Possible Gender-Related Modulation by the ROCK1 Gene in Colorectal Cancer Susceptibility

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Key Words

ROCK1 · ROCK2 · Colorectal cancer · Gene polymorphism · Association study

Abstract

Aim: In view of accumulating evidence supporting a pivotal role of the Rho/ROCK pathway in cancer, we investigated Rho-kinase polymorphisms as potential susceptibility factors in colorectal cancer (CRC) in a representative sample of the Italian population. **Methods:** DNA obtained from the peripheral blood samples of 137 CRC patients and 141 healthy controls was genotyped for four ROCK1 (rs35996865; rs73963110; rs2127958; rs288980) and five ROCK2 (rs12692437; rs7563468; rs35768389; rs17463896; rs16857265) selected single nucleotide polymorphisms. **Results:** None of the allelic variants of the nine selected markers was associated with the occurrence of CRC or with the development of regional lymph node metastasis. By contrast, the ROCK1 rs35996865 G variant allele was significantly more frequent in male patients ($p = 0.028$) than in the control group. **Conclusion:** This finding is, at present, the first that points to a possible gender-related modulation by the ROCK1 gene in CRC susceptibility.

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Introduction

Every year, about 1 million people worldwide develop colorectal cancer (CRC). The majority of CRC cases occur in people who have no family risk factor, whereas 15–20% occur in people with a family history [1]. CRC is by far the most frequent tumor in the Italian population, with nearly 52,000 diagnoses estimated for 2014 by the Ministry of Health, based on data from the Italian Association of Medical Oncology (AIOM) and the Italian Association of Tumor Registration (ARTIUM).

Considering the great incidence of this pathology, many efforts have been made to elucidate the molecular bases of CRC etiology, but the causes still remain unclear. Several studies have been carried out in order to reveal the potential association between polymorphic genetic variants and the risk of CRC [2–5], although the actual genetic contribution to CRC is still not completely understood. In the challenge to establish the molecular basis of individual susceptibility and the factors that initiate the development of the tumor and its progression, ROCKs

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deserve greater insight. ROCK1 and ROCK2, the main downstream effectors of the Rho family of GTPases, are serine-threonine kinases that act as central regulators of actin cytoskeleton dynamics. Through the phosphorylation of a range of target substrates involved in the cytoskeleton organization, such as myosin light chain (MLC) [6, 7], MLC phosphatase (MYPT1) [8, 9], ezrin/radixin/moesin (ERM) [10], adducin [11] and LIM kinase (LIMK) [12], ROCKs control the formation of actin stress fibers, focal adhesions and cellular myosin-based contractility [13]. The two isoforms described, ROCK1 and ROCK2, although encoded by distinct genes, share 65% homology in their amino acid sequences [14]. Despite the extensive homology as well as their substrate promiscuity, the two ROCK isoforms have distinct functions. In particular, ROCK2 depletion has been reported to enhance microfilament bundle assembly into stress fibers and focal adhesion formation, whereas ROCK1-depleted cells show an opposite phenotype, and ROCK1 seems to be required for myosin II-dependent phagocytosis [15–17].

Since actin cytoskeleton dynamics mediate a number of cellular processes involved in the malignant phenotype gain, such as the regulation of morphology, cell-cell adhesion, migration and invasion, the implication of ROCK1 and ROCK2 in cancer is not surprising. Overexpression of ROCK has been described in many solid tumors, including hepatocellular carcinoma [18], osteosarcoma [19, 20], breast cancer [21], testicular cancer [22], bladder cancer [23] and also colon cancer [24]. In addition, several studies have emphasized the antimigratory and anti-invasive activity due to ROCK inhibition [25–28] or silencing [18, 24, 29, 30], reinforcing the association between the Rho-ROCK axis and cancer. Recently, Sari et al. [31] suggested that genetic polymorphisms in the ROCK1 and ROCK2 genes modify individual susceptibility to CRC in the Turkish population. Thus, in the present study we examined the genetic influence of Rho-kinase polymorphisms on CRC susceptibility in a representative sample of the Italian population.

