

EXO1 Variants Occur Commonly in Normal Population: Evidence against a Role in Hereditary Nonpolyposis Colorectal Cancer¹

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ABSTRACT

Mutations in the currently known mismatch repair genes cannot explain all cases of hereditary nonpolyposis colorectal cancer (HNPCC), and novel predisposing genes are actively sought. Recently, mutations in the DNA repair gene *EXO1* have been implicated in HNPCC. One truncating and several missense changes were observed in familial colorectal cancer (CRC) cases but not in controls. We evaluated a series of European CRC patients and population controls to clarify whether *EXO1* variants may indeed predispose to familial CRC. Several variants observed in patients were also observed in controls with similar frequencies, including the truncating variant proposed previously to be a disease-causing mutation. Thus, little evidence was obtained to support a major causative role of *EXO1* in HNPCC, although we cannot exclude a role for *EXO1* as a low penetrance cancer susceptibility or modifying gene.

INTRODUCTION

HNPCC⁴ is the predominant cause of familial CRC. MSI caused by loss of MMR function is characteristic of HNPCC tumors. Germ-line mutations in one of two MMR genes (*MSH2* and *MLH1*)⁵ (1) are often detected in HNPCC families fulfilling the Amsterdam criteria (2), whereas mutations in other MMR genes (*MSH3*, *MSH6*, *PMS2*, and *MLH3*) appear to be much less frequent. However, in families with less prominent clustering, MMR gene germ-line mutations account only for up to 30% of cases (3–5). Thus, the known MMR genes and other colon cancer susceptibility genes are not likely to be responsible for all of the cases of familial clustering of CRC (4) reminiscent of HNPCC, and novel predisposing genes still remain to be discovered.

One candidate HNPCC gene is *EXO1*, a recently cloned human

gene that encodes the homologue of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* exonuclease 1 (6, 7). Exonuclease 1 is a 5' to 3' exonuclease that directly interacts with Msh2, Msh3, and Mlh1 (8–12), and has been suggested to act in MMR by catalyzing 5' to 3' excision and by stabilizing higher order complexes of MMR proteins (9, 11). Consistent with these views, human exonuclease 1 has been shown recently to play a role in both 5' to 3' and 3' to 5' mismatch-dependent excision *in vitro* (12). In *S. cerevisiae*, *Exo1* mutations cause an increased rate of accumulating base substitution and frameshift mutations in mononucleotide runs that is epistatic to a *Msh2* mutation. The increased mutation rate caused by an *Exo1* mutation is only on the order of 1% of the mutation rate caused by *Msh2* mutation (9). Because of the extensive evidence supporting a role of *EXO1* in MMR, the human *EXO1* gene is a candidate HNPCC gene. However, the weak mutator phenotype caused by *Exo1* mutations in *S. cerevisiae* suggests that mutations in human *EXO1* could be less frequent or of lower penetrance than mutations in the other MMR genes like *MSH2* or *MLH1*.

To investigate a possible role of human *EXO1* in HNPCC Wu *et al.* (13) screened a set of 258 colon cancer families, most of which were found previously not to carry mutations in the main HNPCC genes, for the presence of germ-line variants in *EXO1* by DGGE. Putative germ-line mutations were found in 14 patients, including 1 patient from a family fulfilling the Amsterdam criteria for HNPCC. The latter patient carried the only truncating mutation found in this large cohort. The newly characterized variants were not detected in 235 healthy Dutch controls and were thus considered as disease-associated mutations. Surprisingly, 12 of 13 CRCs available for LOH studies displayed loss of the mutant instead of the wild-type allele, a result that seems inconsistent with the two-hit model for tumor suppressor gene inactivation. Six of 13 tumors from patients with *EXO1* variants were MSI-H, whereas the other tumors showed MSS phenotype with the five consensus markers (13). On the basis of the findings it was suggested that *EXO1* could represent a high or moderate penetrance CRC predisposition gene.

For this report, we have combined results from *EXO1* analyses in multiple European populations. A series of 18 Finnish CRC probands and 20 Finnish control samples were first analyzed for *EXO1* variants by direct sequencing. This *EXO1* mutation analyses was extended to a total of 970 CRC patients and 1007 control samples from the Netherlands, Norway, Finland, United Kingdom, and Danish using DGGE, SSCP, CSGE, dHPLC, restriction, and sequence analyses.

