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Association between clinicopathological characteristics and *RAS* mutation in colorectal cancer

Johan Rimbert[1,2,3], Gaëlle Tachon[1,2,4], Audelaure Junca[1,3], Claire Villalva[1,2], Lucie Karayan-Tapon[1,2,4,7] and David Tougeron[1,5,6,7]

[1]Faculty of Medicine and Pharmaceutical Science, University of Poitiers, Poitiers, France; [2]Department of Cancer Biology, Poitiers University Hospital, Poitiers, France; [3]Department of Pathology, Poitiers University Hospital, Poitiers, France; [4]INSERM U-1084, Experimental and Clinical Neurosciences Laboratory, Cellular Therapies in Brain Diseases Group, University of Poitiers, Poitiers, France; [5]Department of Gastroenterology, Poitiers University Hospital, Poitiers, France and [6]Laboratory Inflammation, Tissus Epithéliaux et Cytokines, EA 4331, University of Poitiers, Poitiers, France

In colorectal cancer, *KRAS* (exons 2, 3, and 4) and *NRAS* (exons 2, 3, and 4) mutations are associated with resistance to antiepidermal growth factor receptor monoclonal antibodies, and *BRAF* mutation is a molecular marker of poor prognosis. *KRAS* exon 2 and *BRAF*-mutated colorectal cancers have well-known distinct clinicopathological characteristics. Comparison of tumors with different *RAS* status (exons 2, 3, and 4 of *KRAS* and *NRAS*) based on their clinicopathological characteristics has never been established. All colorectal cancer patients with *RAS* and *BRAF* testing from 2011 to 2015 were included in this observational retrospective study. Patient and tumor characteristics were collected and correlation with *RAS* and *BRAF* status was evaluated. A total of 1735 patients with colorectal cancer were included. *RAS*-mutated colorectal cancers ($n=1002$), compared with *RAS* wild-type colorectal cancers ($n=733$), were significantly associated with male gender, classical adenocarcinoma subtype, well/moderately differentiated tumors, and microsatellite stable phenotype. *KRAS* codon 13-mutated colorectal cancers ($n=171$), compared with *RAS* wild-type colorectal cancers, more frequently presented classical adenocarcinoma subtype and microsatellite stable phenotype. In comparison with other *RAS* mutations, *KRAS* exon 3-mutated colorectal cancers ($n=23$) were associated with mucinous/rare histological subtypes and, most likely to located in the rectum. *KRAS* exon 4-mutated colorectal cancers ($n=33$) were more frequently associated with mucinous/rare histological subtypes. There was no significant association between *NRAS* mutation ($n=37$) and clinicopathological features. Colorectal cancers are associated with different clinicopathological features according to the type of *RAS* mutation. Consequently, these particular characteristics must be considered when assessing the prognostic value of *RAS* status in colorectal cancer.
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Colorectal cancer is the third most common cancer in the world,[1] with nearly 1.4 million new cases diagnosed each year. An estimated, 700 000 people die from colorectal cancer worldwide each year. The 5-year overall survival rate is ~59%.[2] About 45% of colorectal cancers are associated with activating mutations of the *KRAS* gene. Mutations are located mostly in codons 12 (~30%) and 13 (~8%) of exon 2.[3,4] These somatic mutations result in a constitutive activation of the epidermal growth factor receptor (EGFR) pathway and, therefore, confer resistance to anti-EGFR therapy. Previous studies have shown that *KRAS* mutations in exon 2 are associated with no clinical benefit from anti-EGFR monoclonal antibodies in metastatic colorectal cancers.[5,6] *KRAS* mutation has been also associated with worse disease-free survival in adjuvant setting.[7,8] Recently, other mutations in *KRAS* (exons 3 and 4) and *NRAS* genes (exons 2, 3, and 4) have been reported to be associated with resistance to anti-EGFR monoclonal antibodies too.[9] Anti-EGFR monoclonal antibodies, panitumumab and cetuximab, were consequently

Correspondence: Professor D Tougeron, MD, PhD, Department of Gastroenterology, Poitiers University Hospital, 2 rue de la Milétrie, Poitiers 86000, France.
E-mail: davidtougeron@hotmail.fr
[7]Co-authors.
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restricted to patients with metastatic colorectal cancers harboring a complete wild-type *RAS* genotype (*KRAS* and *NRAS* exons 2, 3, and 4 testing).[9–12]

Approximately 10% of colorectal cancer are *BRAF*-mutated.[13] *BRAF* mutation in colorectal cancer is associated with poor prognosis, especially in metastatic colorectal cancer.[14] In addition, mutations in *RAS* and *BRAF* genes are mutually exclusive.[15–19] Colorectal cancers with deficient mismatch repair system account for ~15% of all colorectal cancers. Deficient mismatch repair system is due to germline mutation in a mismatch repair gene (Lynch syndrome) or more commonly due to epigenetic inactivation of the *MLH1* gene (sporadic cases). As compared with proficient mismatch repair colorectal cancers, deficient mismatch repair colorectal cancers are associated with good prognosis in non-metastatic setting[20] and high disease control with drugs that target the immune checkpoints in metastatic setting.[21]

