Mutation screening of the *SMARCA3* gene in Swedish colorectal cancer patients

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Abstract. The *SMARCA3* gene was recently found to be a common target for methylation in colon and gastric cancer, suggesting it has possible tumor suppressor activity. To determine whether *SMARCA3* plays a role in colorectal and/or gastric cancer predisposition, a mutation screening of the gene was performed in affected index cases from 20 Swedish families with colorectal and/or gastric cancer. Notably, one family included in the screening exhibited suggestive linkage to the region on chromosome 3q that harbors the *SMARCA3* gene. In addition to known polymorphisms, nine novel variants - none of them clearly pathogenic - were detected. Seven of these variants were further tested in an additional 287 patients with a family history of the disease, but their frequency was found to not be significantly different from that observed in the controls. In conclusion, although a very low effect of some variants could not be excluded, it seems that the *SMARCA3* gene does not play an important role in the predisposition to colorectal and gastric cancer.

Introduction

Colorectal cancer (CRC) is one of the most common types of malignancies worldwide and has an estimated genetic contribution of approximately 35% (1). A small fraction of CRC cases are found in the setting of highly penetrant inherited syndromes, such as familial adenomatous polyposis (FAP), hereditary nonpolyposis colorectal cancer (HNPCC) and MUTYH-associated polyposis (MAP); however, a significant proportion of CRC families remain in which no predisposing genes have so far been identified (2).

Similarly to CRC, multiple factors play a role in gastric carcinogenesis (1,3,4). Gastric cancer frequently occurs in families affected with HNPCC and FAP, particularly in families of Asian origin (2). In addition, an increased predisposition to gastric cancer has been observed in the Peutz-Jegher (5), Li-Fraumeni (6) and Ataxia Telangiectasia (7) syndromes. The majority of families with an autosomal dominant predisposition to gastric cancer, outside the syndromes mentioned above, have a diffuse type of gastric cancer. In a subset of these families, germline mutations in the E-cadherin (*CDH1*) gene have been identified (8).

The SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 3 (*SMARCA3*) gene (also known as helicase-like transcription factor *HTLF*) is a member of the SWI/SNF family of genes highly conserved in eukaryotes. The SWI/SNF complex is a multiprotein complex involved in chromatin remodeling in an ATP-dependent manner and is implicated in a variety of important cellular functions. Some members of this family, such as SMARCA3, also have sequence-specific domains and can be targeted directly to specific promoters (9). A growing body of evidence suggests that subunits of this complex may be involved in cancer development (10). In particular, mutations in several of the SWI/SNF family genes (*RAD54B*, *hSNF5/INI1*, *BRG1*) have been reported in different tumors and tumor cell lines, suggesting a potential tumor suppressor activity for this gene family (11-15). Moreover, the *SMARCA3* gene has been shown to be a common target for methylation and epigenetic silencing in colon and gastric cancer (16-19).

In the search for novel CRC predisposing genes, a genome-wide scan in 18 non-HNPCC/non-FAP CRC families from Sweden was recently performed (20). In this analysis, suggestive linkage of the disease to chromosome 3q22.1-26.31 was identified in family 242, with the highest location score of 1.99 for the marker D3S1279. In this 3-generation family, an autosomal dominant inheritance of gastric and rectal cancer was observed. Notably, the region of linkage on chromosome 3 harbors the *SMARCA3* gene.

In an attempt to evaluate the role of the *SMARCA3* gene in Swedish CRC patients, mutation screening of all exons, the 5' and 3' untranslated regions (UTR) and a putative promoter region was carried out.

Materials and methods

Patients. Family 242 was included in the previously-performed genome-wide linkage analysis. Three family members presented with gastric cancer and six with rectal cancer, while five family members presented with adenomas and/or hyperplastic polyps. Two affected members from family 242, one

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with gastric cancer (Co-441) and one with rectal cancer (Co-666), as well as index cases from 20 additional colorectal and/or gastric cancer families, were selected for mutation screening of the *SMARCA3* gene. Of these 20 additional families, 18 had a history of both colorectal and gastric cancer, while two had a history of gastric cancer only.

The frequency of the identified variants was subsequently analysed in a set of 287 unrelated patients, index cases of families with at least two family members affected with CRC. Of these 287 patients, 95 had also reported at least one gastric cancer case in the family. The variant frequency was also assessed in a control population of either 95, 380 or 475 anonymous blood donors from the Stockholm region.