MATERIALS AND METHODS

Finnish Sample Material

For sequencing analysis of *EXO1* 18 CRC probands were selected. Seventeen of these had at least one first degree relative with CRC. Age of onset

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⁴ The abbreviations used are: HNPCC, hereditary nonpolyposis colorectal cancer; CRC, colorectal cancer; CSGE, conformation sensitive gel electrophoresis; DGGE, denaturing gradient gel electrophoresis; dHPLC, denaturing high performance liquid chromatography; LOH, loss of heterozygosity; MMR, mismatch repair; MSI, microsatellite instability; MSI-H, microsatellite instability high; MSI-L, microsatellite instability low; MSS, microsatellite stable; SSCP, single strand conformation polymorphism.

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varied between 45 and 84 years. Eight were found to be MSI-positive, although their *MLH1* and *MSH2* mutation status was negative. Eleven samples were selected from a series of CRCs none of which was MSI-H when analyzed with a series of markers, including the polyadenylic acid marker BAT26 (14, 15). As controls, 20 anonymous Finnish blood donors were sequenced for *EXO1* germ-line variations.

To evaluate the frequency of *EXO1* variants among different patients and controls by SSCP, we selected an additional 300 MMR mutation-negative CRC patients using the following criteria: the first 111 patients were chosen from cases with a first-degree relative with CRC. These patients were either MSS or MSI-L (14, 15). Another set of 67 patients was diagnosed with CRC ≤ 50 years of age, with unknown family history and no MSI (MSS). The remaining 122 CRC patients were >50 years of age and had no familial aggregation of CRC, and were thus considered as sporadic. Furthermore, 373 anonymous blood donor samples from Finnish Red Cross were used as controls. Eighty familial CRC samples and 3 CRC ≤ 50 years of age were used to evaluate a variant detectable by restriction analysis only. In addition, 504 anonymous controls were used in this effort.

Dutch/Norwegian Sample Material

The coding sequence of *EXO1* was analyzed by DGGE in 116 Dutch and 39 Norwegian CRC families in which no *MSH2*, *MLH1*, or *MSH6* mutations were found. Fifty-five families fulfilled the revised Amsterdam criteria, 68 were suspected HNPCC families, and 32 families had clustering of late onset CRC. At least 52 spouses of the Dutch families were used as controls to evaluate the significance of the detected variants.

United Kingdom Sample Material

Four hundred and eighteen cases of CRC with a diagnosis age <56 years were ascertained from three regional cancer genetics centers within the United Kingdom. Altogether 208 spouses from this cohort with no family history of CRC were used as controls. These samples were only analyzed for three *EXO1* variants (Ser610Gly, Pro640Ser, and Pro640Ala) found in exon 11 that had been detected previously in other samples (Finnish effort and Wu *et al.*, Ref. 13).

Danish Sample Material

Ninety-seven suspected HNPCC patients and 95 anonymous controls were ascertained from the Danish population. These samples were also only analyzed for three *EXO1* variants (Ser610Gly, Pro640Ser, and Pro640Ala) found in exon 11.

Mutation Screening

Sequencing. Normal genomic DNA from 18 CRC probands and 20 Finnish controls were used for amplification of all 14 exons of *EXO1*. PCR primer information is available from authors on request. PCR reactions were performed using 50 ng of genomic DNA, 1 \times AmpliTaq Gold PCR buffer [Applied Biosystems (AB) Division, Foster City, CA], 250 μ M each deoxynucleotide triphosphate (Amersham Pharmacia Biotech, NJ), 0.4 μ M each primer, 1.5–2.5 mM MgCl₂, and 0.4 units of AmpliTaq Gold polymerase (AB). PCR cycles were 95°C for 10 min, following 35 cycles at 94°C for 45 s, 57–65°C for 45 s, and 72°C for 45 s. The final extension was 72°C for 10 min. Both strands of the detected variants were sequenced with an ABI 3100 DNA sequencer (AB).

SSCP. Candidate mutations Val27Ala, Glu109Lys, Leu410Arg, Ser610Gly, Pro640Ser, Pro640Ala, Gly759Glu, and Pro770Leu detected by Wu *et al.* (13) were screened from Finnish material using SSCP. PCRs were done as above. Primer sequences are available from authors on request. The fragments were run on 0.6 \times mutation detection enhancement gel (MDE) (BioWhittaker Molecular Applications, Rockland, ME), 4 W (2.5 kV, 80 mA), for 20–23 h. The variants were confirmed by sequencing.

