whether there was an association between the presence of the diffuse ring and breast cancer. There were 49 samples from women with breast cancer and 59 from unaffected women. A χ^2 value of 0.86 was obtained. For 1% significance, a value of 6.63 and for 5% significance, a value of 3.84 is required. Thus, it can be concluded that there is no measurable association between the diffuse ring and breast cancer. The trace element (Zn, Cu, Fe, and S) analysis of intact hair showed no correlation with the ring structure in the diffraction pattern or with the subjects' group. The women in the normal population group whose hair had shown the diffuse ring were examined and shown not to have breast cancer.

Our x ray diffraction data do not support the recent claim that hair from breast cancer patients or those at high risk (*BRCA1/BRCA2* mutation carriers) show a distinct diffuse ring. This conclusion for breast cancer diagnosis was also reached on a much smaller study of head hair only.[6] In our study, diffraction patterns from 75% (37 of 49) of the breast cancer patients do not show this ring. Moreover, the χ^2 test shows no association between the diffuse ring and breast cancer and, as such, the claim that x ray diffraction of pubic hair can be used as a screening method for breast cancer or breast cancer predisposition is invalid.

ANTHONY HOWELL*
J GÜNTER GROSSMANN†
KAN C CHEUNG†‡
LALJI KANBI†‡
D GARETH R EVANS§
S SAMAR HASNAIN†‡
*CRC Department of Medical Oncology, Christie Hospital NHS Trust, Wilmslow Road, Manchester M20 4BX, UK
†CLRC Daresbury Laboratory, Warrington WA4 4AD, Cheshire, UK
‡Faculty of Applied Sciences, DeMontfort University, Leicester LE1 9BH, UK
§Department of Medical Genetics, St Mary's Hospital, Manchester M20 8LR, UK

Correspondence to: Dr Evans, Gevans@central.cmht.nwest.nhs.uk

1 Wiesener W, Gorner W, Niese S, Grund W, Hennig M, Mende T. Neutron activation analytical determination of trace elements in human hair. *Isotopenpraxis* 1981;**17**:278-82.
2 Astbury WT, Woods HJ. The X-ray interpretation of the structure and elastic properties of hair keratin. *Nature* 1930;**126**:913-14.
3 James VJ, Yue DK, McLennan SV. Changes in the molecular structure of human hair in insulin-dependant diabetes. *Biochem Biophys Res Commun* 1997;**233**:76-80.
4 James V, Kearsley J, Irving T, Amemiya Y, Cookson D. Using hair to screen for breast cancer. *Nature* 1999;**398**:33-4.
5 Fraser RDB, MacRea TP, Rogers GE, Filshie BK. Lipids in keratinised tissues. *J Mol Biol* 1963;**7**:90-1.
6 Briki F, Busson B, Salicru B, Esteve F, Doucet J. Breast-cancer diagnosis using hair. *Nature* 1999;**400**:226.

Mutation analysis of *SMAD2*, *SMAD3*, and *SMAD4* genes in hereditary non-polyposis colorectal cancer

EDITOR—Transforming growth factor-β (TGF-β) family members are known to be involved in the regulation of cell proliferation, differentiation, and apoptosis.[1] Members of the TGF-β family include TGF-βs, activins, and bone morphogenetic proteins (BMPs). Their signals are mediated to the cell nucleus by a network of transmembrane serine/threonine kinase receptors and their downstream effectors, the SMAD proteins.[2] SMAD proteins play a key role in intracellular TGF-β signalling and inactivating mutations of *SMAD*s, such as *SMAD2*, *SMAD3*, and *SMAD4*, provide resistance of cells to TGF-β induced growth inhibition.

To date, eight human *SMAD*s have been identified. Two of them, *SMAD2* and *SMAD4*, have been reported to be mutated in a subset of colorectal carcinomas.[3-6] Germline mutations of *SMAD4* have been found in patients with juvenile polyposis, a condition predisposing to colorectal cancer.[7-10]

SMAD3 mutations have not been reported in human cancers. In a recent study by Arai *et al*,[11] *SMAD3* mutations were analysed in 35 sporadic colorectal and 15 HNPCC cancers and no mutations were found. Targeted disruption of the *SMAD3* gene in mice has been reported to lead to development of colorectal cancer,[12] though other studies have not detected a clear association.[13 14] No genetic alterations in other *SMAD*s have been reported in malignancy.

Hereditary non-polyposis colorectal cancer (HNPCC) is an autosomal dominantly inherited cancer susceptibility syndrome, associated with germline mutations in five DNA mismatch repair genes: *MLH1, PMS1, PMS2, MSH2*, and *MSH6*.[15-19] Inactivation of both alleles of a mismatch repair gene results in microsatellite instability (MSI) that is a hallmark of HNPCC tumours.[20-23] The genes responsible for microsatellite stable (MSS) HNPCC are still unknown.

