ONLINE MUTATION REPORT

Mutation and LOH analysis of *ACO2* in colorectal cancer: no evidence of biallelic genetic inactivation

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Colorectal cancer (CRC) is the second most common malignancy as a cause of death in the western countries. Some of the genetic changes leading to colorectal cancer development are well characterised, such as frequent loss of chromosomes 5q, 17p, and 18q.[1] These regions contain important tumour suppressor genes: *APC* in 5q, *TP53* in 17p, and *DPC4/SMAD4* in 18q.[2–4] These genes play a role in both somatic and hereditary tumorigenesis. Somatic mutations in *APC*, *TP53*, and *DPC4/SMAD4* are frequently observed in sporadic CRCs. Germline mutations in *APC* predispose to familial adenomatous polyposis (FAP)[2] and germline mutations in *DPC4/SMAD4* underlie juvenile polyposis.[5] Both conditions are associated with CRC susceptibility. A recent twin study suggested that up to 35% of CRCs could have a predisposing genetic component.[6] However, known mutations only account for 2-5% of all CRCs.[7 8] Despite the growing knowledge about the genetic events underlying CRC, many of them are still unclear. Comparative genomic hybridisation (CGH) and loss of heterozygosity (LOH) analyses have shown that besides the above mentioned losses, whole or partial loss of chromosomes/chromosome arms 1p, 4, 8p, and 22 are recurrent aberrations in colorectal tumours.[9–11] This suggests that genes that have a key role in colorectal tumorigenesis reside in these chromosomes.

Recent studies have shown evidence of the role of mutations in genes associated with mitochondrial energy metabolism in the pathogenesis of different tumour types. Germline mutations affecting the B, C, and D subunits of succinate dehydrogenase (SDH) cause hereditary paragangliomas.[12–14] Carriers of mutations affecting the B subunit of SDH can occasionally develop phaeochromocytomas.[14] A novel cancer predisposition syndrome, hereditary leiomyomatosis and renal cell cancer (HLRCC), is caused by germline mutations in the fumarate hydratase gene (*FH*).[15] HLRCC is characterised by skin and uterine leiomyomas and type II papillary renal cell carcinoma.[15 16] Besides predisposing germline mutations in *SDH* and *FH*, somatic defects have also been reported in both enzymes. The B subunit of SDH has been reported to be defective in a sporadic phaeochromocytoma.[14] An example of somatic inactivation of FH was found in a soft tissue sarcoma.[17] Both SDH and FH are tricarboxylic acid cycle (Krebs' cycle) enzymes, which are critical components of cells' energy metabolism in all tissues. The tumour suppressor nature of SDH and FH makes genes encoding proteins related to SDH and FH function candidates for causing tumour susceptibility. One such protein is aconitase 2 (ACO2), nuclear encoded mitochondrial protein, which catalyses the interconversion of citrate to isocitrate via *cis* aconitate in the tricarboxylic acid cycle. The *ACO2* gene characterised by Mirel *et al*[18] in 1998 is located in chromosome 22q13, which is one of the chromosome regions characteristically lost in many CRCs.[9 19 20] Furthermore, somatic mutations in mitochondrial DNA appear to be common in CRC emphasising the possible role of mitochondrial defects in CRC.[21]

To study the possible role of *ACO2* in colorectal tumorigenesis, we performed a mutation analysis in 23 CRCs showing deletion in chromosome 22q13, selected from a series of 104 CRCs genotyped in a whole genome LOH study.[22]

Key points

- Recent studies have shown evidence of the role of genes associated with mitochondrial energy metabolism in the pathogenesis of different tumour types. Chromosome 22q13 is one of the regions characteristically lost in many colorectal tumours. Aconitase 2 (*ACO2*), nuclear encoded mitochondrial protein, which catalyses the interconversion of citrate to isocitrate in the tricarboxylic acid cycle, is located in this chromosomal area.
- The aim of this study was to analyse the role of *ACO2* in colorectal tumorigenesis. Twenty-three colorectal cancer samples showing a deletion in chromosome 22q13 were sequenced for mutations in *ACO2*.
- We observed two silent polymorphisms in exons 3 and 5, but did not detect any pathogenic mutations.
- Our results show that *ACO2* is often deleted in colorectal cancer but is unlikely to be the true target of the deletions.