Recent studies have shown that *KRAS* exon 2 and *BRAF*-mutated colorectal cancers have distinct clinical and pathological characteristics. *KRAS* exon 2-mutated colorectal cancers occur more frequently in older patients, with a predominance of male gender, and are frequently located in the proximal colon compared with *KRAS* wild-type colorectal cancers.[22,23] High frequency of *KRAS*-mutation has been reported especially in the cecum.[22,24] Nevertheless, limited number of patients is a major weakness of those studies, leading to inconsistent conclusions. For instance, some studies have mentioned an association between *KRAS* mutations and mucinous differentiation, whereas others have not.[22,23] Moreover, correlation between *KRAS* mutation subtypes and clinicopathological characteristics was never clearly appraised. Gonsalves *et al.* showed that *KRAS* codon 13 mutations (p.Gly13Asp) are associated with deficient mismatch repair status and poor histologic grade.[15] *BRAF* mutations are significantly associated with advanced age, female gender, poor histologic grade, mucinous differentiation, proximal colon tumor site, and deficient mismatch repair status.[15,25]

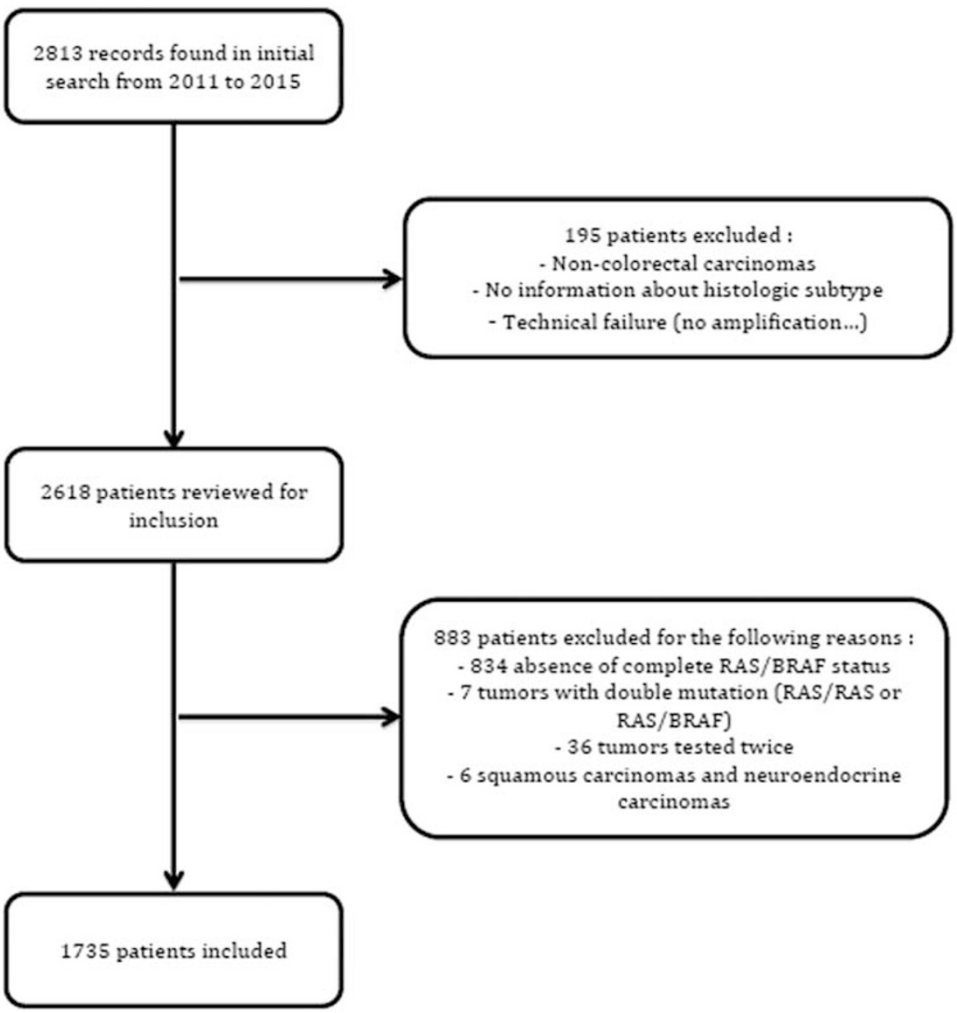

Figure 1 Flow chart.

Up until now, few studies have evaluated the possible correlation between clinicopathological features and complete *RAS* status (exons 2, 3, and 4 of *KRAS* and *NRAS*). A small series of 264 metastatic colorectal cancers in Japanese patients reported some association between *KRAS* exon 2 mutations and others *RAS* mutations with the rectal tumor site.[25] The aim of this study was to identify new distinct subsets of colorectal cancers based on clinicopathological features and complete *RAS* and *BRAF* genotype of a large cohort of 1735 colorectal cancer patients.

