable ISCs.

Mechanisms leading to the marked differences in the frequency of *KRAS* mutation could arise on the level of the tumor, the cell, or the allele. For example: 1) A precursor adenoma with a *KRAS* mutation might fail to progress as frequently as those adenomas without the mutation. The reported frequency of tubulovillous and villous adenomas found on routine colonoscopic screening in a large study of men and women (ages 50-66 years) without a family history of CRC was reported to be 2.4%, while the frequency of CRC detection was 0.88%, or two to three times less frequent [20]. This cross-sectional data suggests that not all adenomas advance to carcinomas, and the difference in frequency is similar to the difference in *KRAS* mutation rates we determined. This would suggest that an equal percentage of adenomas with and without *KRAS* gene mutation are destined to progress to a carcinoma. If this were true, it would imply there is no particular value of a *KRAS* mutation to the neoplastic process. Furthermore, in the cited study, the frequency of finding an ISC was significantly lower than that of cancers, suggesting further that 'failure to progress' is probably not the explanation for our findings.

2) On the cellular level, initially there could be two distinct populations of tumor cells in an adenoma, and progression beyond a certain point might involve loss of the *KRAS* mutated cells and growth of the cells with wild-type *KRAS*. Indeed, studies have demonstrated that not all areas of a colorectal tumor necessarily demonstrate identical *KRAS* results [21]. With regard to cellular growth, a study of melanomas reported that malignant cells with *BRAF* gene mutations do not outgrow the cells with wild-type *BRAF*, and one suggested explanation is that the cells with *BRAF* mutation undergo senescence [22]. Nonetheless, a colorectal tumor with both *KRAS* wild-type and mutated populations of cells is an infrequent finding, suggesting that two competing clones of tumor cells with one mutated and one wild is also an unlikely explanation for the large difference between adenomas and carcinomas with respect to *KRAS* mutations.

3) On the molecular level, the development of additional random changes might result in the loss of the allele with the *KRAS* mutation during the late stages of a tumor becoming a carcinoma. This mechanism is speculative. It implies that early in carcinogenesis all tumor cells in a particular tumor may contain a *KRAS* mutation, without a competing wild-type clone. The *KRAS* mutation confers a selective growth advantage to the cells at this point in tumorigenesis, and it is a 'driver' mutation. Using a mathematical model, Bozic *et al* showed that the selective advantage of a 'driver' mutation is very low, 0.4% [23]. As pointed out by others, this translates into a high 'extinction rate' for those mutations that confer a selective growth advantage: more than 99% of 'driver' mutations that emerge during tumor development will become extinct [24]. We would then postulate that at a later stage in the neoplastic process, the allele with the *KRAS* mutation is lost from the clonal cells, with retention of the wild type allele.

In conclusion, our data show that tubulovillous and villous adenomas, as well as both the benign and malignant parts of *in situ* carcinomas, are more likely to contain a somatic *KRAS* gene mutation than are colorectal carcinomas. This difference is confined to the non-mucinous and the microsatellite stable carcinomas. These epidemiological data support the possibility that non-mucinous and microsatellite stable carcinomas with wild-type *KRAS* gene may have had a mutation in the *KRAS* gene during their earlier stages, with the mutation lost during further growth of the tumor.

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