MATERIALS AND METHODS

Over 1000 fresh frozen colorectal adenocarcinoma specimens were collected in the Department of Medical Genetics, University of Helsinki between May 1994 and June 1998. The microsatellite instability (MSI) status of the samples was previously determined using BAT26 mononucleotide marker.[23 24] We chose all 23 samples which had displayed LOH in chromosome 22q13 in our previous genome wide analysis of 104 MSI negative CRCs with 372 microsatellite markers,[22] and analysed them by genomic sequencing of *ACO2* in tumour and normal DNA. Because LOH at the locus of interest was an inclusion criterion, normal DNA was sequenced in addition to tumour DNA to exclude loss of *ACO2* variants during tumour progression. This approach allowed detection of all putative somatic and germline sequence changes. Tumour DNA was extracted from fresh frozen specimens. A pathologist evaluated the proportion of tumour tissue histologically before DNA extraction. All samples displayed over 60% carcinoma tissue. Normal DNA was obtained either from blood or normal colon mucosa. The specimens representing normal mucosa were always derived from a separate site rather than from tumour margins. Fifteen of the samples were sporadic and eight had one first degree relative affected with CRC. Because of the MSI negativity and mild family history of cancer, these eight familial samples have not been screened for known genes predisposing to CRC. Familial adenomatous polyposis, Peutz-Jeghers syndrome,

Abbreviations: CRC, colorectal cancer; CGH, comparative genomic hybridisation; HLRCC, hereditary leiomyomatosis and renal cell cancer; MSI, microsatellite instability

Table 1 Primer sequences for the 18 coding exons of *ACO2*

Exon	Primer sequence	GenBank accession number	Product size
1	F: ATT GCG TTC ACA GGG TTC TG R: CGG GAC AGG TAC ACG AGA AG	AF086788	305 bp
2	F: CGG GGA TGG ACT CTC CTA AG R: CCT CCT TCT CTG CCT GAC G	AF093081	270 bp
3	F: GTT GCC ACA TGG ACT GAG AG R: GCT CCA GTG TGA GGG TGA G	AF093082	419 bp
4	F: GAG GGT CAC CTG GAC ACA AA R: CCC ATA GGC CTG GAA CAT AC	U87929	296 bp
5	F: TCG GCT GAG GGC TTC TAA AT R: ACT TGT TCC TTT TCC CTC CTG	AF093084	300 bp
6	F: GGC CAT CCT GAC TTC GTG R: CAA CTG GAG ACC CAT CAG G	AF093084	286 bp
7	F: CGC GTA GCA GGT GTG TGG R: TAG CTC TGT CTT GGG GAG GA	U87931	242 bp
8	F: TGA TGA ATC TCT CAA GAA CAG TTT R: CGG CCC TCA GTG AGT ATT TAG	U87932	240 bp
9	F: AGG CAG TGA AAG AGG CTG TC R: GAG CCA CTG GCC CTG AGA	AF093087	234 bp
10	F: GGT GGA GAC CTC TGC TCA CT R: CTG GAG GCA TTA CTC AGC AT	AF093088	278 bp
11	F: CAG ACT CTC ACC CAC CCT TG R:AAT TCC CCC TCC AAC TTC TG	AF093089	196 bp
12	F: GGA CAG GCC AGG TGA CAA G R: TCT GCC TCC TCC TCT GAC C	AF093090	242 bp
13	F: AGA CAG GAG TGG CAA TTG GT R: GGT TGA GAA GGA AGT GAC GTG	AF093091	237 bp
14	F: TCT GTC CTT GTG GGA ACT GA R: CAG GTT CAT GGC CCT TCC	U87936	217 bp
15	F: TGT CAT CCA CCC CTC CAG R: CTA ATG TCC TCC CTG CAC CT	AF093093	329 bp
16	F: GTA GGG CCA ACA GGT GAG G R: GCA GGG AGA GTG ACC TTG AT	AF093094	360 bp
17	F: CCC AGA TGG GTT CAG AAA AT R: AAG CCT CCT GGG AGA AGC TA	AF093095	293 bp
18	F: AGT GCC CTG TCT CCC TGA C R: GGA TCC ACT GAT GGC ACA C	AF093096	280 bp

and juvenile polyposis were, however, clinically excluded. The age of the patients varied from 30 to 89.