All patients were recruited from the Cancer Family Clinic at Karolinska Hospital. Family history was obtained, and all diagnoses were confirmed through medical records or death certificates. None of the patients included in the study had classical or attenuated polyposis. HNPCC was excluded based on family history of the disease, microsatellite instability test or mutation screening of the mismatch repair genes. In addition, mutation screening of the *CDH1* gene was performed in families 24 and 242, and no mutations were detected (unpublished data). Informed consent was obtained from each subject in accordance with the Swedish law concerning the ethical approval of research on human subjects (97/205, 00/291, 03/198).

DNA from all individuals was extracted from peripheral blood lymphocytes using the standard phenol/chloroform extraction method. In addition, total RNA was extracted from EBV-transformed lymphocytes from two members of family 242 (Co-634 and Co-666) using the TRIzol RNA Extraction Kit (Life Technologies Inc., Rockville, MD, USA) as per the manufacturer's protocol.

Mutation analysis of SMARCA3

Mutation screening of the SMARCA3 gene. All exons, including the exon/intron boundaries, the 5' and 3'UTR regions and the putative promoter sequence, were amplified by PCR using the primers listed in Table I. PCR amplifications were carried out in a 25-μl reaction mixture containing 50 ng genomic DNA, 20 pmol of each primer, 100 μM dNTPs, 1X PCR buffer II (Applied Biosystems, Foster City, CA), 1.5 mM $MgCl_2$ and 0.5 U AmpliTaq Gold DNA Polymerase (Applied Biosystems). PCR reactions were in general carried out using a Touchdown PCR program with an initial annealing temperature of 58˚C. However, exons 2, 6, 7, 13, 21 and 25, as well as the UTR regions, were amplified at an initial annealing temperature of 62˚C in order to avoid non-specific PCR amplification. PCR conditions were 95˚C for 9 min and 30 sec, 7 cycles at 95˚C for 45 sec, 58˚C for 45 sec (-1˚C/cycle) and 72˚C for 45 sec, followed by a further 29 cycles of 95˚C for 30 sec, 51˚C for 45 sec and 72˚C for 45 sec, and a final 72˚C for 7 min. Amplified PCR products were cleaned prior to sequencing by the addition of 1 μl ExoSap-IT Clean-up Kit (GE Healthcare) to 5 μl PCR product, followed by incubation at 37˚C for 30 min and enzyme inactivation at 80˚C for 15 min. One microliter of cleaned PCR product was used as a template for a 10-μl sequencing reaction using the ABI Big Dye Terminator v3.1 Kit (Applied Biosystems) according to the manufacturer's instructions. The sequencing reaction products were subsequently precipitated with EDTA and ethanol following the manufacturer's guidelines. Prior to sequencing, 10 μl of Hi-Di Formamide was added to the dried pellets, and samples were electrophoresed in an ABI 3730 XL capillary sequencer (Applied Biosystems). The Chromas program version 1.43 (http://trishul.sci.gu.edu.au/~conor/chromas.html) was used for sequence analysis.

RT-PCR analysis. RT-PCR was carried out using the two available RNA samples from family 242 isolated from EBV-transformed lymphocytes (Co-634 and Co-666). cDNA was prepared using the GeneAmp RNA PCR Kit (Applied Biosystems) employing the random hexamer priming method. SMARCA3 cDNA was amplified in two fragments with fragment one spanning exons 1-14 and fragment two spanning exons 14-25. Primers for both fragments were designed so that at least one of them was covering two exons (primer sequences are shown in Table I). RT-PCR amplifications were carried out using the same PCR protocol as that used for the amplification of each exon. A Touchdown PCR program was used for amplification with an initial annealing temperature of 62˚C. Amplified RT-PCR products were size fractionated on an agarose gel along with the control sample.