Materials and methods

Population

All patients harboring colorectal cancer with complete *RAS* testing, performed at the Molecular Cancer Genetics Platform of Poitiers (French National Cancer Institute (INCa)) from January 2011 to April 2015, were included in the study (Figure 1). Since 2006, INCa has been supporting a national network of 28 hospital molecular genetics platforms throughout France, offering to patients an access to all essential molecular genetics testing for cancers. Overall, 2813 consecutive colorectal cancer cases with a molecular testing were reviewed to implement the cohort. Tumors with multiple testing were included only once ($n = 36$). Tumors with incomplete *RAS*/*BRAF* status ($n = 834$) were excluded. Indeed, before December 2013 there was no complete *RAS* testing (only *KRAS* exon 2 mutations were analyzed). It became systematic for every colorectal cancer after December 2013. Other rare histological types than adenocarcinoma were also excluded ($n = 6$). Tumors with both *RAS* and *BRAF* mutations, or with two different *RAS* mutations, were also excluded ($n = 7$). Finally, 1735 tumors with complete *RAS* and *BRAF* testing were included in the study. Results of microsatellite instability analysis were also recorded ($n = 700$).

Molecular Analyses

Tumoral DNA was extracted from paraffin-embedded tumor sections using KAPA Express Extract Kits (KAPA Biosystems, CliniSciences, Nanterre, France) and GeneAmp PCR System 9700 Thermal Cycler (Applied Biosystems, Waltham, MA, USA).

According to the manufacturer's instructions, *RAS* and *BRAF* molecular testing was performed using polymerase chain reaction (PCR) amplification and pyrosequencing technology Q24 PyroMark system (Qiagen, Hilden, Germany) with CE-IVD-certified allele-specific (Qiagen) or homemade primers.[26] Mutational status of *KRAS* (exon 2: codons 12 and 13, exon 3: codons 59 and 61, and exon 4: codons 117 and 146), *NRAS* (exon 2: codons 12 and 13, exon 3: codons 59 and 61, and exon 4: codons 117 and 146), and *BRAF* (exon 15: codon 600) was analyzed.

Mismatch repair system status was determined with Microsatellite Instability Analysis System (Promega, Charbonnières-les-Bains, France) and multiplex PCR fluorescence assay using a panel of five mononucleotide repeat markers (BAT-25, BAT-26, NR-21, NR-24, and MONO-27) known to be monomorphic in Caucasian population. Samples were analyzed by capillary electrophoresis and data were deciphered using fragment analysis software (GeneMapper Software, Applied Biosystems). Colorectal cancers with two or more unstable loci were classified microsatellite-unstable and, thus, with deficient mismatch repair system.

Pathological Features

The following pathological features were analyzed using a pathological report database: histologic subtype (classical adenocarcinoma, mucinous adenocarcinoma, signet-ring cell adenocarcinoma, medullary adenocarcinoma, adenosquamous carcinoma, and undifferentiated carcinoma), histologic grade (well, moderately, or poorly differentiated), tumor site (proximal colon, transverse colon, distal colon, and rectum), vascular invasion (lymphatic and/or venous invasion/embols), and perineural invasion. Primary tumors located in the cecum and ascending colon were defined as proximal tumors, whereas tumors located in the splenic flexure, descending colon, and sigmoid colon were defined as distal tumors.[27] Mucinous colorectal adenocarcinoma was defined by at least 50% of the tumor volume composed of extracellular mucin.[28,29]

Definition of *RAS* Status and Different Subgroups

RAS and *BRAF* mutations are considered to be mutually exclusive (at 99%).[15–19] Consequently, tumors with a *RAS* mutation were considered *BRAF* wild type, if no *BRAF* testing was performed. In the same way, tumors with *BRAF* mutation were considered *RAS* wild type, if *RAS* testing was incomplete.

Then, different groups of colorectal cancers were set, whether they presented *RAS* mutation or *BRAF* mutation, or none (super wild-type colorectal cancers), and they were compared with each other. Subtypes of *KRAS* mutants (*KRAS* exon 2: *KRAS* codon 12 and codon 13, *KRAS* exon 3 and *KRAS* exon 4 mutants) were also compared with each other.

Statistical Analyses

Clinical, pathological, and molecular variables collected at baseline were described as means and s.d.'s for quantitative variables and percentages for

Table 1 Association between *RAS* status and clinicopathological features

Variables	*Total (%;* n = *1735)*	*RAS mutant (%;* n = *1002)*	*RAS wild-type (%;* n = *733)*	P[a]
Age, median (range)	70.5 (28–98)	70.4	70.6	0.830
Gender (n = 1735)				0.006
Male	1004 (57.9%)	608 (60.7%)	396 (54.0%)	
Female	731 (42.1%)	394 (39.3%)	337 (46.0%)	
Histologic subtype (n = 1735)				0.005
Classical adenocarcinoma	1539 (88.7%)	908 (90.6%)	631 (86.1%)	
Mucinous	175 (10.1%)	87 (8.7%)	88 (12.0%)	
Others	21 (1.2%)	7 (0.7%)	14 (1.9%)	
Histologic grade (n = 1408)				< 0.0001
Well	479 (34.0%)	290 (35.6%)	189 (31.8%)	
Moderate	777 (55.2%)	470 (57.8%)	307 (51.7%)	
Poor	152 (10.8%)	54 (6.6%)	98 (16.5%)	
Tumor site (n = 1479)				0.014
Proximal colon	547 (37.0%)	317 (36.7%)	230 (37.4%)	
Transverse colon	64 (4.3%)	27 (3.1%)	37 (6.0%)	
Distal colon	580 (39.2%)	336 (38.9%)	244 (39.7%)	
Rectum	288 (19.5%)	184 (21.3%)	104 (16.9%)	
Vascular invasion (n = 862)				0.061
Yes	440 (51.0%)	236 (48.3%)	204 (54.7%)	
No	422 (49.0%)	253 (51.7%)	169 (45.3%)	
Perineural invasion (n = 795)				0.260
Yes	255 (32.1%)	152 (33.7%)	103 (29.9%)	
No	540 (67.9%)	299 (66.3%)	241 (70.1%)	
Microsatellite instability (n = 700)				< 0.0001
Yes	94 (13.4%)	18 (4.7%)	76 (24.2%)	
No	606 (86.6%)	368 (95.3%)	238 (75.8%)	