Somatic and germline mutations were searched for by direct sequencing of the 18 coding exons of *ACO2*. Primers were designed for each exon using GenBank sequence (www.ncbi.nlm.nih.gov). Primer sequences and GenBank accession numbers are listed in table 1. The PCR reactions were carried out in a 50 µl reaction volume containing 100 ng genomic DNA, 1 × Ampli*Taq* Gold PCR buffer (Applied Biosystems (AB) Division, Foster City, CA), 300 µmol/l of each dNTP (Finnzymes, Espoo, Finland), 0.8 µmol/l of each primer, and 2 units of Ampli*Taq* Gold DNA polymerase (AB). $MgCl_2$ concentration was 2.25 mmol/l for all exons except for exons 6 and 15. For exon 6 it was 3 mmol/l and for exon 15 1.5 mmol/l. The PCR cycles for exons 1-4, 6-11, and 16 were 35 cycles at 95°C for 45 seconds, 57°C for 45 seconds, and 72°C for one minute. For exons 5, 12-15, 17, and 18 the annealing temperature was 62°C. Predenaturation was performed at 95°C for 10 minutes and final extension was 72°C for 10 minutes for all exons. The PCR products were run on a 2% agarose gel (BioWhittaker Molecular Applications, Rockland, ME) to verify the specificity of the reactions and purified using NucleoSpin Multi-96 Extract Kit (Macherey-Nagel, Düren, Germany).

Direct sequencing of the PCR products was performed using cycle sequencing with Big Dye 3 Terminator chemistry, and reactions were run on an ABI 3100 capillary sequencer (AB) according to the manufacturer's instructions.

RESULTS

Twenty-three colon cancer patients were analysed for somatic and germline mutations of *ACO2* by genomic sequencing. Sequencing showed two silent changes. Eight out of 23 (35%) patients had a 212A>C change (T64T) in exon 3 (fig 1). In one patient the variant was homozygous. Sixteen out of 23 (70%) patients had a 690C>T change (L224L) in exon 5. Two of the patients had a homozygous change. Both polymorphisms have been reported previously.[18] Occurrence of the variants was not associated with family history of CRC. The two variants were not predicted to affect splicing when analysed with Splice Site Prediction by Neural Network and NetGene2 programs (http://www.fruitfly.org/ seq_tools/splice.html, http://genome.cbs.dtu.dk/services/NetGene2/).

All selected cases had showed LOH close to the *ACO2* locus when analysed with an adjacent microsatellite.[22] This allelic loss typically included *ACO2* as evidenced by loss of heterozygosity in most of the informative tumour sequences (fig 1). In 16 tumours at least one of the polymorphic sites was informative, and LOH was observed in 14 of these 16 tumours. All seven samples that had a heterozygous variant in exon 3 displayed LOH in the respective tumour. Five of these had lost the A allele and two had lost the C allele. Ten out of 16 samples, which had a variant in exon 5, showed LOH. Of these 10 patients, five had lost the C allele and five had lost the T allele. In four cases the locus was heterozygous but the tumour did not show unambiguous LOH. Thus, a minimum of 14 of the 104 (13.5%) tumours in the original LOH tumour study of unselected microsatellite stable samples had lost one allele of *ACO2*.

DISCUSSION

Several genes relevant to colorectal cancer development have been identified during the last two decades. The first such genes were *TP53* and *APC,* which were identified in 1989 and 1991, respectively.[2 4] Germline mutations in *APC* predispose to

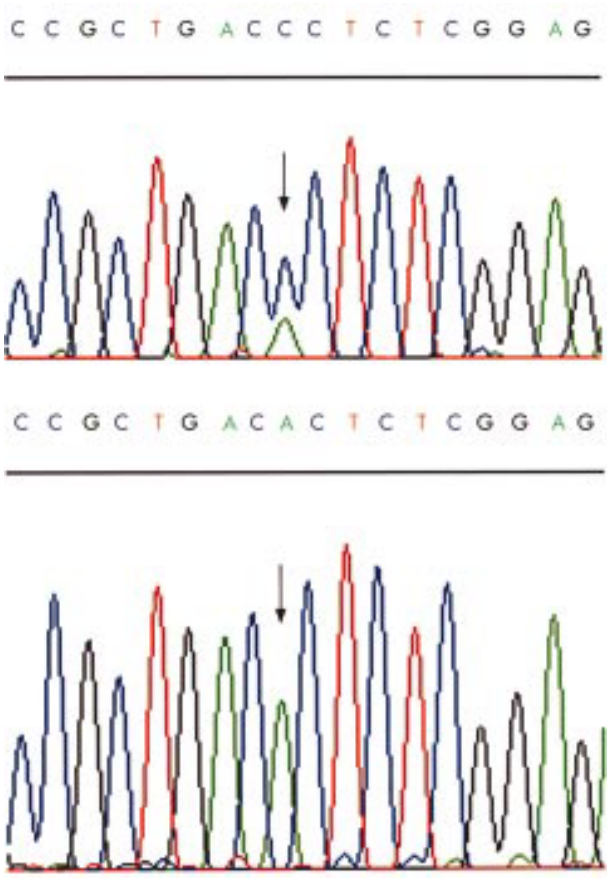

Figure 1 *ACO2* variant in exon 3. The upper panel shows the heterozygous C/A variant in normal DNA. The lower panel shows the deletion of the C allele in the respective tumour DNA.