SNuPE analysis. The allelic difference in mRNA expression in one member of family 242 (Co-666) was tested using the Single Nucleotide Primer Extension (SNuPE) analysis. For this purpose, the coding polymorphism c.909G>A located in exon 8 was used. Fragments used as templates in the SNuPE reaction were a 367-bp RT-PCR product containing exon 8 (primers are shown in Table I) and the amplification product from genomic DNA amplified using the same primers as those used in the mutation screening of exon 8. The SNuPE reaction was carried out in reverse orientation using fluorescently-labeled primer: 5'-ATGGAAGTTGGTAAGGATTA-3'. The reaction mixture of 20 μl contained ~50 ng of template, 50 μM ddCTP, 50 μM dATP, 50 μM dGTP, 50 μM dTTP, 0.025 μM primer, 0.6 units Thermo Sequenase, together with the buffer provided by the manufacturer (Amersham Life Science). PCR conditions were as follows: 95˚C for 2 min, 30 cycles at 95˚C for 30 sec, 55˚C for 30 sec and 72˚C for 1 min, followed by a final 72˚C for 10 min. The reaction products were size fractionated on an ABI PRISM 377 DNA sequencer using 10% denaturing polyacrylamide gel, and the allelic ratios were calculated using the GeneScan 3.1® software program.

Southern blot analysis. For Southern blot analysis, ~10 μg genomic DNA from patient Co-666 was digested with *Bgl*II and blotted to a positively charged Nylon Plus Membrane (Qiabrane) using standard protocols. The membrane was hybridized with two probes; one 1.3-kb probe spanning exons 1-14 and the second 1.4-kb probe spanning exons 14-25 of the *SMARCA3* gene. Both probes were prepared by RT-PCR and labeled with α^{32}P-dCTP. Hybridization was performed using standard conditions.

Results and Discussion

In total, 15 germline sequence variants were detected in the initial mutation screening of the *SMARCA3* gene in 21 patients (Tables II and III). Six of these 15 variants were reported in

Table I. Primers used for mutation screening of the *SMARCA3* gene.

Fragment	Forward	Reverse
Promoter 1	gggacagagcaagactccat	ctccacggtttacgagacct
Promoter 2	catgcatacgctgaggctta	gcactaggaaagcccaatca
5'UTR	ccctcccttctgtgctctgact	tgagtgggatgacaagagga
Exon 1	ggctcgaaaacgatcca	ggaaggtcaggttcatttgg
Exon 2	gagaaaactactcatatggtctttgg	ttctcttattttctaggttaa
Exon 3	ggaaatcccagataaagtataacaaag	agccctaacaagttcaaagat
Exon 4	ttctttcaagtctgcccaac	gccttgagcacaaattagcc
Exon 5	tgagccacagcacccag	aagaccacaaatacccacacg
Exons 6 and 7	cctggcccgtttgacattaac	aagatagtaagatctagtccc
Exon 8	ttttctggtgaactgggtttc	gaaaacacaatttcttg
Exon 9	ttattcaccctccccttcag	tcaatcatctccttcatgaaa
Exon 10	aaaccaaggttttaagagggataatac	cgtgcccagcctctatctc
Exons 11 and 12	gcttttagcagatctgac	tttaaaacccatggttagctc
Exon 13	tgtactctagcacaggagataatttga	gaaaatgcaccaaaaggaaaa
Exon 14	cacattattgccatctcctta	ccccaatgaatgactctttaa
Exon 15	ttggatttgtcacactactgcc	aaagcctgaattttggaacac
Exon 16	agcagtttgtgcttcagttt	ggggcagaatttacacccac
Exon 17	ttgggccaaatagatactcttactg	aagtgccaactggttcaagc
Exon 18	tgttctgtttcgttcttcttgg	ttcagtgaatgggaaacaaag
Exon 19	agatctctcttggttttattagagttg	aagcaatctccatttgacaga
Exon 20	gactttccctactggcaagc	ttaaatccaagtgcaaaactc
Exon 21	cagatgtctcttaaatggatgaaatc	catttcccctcaaattcacc
Exon 22	gggaaataacatttcaggacc	taaaattcttgtgctgccagg
Exon 23	cctgaatttatcagaaaatgac	ttctaaagtttgcctaagaattttcac
Exon 24	tgcaaaacactgaaagatcagg	ttcatgcattacttgcttaattg
Exon 25	attgcggtttttgccattag	caggccacagtatataacggaac
3'UTR-1	tgaacctatttttaatgaaacttcaaa	gcctctcactcccacagact
3'UTR-2	gttcacataaatgacttggagtttt	ggagtcaacccaaattctgc
3'UTR-3	ttttgaacttgagtaattcatcctt	tgtaatgcttgatctaccagg
3'UTR-4	agacctttctgcagggatga	tatggcaccgaggaaggtaa
RT-frag 1	ccatgtcctggatgttcaag	ctgcaagctccctttttc
RT-frag 2	ccgctttctgtgttaagcaa	tcctttacaatgaattttgtgatga
SNUP-RNA	attctgggaacagcgaaatg	gcgggagctagacaattctg

the single nucleotide polymorphism (SNP) database (http://www.ncbi.nlm.nih.gov/SNP) and their frequency, identified among 21 patients in this study, was similar to the reported frequency. These variants were therefore considered to be polymorphisms (Table II). For the nine variants not reported in the SNP database, a heterozygote frequency was determined by testing control individuals (Table II).