[a]Significance threshold at $P < 0.007$.

qualitative variables. Associations between mutational status and patients or tumor characteristics were assessed using the χ^2-test (or Fisher's exact test if appropriate) for qualitative variables and using Student's *t*-test for continuous variables. For all analyses an adjustment for multiple testing has been performed using Bonferroni correction. Statistical analyses were performed using the Statview software (Statview for Windows, SAS Institut, version 5.0).

Results

Patient and Tumor Characteristics

RAS and *BRAF* genotype was available for 1735 patients. The median age was 70.5 years and 57.9% were of male gender (Table 1). Histologic subtypes were 88.7% of classical adenocarcinoma, 10.1% of mucinous adenocarcinoma, and 1.2% of other adenocarcinoma subtypes. The majority of colorectal cancers were moderately differentiated (55.2%). Tumor sites were proximal, transverse, distal colon, and rectum at 37.0%, 4.3%, 39.2%, and 19.5%, respectively.

There was 55.6% of *KRAS* mutation ($n = 965$ /1735), 2.1% of *NRAS* mutation ($n = 37$/1735), 15.5% of *BRAF* mutation ($n = 269$/1735), 26.7% of super wild-type colorectal cancers ($n = 464$/1735), and 13.4% of deficient mismatch repair colorectal cancers ($n = 94$/700).

Comparison of subgroups of super wild-type, *KRAS*, *NRAS*, and *BRAF* mutants showed significant association with several features, namely age, gender, histologic subtype, histologic grade, tumor site, and microsatellite instability (all $P < 0.0001$; Table 2). To highlight differences between mutational status, a 2 by 2 comparison was then conducted (mutated *versus* wild type).

Association Between *RAS*/Frequent *KRAS* Mutations and Clinicopathological Features

Among the 1735 colorectal cancers, 733 were *RAS* wild-type (42.2%) and 1002 were *RAS* mutants (57.8%). There was no age difference among patients according to *RAS* status, 70.4 years for *RAS*-mutated colorectal cancers, and 70.6 years for *RAS* wild-type colorectal cancers (Table 1). Compared with *RAS* wild-type colorectal cancers, *RAS*-mutated colorectal cancers were statistically associated with male gender (60.7% *versus* 54.0%; $P = 0.006$), classical adenocarcinoma subtype (90.6% *versus* 86.1%;

Table 2 Association between *KRAS, NRAS*, *BRAF,* super wild-type status, and clinicopathological features