Materials and Methods

Sample Study

A cohort of 137 unrelated Italian CRC patients was recruited by the Department of Medical and Surgical Sciences of S. Orsola-Malpighi Polyclinic, Bologna University. Diagnosis of CRC was confirmed by histopathological examination. The control group consisted of 141 unrelated healthy volunteers with matching ethnic origin and from the same geographical area (Italy).

The study was approved by the ethics committee of Sant'Orsola-Malpighi General Hospital and complied with the Helsinki Decla-

Table 1. Characteristics of CRC patients

Variable	Cancer cases, n (%)
Age	
<50 years	8 (5.8)
≥50 years	123 (89.8)
NDA	6 (4.4)
Sex	
Male	72 (52.6)
Female	65 (47.4)
Grading	
G1	8 (5.8)
G2	90 (65.7)
G3	25 (18.2)
NDA	14 (10.2)
Lymph node metastasis	
N0	72 (52.6)
N1/2	42 (30.7)
NDA	23 (16.8)
Family history	
Negative	111 (81)
Positive	14 (10)
NDA	12 (8.8)
Smoking history	
Never	79 (57.7)
Active smoker	16 (11.7)
Former	31 (22.6)
NDA	11 (8)
NDA = No data available.	

ration's Ethical Principles for Medical Research Involving Human Subjects. Written informed consent was obtained from all patients and healthy control subjects before study entry. The patient characteristics are summarized in table 1. DNA extraction from peripheral whole blood, collected before primary surgery, was performed using the GenElute™ Blood Genomic DNA Kit (Sigma, Milan, Italy).

Single Nucleotide Polymorphism Genotyping

In order to investigate ROCK1 and ROCK2 genes we chose a total of nine single nucleotide polymorphisms (SNPs). Markers were selected using the HaploTagger tool for maximizing SNP prediction accuracy [32] as well as on the basis of literature data [31].

Table 2 summarizes the details of the investigated polymorphisms. It is noteworthy that the rs35768389 SNP mapping on the ROCK2 gene represents a missense variant (Val601Asp). Each polymorphism was amplified by PCR using flanking primers and the products were incubated with a specific restriction endonuclease (table 2). In a couple of primers a mismatch was inserted in order to create a restriction site for a common enzyme. Restriction fragments were separated by 10% native polyacrylamide gel electrophoresis and then stained with ethidium bromide. To assess the accuracy of the genotyping outputs, one third of the samples selected at random were blindly tested by a second operator. The DNA sample of 1 patient was used up when we genotyped the ROCK2 markers.

Table 2. Details of the SNP investigation

Gene and polymorphism ID	SNP position ¹		Nucleotide change	Primer sequence (5'–3')	Amplimer, bp	Restriction enzyme
ROCK1 rs35996865	5'-UTR	chr18:21112383	T>G	Fw-TACCAGCATTGAGCACCAGT Rev-TTGACTGTATCTTCCGGAGTCA	189	<i>BstX</i> I
ROCK1 rs73963110	intron 1	chr18:21098352	T>C	Fw-AGTGGCATTGTCAACCAACA Rev-TTTCCCTATGTCCAGATCA	212	<i>Fnu4H</i> I
ROCK1 rs2127958	intron 1	chr18:21073648	C>T	Fw-GAGTCAGGTGTAAAACATCATTTAGG Rev-GGGTGGTGTCTCCAAAAAT	190	<i>Dra</i> I
ROCK1 rs288980	intron 9	chr18:21029619	C>T	Fw-CCCACCCCAATATCTCCATA Rev-GGAAGACTTGGTCCACATCAA	204	<i>Fat</i> I
ROCK2 rs12692437	intron 1	chr2:11340435	T>C	Fw-CTTCACATCAACAGTAAGgTCCA ² Rev-TTCTTTATGAAGAGTTTGGCATT	151	<i>Hpy166</i> II
ROCK2 rs7563468	intron 1	chr2:11291149	G>A	Fw-AGCCAAACACCCAGTTTCC Rev-CACCTGGCCAACAACAATAA	192	<i>Dpn</i> II
ROCK2 rs35768389	exon 16	chr2:11214974	T>A	Fw-TAAGCTGGCAGTCTCCAGCA Rev-TGTTGTTTGGTTTTGAATGGA	181	<i>Dpn</i> II
ROCK2 rs17463896	intron 17	chr2:11213800	A>G	Fw-TGCTCATGCTGTTCTCCCTA Rev-ATGCAGTCCTTGCCCTGAAT	217	<i>Fat</i> I
ROCK2 rs16857265	intron 32	chr2:11190286	T>C	Fw-TGCAGATGTACAACAAAATCCA Rev-TTTTGTTCATAGCAACGGTcAGT ³	194	<i>BtsIMut</i> I