familial adenomatous polyposis (FAP)[2] and somatic mutations in *APC* can already be seen in the earliest neoplastic lesions.[25] Like *APC*, many genes which are necessary for cancer development function as tumour suppressor or caretaker genes. The first step in identification of such genes has often been the detection of deletions in the respective chromosomal region. In addition to the well known 5q, 18q, and 17p deletions, losses of chromosomes 1p, 8p, 15, and 22q are frequently observed in colorectal cancer.[9–11] These regions most likely contain key tumour suppressor genes, which have not yet been identified.

Genes associated with mitochondrial energy metabolism have not been considered very relevant to cancer development, since many oncogenes and tumour suppressor genes are part of important signal transduction pathways rather than associated with energy metabolism. More recently, however, the role of mitochondrial DNA and Krebs' cycle genes in tumorigenesis has received increasing attention. In 1997, Polyak *et al*[21] reported a high frequency of somatic mutations in mitochondrial DNA in colorectal cancer cells.[21] Furthermore, germline mutations in nuclear encoded mitochondrial proteins predisposing to hereditary paragangliomas and HLRCC were observed in *SDH* and *FH*, respectively.[12–15] Most mutations affecting *FH* and *SDH* have been found in the germline. Somatic mutations appear to be rare; however, in both enzymes somatic inactivation has been reported as well.[14 17] Both *SDH* and *FH* are components of the tricarboxylic acid cycle and lose function in tumorigenesis like classical tumour suppressor genes, raising the question of how important mutations of housekeeping and mitochondrial proteins are in the pathogenesis of common tumour types. The mechanism by which Krebs' cycle gene defects contribute to tumorigenesis is as yet unclear. It has been shown that a moderately increased level of oxygen radicals is highly mitogenic.[26] Mutations in mitochondrial proteins may lead to increased levels of reactive oxygen, thus giving cells a growth advantage.[21] Another possible explanation is hypermutability resulting from oxidative damage.[15]

Related proteins can play role in very different tumour types. SDH and FH are both components of the tricarboxylic acid cycle. However, mutations in *SDH* predispose to paragangliomas, while mutations in *FH* predispose to leiomyomas and renal cell cancer.[12–15] In addition to hereditary predisposition, these proteins appear to play a role in sporadic tumorigenesis as well.[14 17] It is therefore reasonable to hypothesise that mutations in other Krebs' cycle proteins may also have a role in tumorigenesis. We studied the possible role of *ACO2* in colorectal cancer. Twenty-three patients, who showed deletions near the *ACO2* locus in chromosome 22q13, were screened for somatic and germline variants, but pathogenic mutations were not found. We observed two silent polymorphisms in exons 3 and 5, both of which have been reported previously.[18] A 212A>C (T64T) change in exon 3 was found in 35% (8/23) of the patients. A 690C>T (L224L) change in exon 5 was detected in 70% (16/23) of the patients. Both changes appear to be fairly common in the Finnish population. The respective allele frequencies among the American white population were 31% and 46%,[18] indicating that the 690C>T change is more common among Finns. The occurrence of the variants did not correlate with family history of the disease.

Fourteen out of 16 informative tumour sequences showed LOH at the *ACO2* locus (fig 1). This confirms that *ACO2* resides in the commonly deleted region. The losses were not targeted to a particular allele. In one case, two flanking microsatellite markers showed LOH, but LOH was not detected in the respective tumour sequence. It is possible that this patient has a homozygous deletion at the *ACO2* locus.

Although no pathogenic mutations were found, it is possible that methylation has a role in the inactivation of *ACO2*. To our knowledge, the *ACO2* promoter has not been reported previously. The sequence approximately 5000 bases upstream from the initiation codon ATG, however, is predicted to contain two CpG islands (22-–207 and –295-–519 bp from ATG) when analysed with the CpGPlot program (http://bioweb.pasteur.fr/seqanal/interfaces/ cpgplot.html). It is, therefore, possible that deletion of one allele and promoter CpG hypermethylation of the other might have occurred in some lesions, providing biallelic inactivation of *ACO2*.

Our results show that *ACO2* is often deleted in colorectal cancer but is unlikely to be the true target of the deletions unless haploinsufficiency of ACO2 promotes tumorigenesis. Further work is required to identify the as yet unknown 22q gene(s) inactivated during colorectal cancer development.

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