Variables	*KRAS wild-type (%; n = 770)*	*KRAS mutant (%; n = 965)*	P[a]	*NRAS wild-type (%; n = 1698)*	*NRAS mutant (%; n = 37)*	P[a]	*BRAF mutant (%; n = 269)*	*BRAF wild-type (%; n = 1466)*	P[a]	*RAS or BRAF mutant (%; n = 1271)*	*Super wild-type (%; n = 464)*	P[a]	*Total (%; n = 1735)*	P[a, b]
Age, median (range)	70.7	70.3	0.642	70.4	72.6	0.393	74.7	68.2	< 0.0001	71.3	68.3	< 0.0001	70.5 (28–98)	< 0.0001
Gender (n = 1735)														
Male	421 (54.7%)	583 (60.4%)	0.016	979 (57.7%)	25 (67.6%)	0.227	109 (40.5%)	895 (61.1%)	< 0.0001	717 (56.4%)	287 (61.9%)	0.042	1004 (57.9%)	< 0.0001
Female	349 (45.3%)	382 (39.6%)		719 (42.3%)	12 (32.4%)		160 (59.5%)	571 (38.9%)		554 (43.6%)	177 (38.1%)		731 (42.1%)	
Histologic subtype (n = 1735)														
Classical adenocarcinoma	666 (86.5%)	873 (90.5%)	0.014	1504 (88.6%)	35 (94.6%)	0.49	195 (72.5%)	1344 (91.7%)	< 0.0001	1103 (86.8%)	436 (94.0%)	< 0.0001	1539 (88.7%)	< 0.0001
Mucinous	90 (11.7%)	85 (8.8%)		173 (10.2%)	2 (5.4%)		66 (24.5%)	109 (7.4%)		153 (12.0%)	22 (4.7%)		175 (10.1%)	
Others	14 (1.8%)	7 (0.7%)		21 (1.2%)	0 (0%)		8 (3.0%)	13 (0.9%)		15 (1.2%)	6 (1.3%)		21 (1.2%)	
Histologic grade (n = 1408)														
Well	201 (32.1%)	278 (35.6%)	< 0.0001	467 (34.0%)	12 (36.4%)	0.346	63 (29.3%)	416 (34.9%)	< 0.0001	353 (34.3%)	126 (33.2%)	0.07	479 (34.0%)	< 0.0001
Moderate	327 (52.1%)	450 (57.6%)		757 (55.0%)	20 (60.6%)		84 (39.1%)	693 (58.1%)		554 (53.8%)	223 (58.9%)		777 (55.2%)	
Poor	99 (15.8%)	53 (6.8%)		151 (11.0%)	1 (3.0%)		68 (31.6%)	84 (7.0%)		122 (11.9%)	30 (7.9%)		152 (10.8%)	
Tumor site (n = 1479)														
Proximal colon	238 (37.0%)	309 (37.0%)	0.046	539 (37.2%)	8 (27.6%)	0.358	163 (68.2%)	384 (31.0%)	< 0.0001	480 (43.5%)	67 (17.8%)	< 0.0001	547 (37%)	< 0.0001
Transverse colon	37 (5.7%)	27 (3.2%)		64 (4.4%)	0		23 (9.6%)	41 (3.3%)		50 (4.5%)	14 (3.7%)		64 (4.3%)	
Distal colon	257 (39.9%)	323 (38.7%)		567 (39.1%)	13 (44.8%)		39 (16.3%)	541 (43.6%)		375 (34.0%)	205 (54.5%)		580 (39.2%)	
Rectum	112 (17.4%)	176 (21.1%)		280 (19.3%)	8 (27.6%)		14 (5.9%)	274 (22.1%)		198 (18.0%)	90 (23.9%)		288 (19.5%)	
Vascular invasion (n = 862)														
Yes	212 (54.6%)	228 (48.1%)	0.056	432 (51.0%)	8 (53.3%)	0.858	84 (57.5%)	356 (49.7%)	0.118	320 (50.4%)	120 (52.9%)	0.523	440 (51.0%)	0.218
No	176 (45.4%)	246 (51.9%)		415 (49.0%)	7 (46.7%)		62 (42.5%)	360 (50.3%)		315 (49.6%)	107 (47.1%)		422 (49.0%)	
Perineural invasion (n = 795)														
Yes	109 (30.4%)	146 (33.5%)	0.348	249 (31.9%)	6 (40.0%)	0.507	37 (27.0%)	218 (33.1%)	0.338	189 (32.1%)	66 (31.9%)	0.945	255 (32.1%)	0.484
No	250 (69.6%)	290 (66.5%)		531 (68.1%)	9 (60.0%)		100 (73.0%)	440 (66.9%)		399 (67.9%)	141 (68.1%)		540 (67.9%)	
Microsatellite instability (n = 700)														
Yes	76 (23.2%)	18 (4.8%)	< 0.0001	94 (13.7%)	0 (0%)	0.137	57 (57.0%)	37 (6.2%)	< 0.0001	75 (15.4%)	19 (8.9%)	0.019	94 (13.4%)	< 0.0001
No	252 (76.8%)	354 (95.2%)		592 (86.3%)	14 (100%)		43 (43.0%)	563 (93.8%)		411 (84.6%)	195 (91.1%)		606 (86.6%)	

[a]Significance threshold at $P < 0.007$.
[b]Comparison of super wild-type, *KRAS*, *NRAS*, and *BRAF* mutants.

Table 3 Association between *KRAS*-mutant status and clinicopathological features