¹ UCSC Genome Browser assembly ID: hg38. ² Insertion of mutation T>G. ³ Insertion of mutation A>C.

Statistical Analysis

The distribution of genotypes in the patient and control groups was tested for deviation from the Hardy-Weinberg equilibrium (HWE) using Pearson's χ^2 test. A borderline disequilibrium ($p = 0.045$) was noted for the rs35996865 SNP in the control group. Since the deviation from HWE was marginally significant, we did not exclude that particular SNP from the investigation. Genetic association was firstly investigated using a likelihood ratio approach by Unphased software v3.1.5 for Windows Vista [33]. Odds ratios were calculated in order to estimate the level of association of the rare allele carriers, i.e. heterozygotes versus noncarriers, as well as homozygotes versus noncarriers.

Linkage disequilibrium between SNPs was calculated using the Haploview program. Haplotype association analysis was performed to further investigate the association between CRC and ROCK1 or ROCK2 polymorphisms. The haplotype association analysis was performed with the aid of Unphased software. A specific test for each haplotype was carried out. This test is a score test for a difference in risk between one haplotype and all the others pooled together. This is in contrast with the odds ratios, which were calculated in relation to a single reference haplotype. A permutation test was performed with 10,000 sample replicates to allow for multiple testing corrections over all the performed tests. In each replicate the trait values were randomly shuffled between all subjects.

Results

Only a single genotype was missing in the genotyping step (call rate >99.9%). The assay for the ROCK1 polymorphism rs73963110 did not provide any evidence of

variant alleles in our data set; therefore, the SNP was excluded from the statistical analysis. A case-control association study was performed in order to test whether genetic variants in ROCK1 or ROCK2 genes were associated with CRC as a whole, with CRC in a specific gender, or with the occurrence of lymph node metastasis (tables 3, 4). Variant alleles appeared not to be associated with the occurrence of CRC or with regional lymph node metastasis for any of the investigated genes; however, a significant association was observed with CRC occurrence in males. Indeed, the variant allele of the rs35996865 mapping in ROCK1 was more frequent in male patients ($p = 0.028$) than in the control group. The calculated odds ratio (OR) for rs35996865 variant allele (G) heterozygote carriers versus noncarriers (OR_{het}) was 1.88 (95% CI 1.02–3.49), while for homozygote carriers versus noncarriers (OR_{hom}) it was not significant (OR 1.977, 95% CI 0.76–5.13), probably due to the small number of individuals with that genotype. However, considering the genotypes presenting the variant together, the OR was 1.9 (95% CI 1.07–3.38). The variant allele was more frequent in male patients (minor allele frequency, MAF = 0.33) with respect to controls (MAF = 0.23), while the female patient frequency (MAF = 0.23) was close to controls. No difference in allelic frequency was observed between genders within the control group ($p = 0.45$). To further investigate the genetic association between