Restriction Analysis. NlaIV (New England Biolabs, Beverly, MA) restriction enzyme was used to screen truncating variant IVS12–1 (G/C) from Finnish sample material. Restriction reactions were done according to the manufacturer's instructions.

DGGE. Screening of the whole *EXO1* gene of the 155 Dutch/Norwegian families was performed by DGGE (16) with identical primers as described by

Wu *et al.* (13). Both strands of the detected variants were sequenced with an ABI 3100 DNA sequencer (AB).

CSGE. Screening of the three candidate mutations in exon 11 from the United Kingdom cohort was undertaken by CSGE (17) with primers as described by Wu *et al.* (13). Both strands of samples showing a shift were sequenced on an ABI 3100 DNA sequencer (AB).

dHPLC. Screening of the 97 suspected HNPCC samples and 95 anonymous controls collected from the Danish population for the three candidate mutations present in exon 11 were performed by dHPLC. Genomic DNA was used for amplification of part of exon 11 of *EXO1*. PCRs were performed using 50 ng of genomic DNA, 1 \times Optimase PCR buffer (Transgenomic, Cheshire, United Kingdom), 250 μ g each deoxynucleotide triphosphate, 0.4 μ M each forward and reverse primer (forward: 5'GACCATTTCCACCACCACTTT 3'; reverse: 5'TGAGACTCATCGTCTGGACTC 3'), 2.5 mM MgCl₂, and 0.5 μ l of Optimase (Transgenomic). PCR cycles were 95°C for 5 min, followed by 40 cycles at 68°C for 2 min and 94°C for 30 s. The final extension was 72°C for 10 min. dHPLC was carried out on the Transgenomic Wave genetic analyser (Transgenomics) with the temperature of the dHPLC column set at 59°C for the Ser610Gly mutation, and 62.5°C for the Pro640Ser and Pro640Ala mutations. The gradient was formed by mixing buffer A (0.1 mM triethyl ammonium acetate (TEAA)) and buffer B (0.1 mM TEAA, 25% acetonitril) going from 56% buffer B at the start to 62% buffer B at the end.

Sequence Alignment

EXO1 protein sequences of *Homo sapiens* (GenBank accession no. NP_569082), *Mus musculus* (NP_036142), *Drosophila melanogaster* (CAA61431), *S. cerevisiae* (P39875), and *S. pombe* (P53695) were obtained from the National Center for Biotechnology Information protein database. Sequences were aligned using Clustal W (1.82) multiple sequence alignment program (18).

Statistical Analysis

Comparison of *EXO1* variants in cases and controls was assessed by Fisher's exact test, with a *P* of 0.05 being considered statistically significant.

RESULTS

Study Design. An extensive series of suspected HNPCC cases collected from Finland, Denmark, the United Kingdom, Norway, and the Netherlands were screened for the presence of germ-line mutations in the *EXO1* gene. Most of these samples were shown previously not to have germ-line mutations in other MMR genes (*MLH1*, *MSH2*, and *MSH6*). The combined results are presented Table 1.

Sequencing. As the first step of our study, 18 Finnish CRC probands and 20 controls were sequenced to get an overview on the *EXO1* variants and their possible significance in CRC predisposition. Only the Pro640Ser variant appeared as a possible mutation. The remaining variants were detected both in CRC and control samples, and were regarded as polymorphisms.

SSCP, DGGE, CSGE, dHPLC, and Restriction Analyses. To additionally evaluate the significance of the candidate *EXO1* mutations reported by Wu *et al.* (13), SSCP, DGGE, CSGE, dHPLC, and restriction analysis were used to analyze a larger cohort of familial and sporadic CRC samples. The whole *EXO1* gene was screened when evaluating 155 Dutch/Norwegian CRC kindreds using DGGE. SSCP, CSGE, dHPLC, and restriction analysis were used to screen for the previously reported candidate mutations (13). Altogether, 970 CRC patients and 1007 controls from the Netherlands, Norway, Finland, United Kingdom, and Denmark were used. Five of nine mutations proposed by Wu *et al.* (13) were detected in both CRCs and controls including the only truncating variant IVS12–1 (G/C), which was detected in a Dutch CRC sample as well as in three Dutch controls, with equal frequency (Table 1). Two of the Dutch controls with the truncating variant IVS12–1 (G/C) had no family history of any type of cancer, and they were 61 and 50 years of age, respectively.