Variables	*BRAF mutant* (n = *269*)	*NRAS mutant* (n = *37*)	*KRAS mutant* (n = *965*)	*KRAS codon 12 mutant* (n = *738*)	*KRAS codon 13 mutant* (n = *171*)	*KRAS exon 3 mutant* (n = *23*)	*KRAS exon 4 mutant* (n = *33*)	P[a]
Age, median (range)	74.7	72.6	70.3	70.4	69.4	69.2	73.9	0.222
Gender (n = 965)								
Male	109 (40.5%)	25 (67.6%)	583 (60.4%)	451 (61.1%)	99 (57.9%)	13 (56.5%)	20 (60.6%)	0.861
Female	160 (59.5%)	12 (32.4%)	382 (39.6%)	287 (38.9%)	72 (42.1%)	10 (43.5%)	13 (39.4%)	
Histologic subtype (n = 965)								
Classical adenocarcinoma	195 (72.5%)	35 (94.6%)	873 (90.5%)	665 (90.1%)	163 (95.3%)	19 (82.7%)	26 (78.8%)	0.002
Mucinous	66 (24.5%)	2 (5.4%)	85 (8.8%)	70 (9.5%)	7 (4.1%)	3 (13.0%)	5 (15.1%)	
Others	8 (3.0%)	0 (0%)	7 (0.7%)	3 (0.4%)	1 (0.6%)	1 (4.3%)	2 (6.1%)	
Histologic grade (n = 781)								
Well	63 (29.3%)	12 (36.4%)	278 (35.6%)	226 (37.8%)	37 (26.4%)	6 (33.3%)	9 (36.0%)	0.083
Moderate	84 (39.1%)	20 (60.6%)	450 (57.6%)	339 (56.7%)	87 (62.1%)	10 (55.6%)	14 (56.0%)	
Poor	68 (31.6%)	1 (3.0%)	53 (6.8%)	33 (5.5%)	16 (11.5%)	2 (11.1%)	2 (8.0%)	
Tumor site (n = 835)								
Proximal colon	163 (68.2%)	8 (27.6%)	309 (37.0%)	215 (33.8%)	76 (50.3%)	6 (28.6%)	12 (44.5%)	0.014
Transverse colon	23 (9.6%)	0	27 (3.2%)	23 (3.6%)	4 (2.6%)	0	0	
Distal colon	39 (16.3%)	13 (44.8%)	323 (38.7%)	263 (41.4%)	45 (29.8%)	8 (38.1%)	7 (25.9%)	
Rectum	14 (5.9%)	8 (27.6%)	176 (21.1%)	135 (21.2%)	26 (17.2%)	7 (33.3%)	8 (29.6%)	
Vascular invasion (n = 474)								
Yes	84 (57.5%)	8 (53.3%)	228 (48.1%)	170 (47.1%)	46 (53.5%)	5 (45.5%)	7 (43.8%)	0.729
No	62 (42.5%)	7 (46.7%)	246 (51.9%)	191 (52.9%)	40 (46.5%)	6 (54.5%)	9 (56.2%)	
Perineural invasion (n = 436)								
Yes	37 (27.0%)	6 (40.0%)	146 (33.5%)	107 (32.5%)	35 (43.8%)	2 (18.2%)	2 (12.5%)	0.041
No	100 (73.0%)	9 (60.0%)	290 (66.5%)	222 (67.5%)	45 (56.2%)	9 (81.8%)	14 (87.5%)	
Microsatellite instability (n = 372)								
Yes	57 (57.0%)	0 (0%)	18 (4.8%)	10 (3.6%)	6 (9.1%)	1 (6.2%)	1 (6.7%)	0.304
No	43 (43.0%)	14 (100%)	354 (95.2%)	265 (96.4%)	60 (90.9%)	15 (93.8%)	14 (93.3%)	

[a]Comparison between *KRAS* codon 12 mutant, *KRAS* codon 13 mutant, *KRAS* exon 3 mutant, and *KRAS* exon 4 mutant; significance threshold at $P < 0.007$.

$P = 0.005$), well/moderately differentiated tumors (93.4% *versus* 83.5%; $P < 0.0001$), and microsatellite-stable phenotype (95.3% *versus* 75.8%; $P < 0.0001$).

Secondly, we looked at the association between *RAS* mutation subtypes and clinicopathological features. Among *RAS*-mutated colorectal cancers, 965 were *KRAS* mutants (exons 2, 3, or 4; 96.3%) and 37 were *NRAS* mutants (exons 2, 3, or 4; 3.7%). Like *RAS*-mutated colorectal cancers, well/moderately differentiated tumors ($P < 0.0001$) and microsatellite-stable phenotype ($P < 0.0001$) were found to be associated with *KRAS*-mutated colorectal cancers as compared with *KRAS* wild-type colorectal cancers (Table 2). Moreover, results tended to show a possible association with male gender ($P = 0.016$) and classical adenocarcinoma subtype ($P = 0.014$). Among the colorectal cancer cohort, there were 909 *KRAS* exon 2 mutations (52.4%) distributed between 738 *KRAS* codon 12 mutants (42.5%) and 171 *KRAS* codon 13 mutants (9.9%; Table 3). *KRAS* exon 2 and *KRAS* codon 12 mutants, in comparison with *RAS* wild-type colorectal cancers, were also significantly associated with the same clinicopathological features as *RAS*-mutated colorectal cancers (classical adenocarcinoma subtype, well/moderately differentiated tumors and microsatellite stable phenotype; data not shown). Tumors with *KRAS* codon 13 mutants, in comparison with *RAS* wild-type colorectal cancers, were more frequently classical adenocarcinoma (95.3% *versus* 86.1%; $P = 0.004$), with microsatellite-stable phenotype (90.9% *versus* 75.8%; $P < 0.0001$), with perineural invasion (43.8% *versus* 29.9%; $P = 0.018$) and located in the proximal colon (50.3% *versus* 37.4%; $P = 0.011$).

KRAS codon 13-mutated colorectal cancers, compared with *KRAS* codon 12-mutated colorectal cancers, were more frequently poorly differentiated (11.5% *versus* 5.5%; $P = 0.005$) and were located in the proximal colon (50.3% *versus* 33.8%; $P = 0.002$; Table 3).

Association Between Rare *KRAS*/*NRAS* Mutations and Clinicopathological Features

Rare *KRAS* mutations were either *KRAS* exon 3 mutants ($n=23$, 1.3%) or *KRAS* exon 4 mutants ($n=33$, 1.9%; Table 3). Compared with *RAS* wild-type colorectal cancers, results tended to show that rare *KRAS*-mutated colorectal cancers ($n=56$) were associated with rectal site (31.2% *versus* 16.9%; $P=0.043$) and microsatellite-stable phenotype (93.5% *versus* 75.8%; $P=0.024$).