Table 3. Association analysis of ROCK1 *locus*

SNP information		Sample	Genotype, n			MAF	p value	Relative risk (95% CI)	
SNP ID	alleles ¹		11	12	22			OR _{het}	OR _{hom}
rs35996865	T/G	Control	87	42	12	0.23	ref.	ref.	ref.
		Case	71	54	12	0.28	0.173	1.575 (0.945–2.626)	1.225 (0.519–2.894)
		Case male	33	30	9	0.33	0.0285	1.883 (1.017–3.488)	1.977 (0.763–5.126)
		Case female	38	24	3	0.23	0.9418	1.308 (0.697–2.456)	0.572 (0.153–2.145)
		Case N0	37	29	5	0.27	0.3606	1.624 (0.882–2.988)	0.98 (0.322–2.979)
		Case N1/2	20	16	6	0.33	0.0675	1.657 (0.78–3.521)	2.175 (0.728–6.494)
rs2127958	C/T	Control	22	72	47	0.41	ref.	ref.	ref.
		Case	22	56	59	0.36	0.2619	0.778 (0.392–1.545)	1.255 (0.621–2.539)
		Case male	11	30	31	0.36	0.3156	0.833 (0.360–1.930)	1.319 (0.562–3.099)
		Case female	11	26	28	0.37	0.417	0.722 (0.308–1.692)	1.191 (0.503–2.821)
		Case N0	11	29	32	0.35	0.2528	0.806 (0.347–1.870)	1.362 (0.581–3.192)
		Case N1/2	8	16	17	0.39	0.732	0.611 (0.231–1.618)	0.995 (0.373–2.653)
rs288980	C/T	Control	40	72	29	0.46	ref.	ref.	ref.
		Case	38	66	33	0.48	0.6239	0.965 (0.554–1.682)	1.198 (0.614–2.336)
		Case male	17	38	17	0.5	0.4456	1.242 (0.623–2.476)	1.379 (0.605–3.147)
		Case female	21	28	16	0.46	0.9918	0.741 (0.373–1.470)	1.051 (0.469–2.356)
		Case N0	22	34	15	0.45	0.8409	0.859 (0.443–1.663)	0.94 (0.418–2.118)
		Case N1/2	12	17	13	0.51	0.412	0.787 (0.342–1.812)	1.494 (0.596–3.745)

p values are for association analysis. N0 = Tumor cells absent from regional lymph nodes; N1/2 = regional lymph node metastasis present. ¹ Ancestral allele first (in NCBI dbSNP database).

ROCK1 or ROCK2 and CRC, a haplotype association analysis was performed. No haplotype showed a higher level of association than alleles or among sex strata (data not shown).

Discussion

Given the wide spectrum of actin cytoskeleton activities influenced by ROCKs, it is not surprising that the expression and activity of these kinases in CRC has been extensively explored. Sari et al. [31] showed that protein expression of both ROCK1 and ROCK2 was markedly augmented in CRC patients. In addition, it has been demonstrated that the inhibition of ROCKs by Y-27632, a compound that simultaneously targets the two kinase isoforms, significantly reduced the motility of colon adenocarcinoma cells [27] and that ROCK2 knockdown decreased invasiveness in highly malignant intestinal epithelial cell lines [24]. Conversely, several reports showed that the inhibition of Rho-kinase stimulates colon cancer cell proliferation, progression and migration [34–36]. Thus, the precise contribution of ROCK kinase to CRC progression and invasion remains controversial and needs more clarification.

Very little is known about the relationship between ROCK1 and ROCK2 genetic variants and the susceptibility to CRC and, at present, only one other group has investigated a possible association between ROCK1 and ROCK2 polymorphisms and the risk of CRC [31]. For the present study, we genotyped a total of nine SNPs: four of them mapping on ROCK1 gene and the remaining five on ROCK2, in order to explore the possible involvement of these two kinases in colorectal carcinogenesis in a subset of the Italian population.