Table 1 Analysis of *EXO1* variants in CRC probands and controls
 Results of the Finnish, Dutch/Norwegian, United Kingdom, Danish, and combined data sets. The last column lists the 9 candidate *EXO1* mutations reported previously (13).

Exon	Nucleotide change	Protein change	Finnish CRC probands	Finnish CRC controls	Dutch/Norwegian CRC probands	Dutch controls	United Kingdom CRC probands	United Kingdom controls	Danish CRC probands	Danish controls	Combined CRC probands	Combined controls	Wu <i>et al.</i> (13) CRC probands
2	T to C	Val27Ala	-/109	-/112	-/155	-/92					-/264	-/204	1/258
4	G to A	Glu109Lys	-/18	-/20	-/155	-/92					-/173	-/112	2/258
5	G to T	Ala137Ser	-/18	-/20	1/142	-/71					1/160	-/91	
9	T to G	Leu410Arg	-/109	-/112	1/154	-/114					1/263	-/226	2/258
10	T to G	Phe438Cys	-/18	-/20	1/154	-/104					1/172	-/124	
11	A to G	Ser610Gly	1/300	-/373	1/155	-/52	2/418	1/208	1/97	-/95	5/970	1/728	3/258
11	C to T	Pro640Ser	3/300	3/373	3/150	-/52	5/418	2/208	1/97	2/95	12/965	7/728	1/258
11	C to G	Pro640Ala	1/300	1/373	1/150	1/52	3/418	2/208	2/97	-/95	7/965	4/730	1/258
11	C to T	Asp661Asp	-/18	-/20	2/150	-/52					2/168	-/72	
13	IVS12-1	G to C	-/83	-/504	1/149	3/200					1/232	3/704	1/258
13	IVS12-1	G to A	-/83	1/504	-/149	-/200					-/232	1/704	
13	G to A	Gly759Glu	3/109	11/112	1/149	1/200					4/258	12/312	2/258
13	C to T	Pro770Leu	-/109	-/112	-/149	-/200					-/258	-/312	1/258
14	C to T	Ala827Val	-/18	-/20	-/89	1/90					1/107	1/110	

The third control with the same variant had sporadic polycystic kidney disease. No additional information from the control was available. The restriction analysis from Finnish controls revealed an additional G/A substitution at this same position probably resulting in truncation of the protein as well. However, we were not able to demonstrate the expected splicing defect, because RNA was not available. One of the Pro640Ser variants and the only Pro640Ala variant in the Finnish CRC probands were detected in 122 sporadic samples. Four additional candidate *EXO1* mutations (13) were not found in our combined sample sets: Val27Ala, Glu109Lys, Leu410Arg, and Pro770Leu (Table 1). The Leu410Arg mutation was identified in a patient from a suspected HNPCC family. The index patient is also carrier of a *MSH2* missense mutation (Tyr165Asp) of unknown pathogenicity (data not shown). DGGE analysis of the Dutch cohort also revealed four previously unreported *EXO1* variants; Ala137Ser, Phe438Cys, and the silent Asp661Asp variant were detected exclusively in CRC samples, whereas Ala827Val was found also in a control sample.

Sequence Alignment. The comparison of *H. sapiens*, *M. musculus*, *D. melanogaster*, *S. cerevisiae*, and *S. pombe EXO1* protein sequences is depicted in Fig. 1. These proteins show a high degree of homology in the NH₂-terminal region where the exonuclease active site is located and less homology in the COOH-terminal region, which is not required for exonuclease activity (7, 9, 12, 19, 20). The five amino acid changes that were found in both cases and controls did not alter an amino acid that was conserved in the five *EXO1* protein sequences or the subset of the five sequences when gaps were present. In one case (Pro640Ser), the amino acid substitution was the same as found at the equivalent position in at least one *EXO1* protein sequence. The remaining amino acid substitutions (Ser610Gly, Pro640Ala, Gly759Glu, and Ala827Val) were conservative substitutions in comparison to the amino acids found at the equivalent positions in the five *EXO1* protein sequences. In addition, Ala827Val is located in the region encoded by exon 14, which is an alternately spliced exon that encodes sequences that are apparently not required for EXO1 activity (7, 12). Six amino acid changes detected only in CRC probands and not controls (Val27Ala, Glu109Lys, Ala137Ser, Leu410Arg, Phe438Cys, and Pro770Leu) all affected nonconserved amino acids of the protein (Fig. 1), although they may cause significant changes. Thus, Val27Ala changes a large hydrophobic residue to a small hydrophobic residue, Glu109Lys changes the charge of the residue, Ala137Ser changes a small hydrophobic residue to a small polar residue, Leu410Arg changes a hydrophobic residue to a charged residue, Phe438Cys changes a large hydrophobic residue to a small hydrophobic residue, and Pro770Leu changes a small hydrophobic residue to a large hydrophobic residue. In addition, Val27Ala, Glu109Lys, and Ala137Ser alter residues in the region of the protein where the exonuclease active site is located, although none of these changes alter any of the known critical active site residues (7, 19, 20).