When comparing the different *KRAS* mutation groups to each other, regarding distribution of histologic subtypes and tumor site, we found significant discrepancies (Table 3). *KRAS* exon 3-mutated colorectal cancers were more frequently associated with mucinous/rare histological subtypes (17.3% *versus* 9.5%; $P=0.002$) and tended to be associated with rectal tumor site (33.3% *versus* 21.1%; $P=0.009$). *KRAS* exon 4-mutated colorectal cancers were associated with mucinous/rare histological subtypes (21.2% *versus* 9.5%; $P=0.002$). *NRAS* mutation was present in 37 cases (2.1%). Clinicopathological features in *NRAS*-mutated colorectal cancers were not significantly different compared with *NRAS* wild-type colorectal cancers (Table 2). When comparing *NRAS*-mutated and *KRAS*-mutated groups, no significant disparity was observed (data not shown).

Association Between *BRAF* Status and Clinicopathological Features

Patients with *BRAF*-mutated colorectal cancers were significantly older than patients with *BRAF* wild-type colorectal cancers (respectively, 74.7 and 68.2 years) and were associated with female gender (respectively, 59.5% and 38.9%; all $P<0.0001$; Table 2). Moreover, *BRAF*-mutated colorectal cancers were statistically associated with proximal tumor site (68.2% *versus* 31.0%), mucinous differentiation (24.5% *versus* 7.4%), poorly differentiated tumors (31.6% *versus* 7.0%), and microsatellite instability (57.0% *versus* 6.2%; all $P<0.0001$).

Clinicopathological Features of *RAS* and *BRAF* Wild-Type Colorectal Cancers

Patients with super wild-type colorectal cancers were significantly younger than patients with *RAS* or *BRAF* mutations (68.3 *versus* 71.3 years; $P<0.0001$). Moreover, super wild-type tumors were significantly associated with classical adenocarcinoma subtype (94.0% *versus* 86.8%; $P<0.0001$) and distal tumor site (54.5% *versus* 34.0%; $P<0.0001$) compared with *RAS*- or *BRAF*-mutated tumors (Table 2).

Discussion

To our knowledge, this retrospective study is one of the first to analyze associations between complete *RAS* and *BRAF* mutational status and clinicopathological features in a large cohort of colorectal cancers. As compared with *RAS* wild-type tumors, *RAS* mutants, *KRAS* mutants, *KRAS* exon 2 mutants, and *KRAS* codon 12 mutants tended to be associated with the same clinicopathological features, ie, male gender, classical adenocarcinoma subtype, well/moderately differentiated tumors, and microsatellite stable phenotype. *KRAS* codon 13 mutant colorectal cancers were more frequently classical adenocarcinoma subtype and were with microsatellite stable phenotype. For the first time, we highlighted some associations between rare *RAS* mutations (*KRAS* exons 3 and 4; *NRAS* mutations) and clinicopathological features. As compared with other *KRAS* mutations, *KRAS* exon 3-mutated colorectal cancers were more frequently associated with mucinous/rare histological subtypes and, most likely to the rectal tumor site, whereas *KRAS* exon 4-mutated colorectal cancers were associated only with mucinous/rare histological subtypes. In contrast, there was no significant association between *NRAS* mutation and any clinicopathological feature.

Mutation rates of *RAS*, *KRAS*, *NRAS*, and *BRAF* genes were 57.7%, 55.6%, 2.1%, and 15.5%, respectively. Among all of them, as expected, *KRAS* exon 2 (codons 12/13) mutation was the most frequent with 52.4%. These mutation rates were slightly above data in the literature. In recently published studies, around 50% of colorectal cancers presented a *RAS* mutation with an average of 45% of *KRAS* mutants and 40% of *KRAS* exon 2 mutants.[3,7,8,30] *BRAF* mutation rate in the literature is around 10%, which is slightly lower than the *BRAF* mutation rate found in our cohort of patients (15.5%).[14,31,32] Sensitivity of the different molecular techniques used could explain the disparity. Since 2010, our genomic platform has been using pyrosequencing, a robust technique known to be more sensitive than Sanger sequencing used in some other platforms. In addition, our population may be enriched in *RAS*/*BRAF* mutants as colorectal cancers with incomplete *RAS* testing or colorectal cancers with *RAS* wild-type status but no *BRAF* testing or colorectal cancers with *BRAF* wild-type status but no *RAS* testing were automatically excluded (colorectal cancers analyzed between 2011 and 2013). Concerning rare *RAS* mutations (*KRAS* exons 3 and 4 and *NRAS* exons 2, 3, and 4), 5.4% colorectal cancers were concerned in our study. Rare *RAS* mutations were scattered between *KRAS* exon 3 mutants (1.3%), *KRAS* exon 4 mutants (1.9%), and *NRAS* mutants (2.1%). These rates are lower than the 10% rate previously reported by various studies.[3,9,25,33] Apart from the exclusion of colorectal cancers with incomplete *RAS* testing and from the use of different techniques with different specificity and sensitivity, no clear explanation can be drawn. The 13.4% of colorectal cancers with deficient mismatch repair system was in accordance with data found in the literature (≈12%).[34,35] In our study, patients and

tumor characteristics were consistent with previously published data.[30,36–40]