Loss of growth inhibition by TGF-β is an important step in colon tumorigenesis and in HNPCC tumours with MSI this is mainly the result of frameshift mutations within a polyadenine sequence repeat in the TGF-β type II receptor (*TGFβRII*) gene.[24] It has been proposed that mutations in *TGFβRII* could underlie the cancer predisposition in MSS HNPCC,[25] and also that other genes involved in the TGF-β pathway are candidates for MSS HNPCC.[26]

Chromosomal deletions are common genetic alterations in cancer and they are targeted at tumour suppressor loci.[27 28] Previous studies have shown that one copy of chromosome 18q is lost in over 70% of sporadic colorectal cancers.[29-32] The *DCC* (deleted in colorectal cancer) gene has been suggested as a candidate target gene in this region and loss of expression of *DCC* has also been reported in colorectal cancers.[33] However, mutations in the coding region of *DCC* seem to be rare[34] and the position of *DCC* as a candidate tumour suppressor is not clear. Two other candidate genes, *SMAD4* and *SMAD2*, have recently been identified at the same 18q region[3 35] emphasising the possible role of the *SMAD* genes in colorectal tumorigenesis. The aim of the present study was to investigate whether germline mutations in *SMAD2, SMAD3*, and *SMAD4* underlie microsatellite stable HNPCC.

Mutation screening was performed in 14 Finnish HNPCC kindreds from which lymphoblastoid cell lines were available. Based on genealogical evidence the families are unrelated, though the existence of early common ancestors cannot be excluded. One affected subject per family was included in the study. Of the kindreds, six fulfilled the Amsterdam criteria for HNPCC.[36] Other patients represent familial HNPCC-like colorectal cancer (CRC); the number of patients with CRC or endometrial cancer ranged from two to six per family (average three) (table 1). All kindreds selected for this study have previously been shown to be *MLH1* and *MSH2* mutation negative.[37] In three kindreds, DNA from tumour tissue had not been available. From 10 families one and in one family two colorectal cancer samples were available and no evidence of MSI had been detected (table 1). The study

HNPCC family	Criteria for diagnosis	MSI/MSS status	Sites of cancers and age of diagnosis in proband and first degree relatives
F 31	Amsterdam criteria	MSS	CRC (38, 39, 48, 62) small intestine (39)
F 33	Familial CRC	Not known	CRC (34, 40, 44, 50), cervix (40), liver (71)
F 42	Familial CRC	MSS	CRC (61, 67), stomach (56), breast (69)
F 44	Familial CRC	MSS	CRC (33, 53)
F 46	Familial CRC	MSS	CRC (54), cervix (?)
F 56	Amsterdam criteria	MSS	CRC (24, 43, 51, 52, 76)
F 65	Amsterdam criteria	Not known	CRC (32, 41, 54), liver (?)
F 68	Familial CRC	MSS	CRC (66, 68)
F 70	Familial CRC	MSS	CRC (?)
F 74	Amsterdam criteria	Not known	CRC (64, 67)
F 75	Familial CRC	MSS	CRC (?)
F 76	Familial CRC	MSS	CRC (41, 45)
F 80	Amsterdam criteria	MSS	CRC (47, 67), melanoma (70)
F 84	Amsterdam criteria	MSS	CRC (43, 56, 59), breast (44)

CRC = colorectal cancer.

was approved by the ethical committee of the Department of Medical Genetics, University of Helsinki.

Total cellular RNA was extracted from lymphoblasts by RNA extraction kit (QIAGEN). The *SMAD2*, *SMAD3*, and *SMAD4* genes were amplified from RNA using an RT-PCR procedure. First, 20 µl cDNA was created from 0.8 µg of RNA using standard random priming methods with Mu-MLV reverse transcriptase (Promega) and RNAse inhibitor (Promega). The cDNA sequences for *SMAD2*, *SMAD3*, and *SMAD4* were derived from GenBank database (accession numbers U65019, U76622, and U44378, respectively). PCR primers for cDNA amplification were designed using the Primer3 server (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi).