LOH. No LOH was seen in the matched pairs of normal and tumor samples from 7 Finnish CRC patients displaying *EXO1* variants in exon 11 (Ser610Gly, Pro640Ser, and Gly670Gly). The signal intensities of the alleles at the site of the variants were quantified by sequencing.

Statistical Analysis. In each of the populations studied there was no evidence of overrepresentation of specific or collective *EXO1* variants in cases compared with controls (Table 1). Furthermore, there was no evidence of overrepresentation when pooling data from all of the populations, with all of the *Ps* falling below the 0.05 significance threshold.

DISCUSSION

Recently, a novel human homologue to yeast exonuclease 1 was cloned (6, 7). On the basis of its function in yeast (11) and a mutation screening effort in 258 familial CRC cases, Wu *et al.* (13) proposed that *EXO1* could be associated with CRC predisposition. This report combines *EXO1* data created in five European populations: the Netherlands, Norway, Finland, United Kingdom, and Denmark.

We found five of the nine candidate mutations reported previously (13) in population controls, including the splice site variant predicted to truncate the *EXO1* protein (Table 1). The change was identified in a CRC patient with a MSI-L (two of nine microsatellites unstable) colorectal tumor from a family fulfilling the Amsterdam criteria. The brother of the patient, affected with CRC at age 50, tested positive for the variation as well. Unfortunately, no additional segregation analysis could be performed in the family. However, the same variant was detected in three control samples, indicating the polymorphic nature of the variation. From four additional candidate *EXO1* mutations (13) detected exclusively in our CRC samples, the Leu410Arg mutation was identified in a carrier of a *MSH2* missense mutation of unknown pathogenicity from a suspected HNPCC family.

We found additional *EXO1* germ-line missense variants not reported before. Three of these variants, including one silent change and two missense changes, were exclusively found in the CRC patients and not in controls. The Ala137Ser alteration was detected in a Norwegian patient from a late onset CRC family. Notably, this GCC/TCC nucleotide change in exon 5 of *EXO1* introduces a cryptic splice site with a value of 0.72 calculated with the splice prediction software used.⁶ Unfortunately, no additional material was available to test the consequences of the base alteration on RNA splicing or cosegregation of the variant with the disease. The Phe438Cys mutation was found in a Norwegian patient from a family fulfilling the revised Amsterdam criteria. Also here no additional material was available for additional studies. We also found one variant that was present in both a CRC family and normal controls, and one variant that was only present in a normal control.

Altogether, seven *EXO1* variants have been detected in CRC probands but not in controls. Six of these variants caused amino acid change in the protein. None of the variants changed a conserved amino acid of the *EXO1* protein, although they could still represent significant amino acid substitutions (Fig. 1). The hypothesis that *EXO1* is a HNPCC gene is in good part based on the detection of a truncating mutation in a single HNPCC family. The same mutation was detected in our study in several Dutch controls and, therefore, seems unlikely to represent a disease-causing mutation. In this context, it should also be noted that there are two enzymatically active alternate splice forms of *EXO1*, one of which contains exon 14 and one of which is truncated after exon 13, indicating that the COOH terminus of *EXO1* may not be essential for activity (7, 12). In addition, it is possible that some variant alleles not detected in controls are so rare in populations that we would have needed more controls to detect those. Similarly, the potentially truncating IV12-1 G/A change was seen only in a control sample.