None of the identified sequence variants were clearly pathogenic. However, for seven novel variants a slightly increased frequency was observed in the patients compared to the controls. To estimate the significance of these variants, their frequency was determined in a set of 287 unrelated patients with a family history of CRC, 95 of whom also had a family history of gastric cancer. No significant difference was observed in the frequency of the variants between the patients and the controls, either in the entire group or in the group of 95 patients with a history of both colorectal and gastric cancer (Table II). The frequency among patients was only slightly higher for the c.2440C>T variant (p=0.12; OR=2.39; 95% CI 0.75-7.61) located within exon 21. The Pro814Ser amino acid change caused by c.2440C>T was predicted to be possibly damaging using the PolyPhen program (http://coot.embl.de/PolyPhen/) with a PSIC score difference of 1.800. The c.2440C>T sequence variant was found in nine patients, two from the set of 20 families and seven from the second set of 287 patients. However, in only three of these nine families were additional samples available for segregation analysis (family 89, family 106, and family 258), and a possible co-

Table II. Sequence alterations observed in the *SMARCA3* gene.

Location	Nucleotide change	Amino acid change	Carrier frequency[a]			Status[b]
			% First set of patients	% Second set of patients	% Controls	
Promoter	-454G>A		5 (1/22)	0.38 (1/261)	0 (0/367)	Novel
Promoter	-291C>T		5 (1/22)	0 (0/261)	0 (0/366)	Novel
Intron 6	IVS6-24C>T		9 (2/22)	2.14 (5/234)	1.58 (7/444)	Novel
Exon 8	c.909G>A	T303T	50 (11/22)		38[c]	Reported
Exon 8	c.932A>G	N311S	5 (1/22)		2[c]	Reported
Intron 12	IVS12+56C>T		5 (1/22)	1.95 (5/256)	1.49 (5/336)	Novel
Exon 16	c.1740A>G	R580R	14 (3/22)		11 (9/80)	Novel
Exon 20	c.2328A>G	A776A	40 (9/22)		37[c]	Reported
Exon 21	c.2411T>C	I804T	5 (1/22)	0.38 (1/260)	0.92 (4/437)	Novel
Exon 21	c.2440C>T	P814S	9 (2/22)	2.69 (7/260)	1.14 (5/437)	Novel
Exon 21	c.2456G>A	R819H	5 (1/22)	3.85 (10/260)	5.95 (26/437) and 5[c]	Reported
3'UTR	c.3416A>G		50 (11/22)		44[c]	Reported
3'UTR	c.3538insTA		5 (1/22)	0 (0/274)	0.3 (1/335)	Novel
3'UTR	c.4657A>G		50 (11/22)		42 (39/89) and 42[c]	Reported
3'UTR	c.4775insGAC		40 (9/22)		53 (47/89)	Novel

[a]Heterozygotes. [b]Novel variants or variants reported in the single nucleotide polymorphism database. [c]Average estimated heterozygosity reported in the SNP database.

Table III. Variants identified in the patients.