Complete *RAS* status with analyses of exons 2, 3, and 4 underlined some distinct clinicopathological and molecular characteristics. *RAS*-mutated colorectal cancers, and more precisely *KRAS* exon 2 and *KRAS* codon 12 mutants, were significantly associated with classical adenocarcinoma subtype, well/moderately differentiated tumors, and microsatellite stable phenotype. Most of our results were consistent with the literature concerning *KRAS* exon 2, notably the association with well/moderately histologic grade.[25] Nevertheless, the data found in literature present some disparities, and some dissimilarity can be observed concerning *KRAS* mutations and tumor sites or histologic subtypes. Some studies have shown an association between *KRAS* mutation and either right colon[8] or rectal tumor sites.[25] Other studies precisely determined that cecal cancers exhibited significantly higher frequency of *KRAS* mutations than other tumor sites.[22,24] Rosty *et al.* has also observed gender-related distribution of *KRAS*-mutated carcinoma between different colonic segments.[22] Association between *KRAS* mutation and mucinous differentiation was affirmed in some studies but denied in others.[19,20] In the same way, two studies observed that *KRAS* exon 2-mutated colorectal cancers seemed to occur more frequently in elderly patients, but other studies did not.[22,23] These inconsistent results could arise from a low patient number and lack of robustness of some studies. We analyzed a large cohort and found no association between *KRAS* exon 2 mutations with tumor site, neither with age nor with mucinous differentiation. No significant association between *KRAS* mutations and tumor site in our study could be explained by the fact that no data on cecum tumor site were available. Yamauchi *et al.* introduced a new concept of continuum colorectal cancer characterized by linear gradual changes of key tumor molecular frequencies from the rectum to the ascending colon.[41] However, cecal cancers did not follow the same continuum trend and stand for a unique colorectal cancer subtype characterized by high frequency of *KRAS* mutation.[24] Indeed, there are some differences among proximal colorectal cancers, which is a heterogeneous subgroup.

A recent publication showed that, when compared with other *KRAS* mutations, *KRAS* codon 13 mutation was associated with deficient mismatch repair phenotype and poor histologic grade.[15] In our population, *KRAS* codon 13 mutation was correlated with poor tumor differentiation, but also with the proximal colon site. To our knowledge, there exist no published data concerning the association of rare *RAS* mutations (*KRAS* exons 3 and 4 and *NRAS* mutations) and clinicopathological features. We demonstrated that, in comparison with colorectal cancers with other *KRAS* mutations, *KRAS* exon 3-mutated colorectal cancers and *KRAS* exon 4-mutated colorectal cancers were both associated with mucinous/rare histological subtypes. Moreover, *KRAS* exon 3-mutated colorectal cancers tended to be associated with rectal tumor site. Finally, there was no significant association between *NRAS* mutations and clinical or pathological features. Therefore, correct and complete determination of the *RAS* genotype is required as clinicopathological features are associated with colorectal cancer prognosis value and varied according to *RAS* mutation.

According to previous studies, patients with *BRAF*-mutated colorectal cancers are aged, mostly women, with right colon tumor site, either poorly differentiated or mucinous tumors, and had tumor with deficient mismatch repair status.[15,25,42,43] Our results were in complete accordance with the literature.

Concerning super wild-type colorectal cancers, they were significantly associated with younger age, classical adenocarcinoma subtype, and distal colon tumor site. It is worth noting that no other study has ever evaluated the correlation between clinicopathological features and super wild-type colorectal cancers. As expected, the correlations found for clinicopathological features were opposite to those discovered for *KRAS* and *BRAF* mutated-colorectal cancers.

Part of our study limitations is because of missing clinical, histological, molecular data, or incomplete information on tumor site, explained by the fact that it is an observational retrospective study. Nevertheless, aside from vascular and perineural invasions, the major data were efficiently collected with less than 20% of missing information. Having no data on the exact proximal location site (ie, cecum or not) is one of the limitations of our study. Because of unique molecular status of cecal tumors, detailed site information in future molecular studies in colorectal cancers is necessary. Our study can be criticized on the choice made to consider *RAS* and *BRAF* mutations as mutually exclusive. However, only 1–2% colorectal cancers may carry both mutations, which is a too small percentage to change the statistical results obtained, considering the size of our study.[15–19] On the other hand, the strength of our study was to collect and statistically correlate data from one of the largest cohorts of colorectal cancers ($n = 1735$). To take into account multiple testing, we used Bonferroni correction but some *P*-value are above the alpha level and we cannot exclude true association. Above all, this is the first report correlating complete *RAS* and *BRAF* analysis with colorectal cancers' clinicopathological features.

In conclusion, this study provides a novel broader approach of clinicopathological features according to mutational status in colorectal cancers. In particular, *KRAS* exon 2 (codon 13), exon 3, and 4 mutations have distinct clinical, pathological, and molecular characteristics, and must be carefully considered when assessing the prognosis value of *RAS* status and in clinical trials in colorectal cancers.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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