In conclusion, the combined results of this study and that performed by Wu *et al.* (13) have detected missense variants and a splice site change in the *EXO1* gene in HNPCC cases, and similar changes in population controls. These studies also detected missense changes and one silent change in HNPCC cases but not in controls. It is possible that functional studies may show that some of these amino acid substitutions actually alter or inactivate the function of *EXO1*. However, given the many HNPCC cases lacking mutations in other MMR

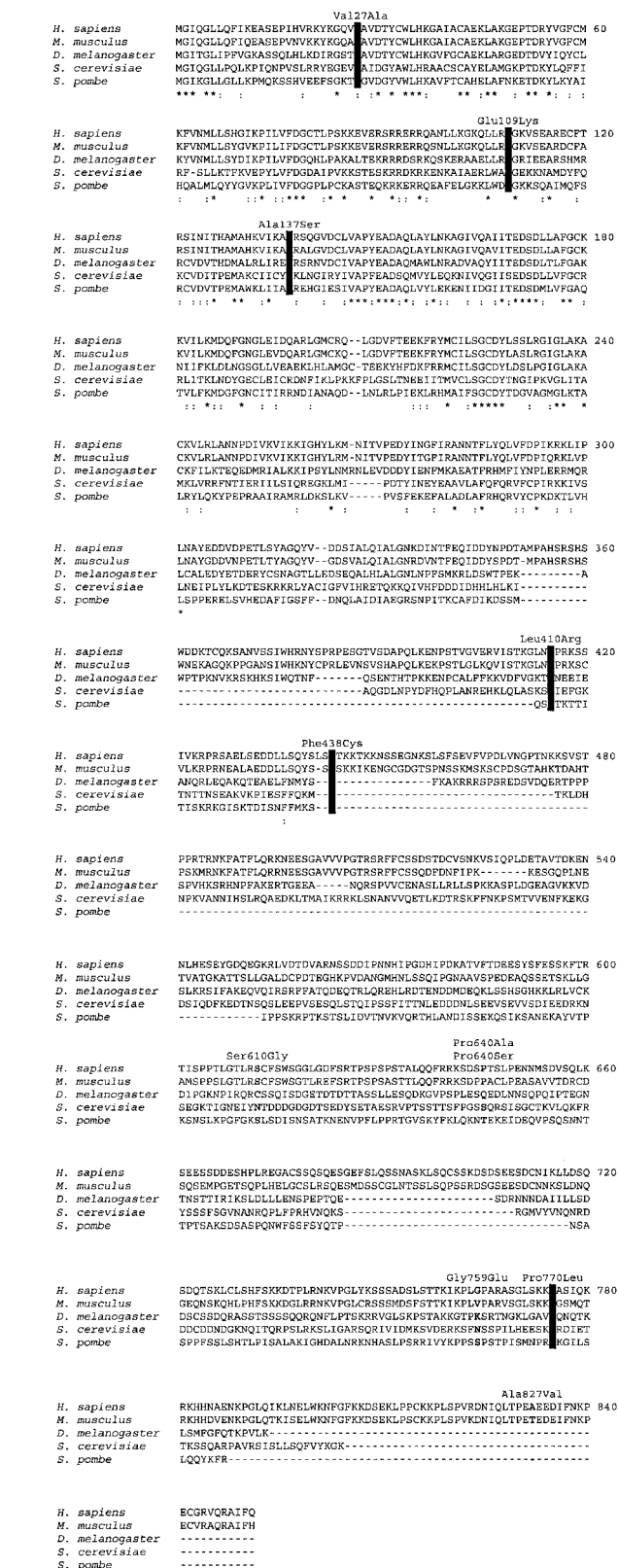


Fig. 1. Protein sequence alignment of *EXO1* proteins of *H. sapiens*, *M. musculus*, *D. melanogaster*, *S. cerevisiae*, and *S. pombe*. The fully conserved amino acid residues are indicated by * whereas similar amino acids are indicated by ;. The shaded and bolded amino acids indicate the changes detected only in CRC probands, and changes detected both in CRCs and controls, respectively.

⁶ Internet address: http://www.fruitfly.org/seq_tools/splice.html.

genes like *MLH1*, *MSH2*, and *MSH6* that were analyzed here and by Wu *et al.* (13) to identify a few cases with a potential germ-line mutation in *EXO1*, it seems likely that *EXO1* variants either do not predispose to an HNPCC-like familial predisposition to CRC or account for only a very small proportion of such cases. Compatible with this view, it has been shown that germ-line *EXO1* deletions are not associated with clinically manifested colorectal tumors in two United Kingdom families.⁷ However, because the studies performed to date have only focused on suspected HNPCC cases, we cannot exclude a role of *EXO1* as a low penetrance cancer susceptibility or modifying gene.

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⁷ I. P. M. Tomlinson, unpublished observations.