None of the nine selected polymorphisms showed significant allele or genotype association with the occurrence of CRC, suggesting that these SNPs are not directly involved in CRC onset in the studied population. It is noteworthy that no evidence of an association was found for ROCK2 rs35768389 or for ROCK1 rs73963110 variants, which have been previously described as relevant to CRC development in a Turkish cohort [31]. Specifically, in our study no statistically significant difference was found in genotype and allele frequencies for the rs35768389 polymorphism between the control and patient groups, whereas the genotyping of the ROCK1 polymorphism rs73963110 did not provide any evidence of a variant allele in our entire data set. These conflicting re-

Table 4. Association analysis of *ROCK2* locus

SNP information		Sample	Genotype, n			MAF	p value	Relative risk (95% CI)	
SNP ID	alleles ¹		11	12	22			OR _{het}	OR _{hom}
rs12692437	T/C	Control	119	21	1	0.08	ref.	ref.	ref.
		Case	108	26	2	0.11	0.2504	1.364 (0.726–2.565)	2.204 (0.197–24.565)
		Case male	56	15	1	0.12	0.2219	1.518 (0.728–3.165)	2.125 (0.131–34.595)
		Case female	52	11	1	0.1	0.5072	1.199 (0.539–2.665)	2.288 (0.140–37.292)
		Case N0	61	11	0	0.08	0.8522	1.022 (0.463–2.256)	0.648 (0.026–16.135)
		Case N1/2	33	8	1	0.12	0.2924	1.374 (0.558–3.382)	3.606 (0.22–59.212)
rs7563468	G/A	Control	3	30	108	0.13	ref.	ref.	ref.
		Case	3	37	95	0.16	0.2892	1.233 (0.232–6.559)	0.880 (0.173–4.462)
		Case male	2	21	48	0.18	0.1802	1.050 (0.161–6.841)	0.667 (0.108–4.120)
		Case female	1	16	47	0.14	0.719	1.600 (0.154–16.661)	1.306 (0.132–12.879)
		Case N0	1	18	53	0.14	0.7456	1.800 (0.174–18.638)	1.472 (0.15–14.493)
		Case N1/2	1	10	30	0.15	0.6599	1 (0.093–10.736)	0.833 (0.084–8.304)
rs35768389	T/A	Control	140	1	0	0.004	ref.	ref.	ref.
		Case	136	0	0	0	13.793	0.343 (0.014–8.495)	1.029 (0.020–52.242)
		Case male	72	0	0	0	n.c.	n.c.	n.c.
		Case female	64	0	0	0	n.c.	n.c.	n.c.
		Case N0	72	0	0	0	n.c.	n.c.	n.c.
		Case N1/2	42	0	0	0	n.c.	n.c.	n.c.
rs17463896	A/G	Control	44	65	32	0.46	ref.	ref.	ref.
		Case	40	60	36	0.49	0.5115	1.015 (0.584–1.766)	1.238 (0.652–2.348)
		Case male	24	32	16	0.44	0.7987	0.903 (0.470–1.734)	0.917 (0.420–1.999)
		Case female	16	28	20	0.53	0.1657	1.185 (0.575–2.443)	1.719 (0.773–3.824)
		Case N0	16	34	22	0.54	0.0999	1.438 (0.71–2.916)	1.891 (0.859–4.16)
		Case N1/2	17	15	10	0.42	0.509	0.597 (0.27–1.32)	0.809 (0.327–1.998)
rs16857265	T/C	Control	118	18	5	0.1	ref.	ref.	ref.
		Case	120	15	1	0.06	0.113	0.819 (0.395–1.702)	0.197 (0.023–1.709)
		Case male	62	9	1	0.08	0.4381	0.952 (0.404–2.242)	0.381 (0.044–3.330)
		Case female	58	6	0	0.05	0.0745	0.678 (0.256–1.800)	0.184 (0.010–3.387)
		Case N0	63	8	1	0.07	0.3066	0.832 (0.343–2.021)	0.375 (0.043–3.277)
		Case N1/2	37	5	0	0.06	0.264	0.886 (0.308–2.55)	0.287 (0.016–5.317)

p values are for association analysis. N0 = Tumor cells absent from regional lymph nodes; N1/2 = regional lymph node metastasis present; n.c. = not calculable. ¹ Ancestral allele first (in NCBI dbSNP database).

sults could be justified considering first that the influence of genetic factors on colon cancer disease may significantly differ among populations with various origins. The discrepancy may also depend on the different allelic frequencies observed for the two studied groups. Notably, the allele frequency for the above-mentioned markers in the Italian cohort reaches values more consistent with the NCBI database than with the Turkish ones.