Family	Patient	Variants
12	Co-89	c.909G>A, c.3416A>G, c.4775insGAC
24	Co-166	-291C>T, c.909G>A, c.3416A>G, c.4775insGAC
26	Co-135	c.909A, c.2328A>G, c.3416A>G, c.4657A>G, c.4775insGAC
81	Co-207	c.909A, c.2328G, c.2411T>C, c.4657G
242	Co-441	c.909G>A, c.3416A>G
242	Co-666	c.909G>A, c.2328A>G, c.4657A>G
293	Co-772	c.909A, IVS9+78G>T, c.3416A
303	Co-762	c.909G>A, c.2328A>G, c.2440C>T, c.3416A, c.4657A>G
398	Co-1124	c.909A, c.1740A>G, c.2328A>G, c3416A>G, c.4657A>G, c.4775insGAC
436	Co-1107	-454G>A, c909A, c.2328A>G, c3416A>G, c.4657A>G
485	Co-1315	c.909G>A, c.2456G>A, c.3416A
488	7/03	c.909A, c.932A>G, c.1740A>G, c.2328A>G, c.3416A>G, c.3538insTA, c.4657A>G, c.4775insGAC
506	45/04	IVS6-24C>T, c.909G>A, c.3416A, c.4657A>G
550	443/04	c.909A, IVS12+56C>T, c.3416A
552	349/04	c.909G>A, c.3416A>G, c.4775insGAC
569	Co-1296	c.909A, c.3416A>G, c.4657A>G, c.4775insGAC
578	531/04	c.909A, c.1740A>G, c.3416G, homozygous c.4775insGAC
590	593/04	c.909G>A, c.3416A>G, c.4775insGAC
600	601/04	c.909A, c.2328A>G, c.3416A>G, c.4657A>G, c.4775insGAC
615	525/04	c.909G>A, c.2328AG, c.2440C>T, c.3416A, c.4657A>G
677	687/04	IVS6-24C>T, c.909A

segregation of the variant with the disease was observed in family 258 only. Although this variant most probably represents a rare polymorphism, the possibility that it might act as a low risk or modifying variant could not be excluded.

Two identified promoter variants were not found among the tested control individuals. The variant -454G>A was detected in a total of 2 patients, one from the first set of 20 families screened and the second among the set of 287 patients. Due to the lack of additional DNA material from these two families, segregation analysis of this variant could not be determined. The variant -291G>A was found only in one patient, Co-166, from family 24. However, subsequent segregation analysis performed in this family showed no co-segregation of -291G>A with the disease. Both promoter variants need to be tested in a larger sample of DNA material in order to evaluate their role in colorectal and gastric cancer predisposition.

Family 242 was included in the recently-performed genome-wide linkage analysis in CRC families (20), and was shown to be possibly linked to the region on chromosome 3q22.1-26.31 which harbors *SMARCA3*. Two members from family 242, one affected with rectal cancer and one with gastric cancer, were selected for mutation screening of the gene. Altogether, four variants were identified in these two family members (c.909G>A, c.2328A>G, c.3416A>G, c.4657A>G) (Table III). However, only the silent variant c.909G>A was shared by both family members. Furthermore, RT-PCR, SNuPE and Southern blot analyses excluded the *SMARCA3* gene as the disease-causing gene in this family as no abnormalities were detected.

The methylation of CpG regions is known to cause the silencing of a number of tumor suppressor genes. Therefore, the identification of genes whose promoters are common targets for methylation may point to their possible role as tumor suppressor genes. Moinova *et al* first reported *SMARCA3* promoter methylation in 43% of primary colon cancers and 26% of colon cancer cell lines. It was also suggested that loss of expression of *SMARCA3* confers a growth advantage in some colon cancers (16). Moreover, promoter methylation of this gene was found in primary gastric cancer and gastric cancer cell lines (17-19), as well as in oesophageal cancer (18).

Only one mutation in *SMARCA3* has been reported previously. A hemizygous nonsense mutation at codon 979 was found in one colon cancer cell line matched to the primary tumor (16). The present data suggest that obviously pathogenic mutations in *SMARCA3* are rare and may be poorly tolerated. The discovery of gene silencing caused by promoter hypermethylation rather than mutations found in tumors also suggests that this gene plays a more important role in tumor progression than tumor initiation.

To the best of our knowledge, this is the first report on screening for germline mutations in the *SMARCA3* gene. The lack of clearly pathogenic mutations suggests that *SMARCA3* is not a common target for mutations in familial colorectal and gastric cancer. In addition to known polymorphisms reported in the SNP database, nine novel variants were also detected. All identified variants demonstrated similar frequencies in patients and controls; however, their significance remains to be determined by future studies.

The region of linkage identified in family 242 spans 43 cM and contains close to 200 genes. Evaluation of candidate genes within a region of this size is beyond the scope of this study; however, the confirmation of the linkage in an independent family sample of DNA material with the same phenotype would be of great importance as it would help in narrowing down the region and reducing the number of candidate genes. Recently, novel evidence for a CRC predisposing locus on chromosome 3q21-q24 has been provided (21). There is therefore a need to combine the results of the two studies and determine the candidate CRC predisposing genes.

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