When a case-control association study was performed in order to assess the association of the selected genetic variants in *ROCK1* or *ROCK2* genes with CRC in a specific gender, we found that the variant allele of the rs35996865 SNP mapping in *ROCK1* was more frequent

in male patients ($p = 0.028$) than in the control group. To our knowledge, this represents the first evidence of a possible correlation between polymorphic genetic variants of *ROCK1* and the increased risk in CRC male patients. The risk of CRC shows sexual dimorphism, with a significantly higher incidence rate in males than in females (2:1) [37]. It has been proposed that male hormones play a key role in the development of colonic adenomas [38]. Moreover, several reports describe a link between androgens and the *ROCK* signaling pathway [39–41]. On the other hand, cytokines have been associated with the diffusion of human cancer cells of an epithelial origin and, in particular, IL-6 was observed to induce gastric cancer pro-

gression via activation of the c-Src/RhoA/ROCK signaling pathway [42]. Notably, estrogens inhibit IL-6 production and the lower concentration of this hormone in males seems to justify the disparity of occurrence of hepatocellular carcinoma in males compared to females [43]. In the light of these considerations, we propose that this relationship between sexual hormones and RhoA/ROCK signaling may be influenced by gender-dependent differences in ROCK1 gene expression control. Remarkably, the ROCK1 rs35996865 G variant we found associated with CRC in males maps on 5'-UTR, a putative regulatory region that could be influenced by a different upstream modulation, depending on gender. As a last consideration, the rs35996865 polymorphism was recently associated with the risk of developing metabolic syndrome in a Turkish population [44] and clear cell renal cell carcinoma in a Chinese population [45]. Data about this SNP in the literature are rare, so it is impressive that it could play a role in both obesity, a risk factor for CRC, and in another type of carcinoma.

Taking into account the involvement of ROCK isoforms in invasiveness, we analyzed the relationship between the selected polymorphisms and the presence of regional lymph node metastasis. Despite ROCKs being attractive candidate genes, our allelic association study did not support the selected variants in ROCK1 and ROCK2 having an effect on colon cancer lymph node diffusion. However, considering the pivotal role played by ROCK1 and 2 in migration [46], caution should be adopted in completely ruling out their biological role in CRC invasiveness, and we believe that further investigation in patients presenting metastasis in distal organs could help to better clarify the real involvement of ROCK

polymorphisms in influencing the invasive and metastatic properties of CRC cells.

These overall results suggest a potential minor role for Rho-kinase polymorphisms in CRC development in the Italian population, limited to the male subgroup of patients. It should be considered that although the selected markers tag the large majority of polymorphisms presenting an MAF >0.05, the degree of information provided for rarer polymorphisms is very low. Therefore, the absence of an association with CRC for our whole sample does not necessarily rule out a role for ROCK1 or 2 in CRC etiology, since other untyped rare polymorphisms could represent susceptibility factors. In addition, two of the genotyped SNPs, selected because they were previously seen to be involved in a predisposition for CRC [31], were not polymorphic in our sample study, rendering them no longer informative to us. Moreover, it is helpful to recall that ROCK1 and ROCK2 are central players in the cytoskeleton remodeling pathway, a complex genetic network that includes a large number of ROCKs upstream [47]. It is likely that the effect of such a limited number of SNPs could be masked by the relationship among ROCKs and their numerous specific interaction partners. Therefore, further explorations both in the genetic and the epigenetic field are advisable in order to investigate the key role of Rho-kinases in CRC occurrence in greater detail.

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