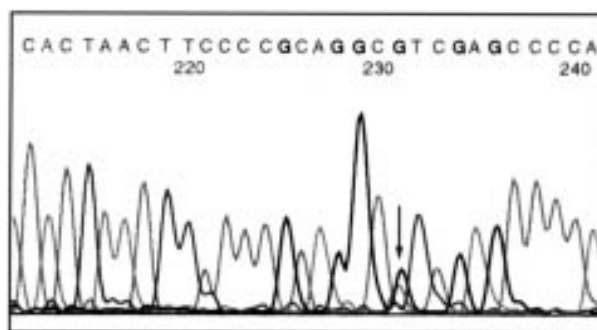| Gene/fragment	Primer sequence (5'→3')	Product size (bp)
SMAD2 Fragment 1	F: ATGTCGTCCATCTTGCCATT R: CCTTTTCGATGGGATACCTG	365
SMAD2 Fragment 2	F: CCAGGTCTCTTGATGGTCGT R: TATATCCAGGAGGTGGCGTT	354
SMAD2 Fragment 3	F: TATTCCAGAAACGGCCACCTC R: GCACTCAGCAAAAACTTCCC	400
SMAD2 Fragment 4	F: TGCCACGGTAGAAATGACAA R: AAGGGGATCCCATCTGAGTT	413
SMAD2 Fragment 5	F: AATGTGCACCATAAGAATGAGTT R: TTCCATGGGACTTGATTGGT	202
SMAD3 Fragment 1	F: CCAGCCATGTCGTCCATC R: AAGGCGAACTCACACAGCTC	344
SMAD3 Fragment 2	F: ATGTCATCTACTGCCGCCTG R: ATTCGGGGATAGGTTTGGAG	377
SMAD3 Fragment 3	F: TGGCTACCTGAGTGAAGATGG R: CCTCCGATGTAGTAGAGCCG	357
SMAD3 Fragment 4	F: TAGGGCTGCTCTCCAATGTC R: TGTCTCCTGTACTCCGCTCC	349
SMAD3 Fragment 5	F: GGCTTTGAGGCTGTCTACCA R: AACATCCACCTCTGGGTTTG	382
SMAD4 Fragment 1	F: TTTCCAAAGGATCAAAATTGC R: TTGTGAAGATCAGGCCACCT	385
SMAD4 Fragment 2	F: GATCTATGCCCGTCTCTGGA R: GTGGAAGCCACAGGAATGTT	387
SMAD4 Fragment 3	F: CTGCCAACTTTCCCAACATT R: GGGTCCACGTATCCATCAAC	436
SMAD4 Fragment 4	F: CCATTTCCAATCATCCTGCT R: CGATGACACTGACGCAAATC	480
SMAD4 Fragment 5	F: GATTTGCGTCAGTGTCATCG R: TGATAAGGTTAAGGGCCCCA	378

Figure 1 *SMAD3 polymorphism identified by sequencing. Adenine has changed to guanine (marked by an arrow), which is predicted to convert isoleucine to valine at amino acid 170.*

Each gene was divided into five fragments, covering the whole coding region of the gene. The forward (F) and reverse (R) primers and size of each PCR product are listed in table 2.

The PCR reactions were carried out in a 50 µl reaction volume including 2 µl of cDNA, 1 × PCR reaction buffer (Perkin Elmer Applied Biosystems Division), 200 µmol/l of each dNTP (Finnzymes), 0.8 µmol/l of each primer, and 2 units of Ampli*Taq* GOLD polymerase (PE/ABI). The MgCl$_2$ concentration was 1.5 mmol/l for *SMAD2* fragments 1 and 2, *SMAD3* fragment 3, and all *SMAD4* fragments. For all other fragments the MgCl$_2$ concentration was 2.5 mmol/l. *SMAD3* fragment 2 reaction also included 10% of DMSO. The PCR conditions are available upon request.

After PCR, 5 µl of the PCR product was run on a 3% agarose (NuSieve) gel to verify the specificity of the PCR reaction. The rest of the PCR product was purified using QIAquick PCR purification Kit (QIAGEN). Direct sequencing of the PCR products was performed using the ABI PRISM Dye Terminator or ABI PRISM dRhodamine cycle sequencing kits (PE/ABI). Cycle sequencing products were electrophoresed on 6% Long Ranger gels (FMC Bioproducts) and analysed on an Applied Biosystems model 373A or 377 DNA sequencer (PE/ABI).

To screen for the presence of a base substitution in *SMAD3* in controls, restriction enzyme digestion was performed. *Hga*I (New England BioLabs) digestion was used to detect A to G change in *SMAD3* exon 3 at codon 170. New PCR primers for genomic exon 3 amplification were designed using the Primer3 server. The primers were: 5'-ATCGACACTGAGCCACCTCT (forward) and 5'-CCCACGTGCCTACCTCTG (reverse). The PCR reactions were carried out in a 50 µl reaction volume including 100 ng genomic DNA, 1 × PCR reaction buffer (PE/ABI), 200 µmol/l of each dNTP (Finnzymes), 0.8 µmol/l of each primer, 2 units of Ampli*Taq* GOLD polymerase (PE/ABI), and 1.5 mmol/l of MgCl$_2$. The following PCR cycles were used for amplification: 10 minutes at 95°C, 40 cycles of 45 seconds at 95°C, 45 seconds at 56°C, one minute at 72°C, and final extension for 10 minutes at 72°C. *Hga*I cuts the PCR fragment (187 bp) that contains the substitution into two fragments (134 bp and 53 bp in size) whereas the wild type fragment lacks the restriction site and is not digested. The digestion was performed in 1 × NEBuffer (New England BioLabs) at 37°C overnight. After digestion, the PCR products were electrophoresed through a 3% agarose gel.

In this work we analysed *SMAD2*, *SMAD3*, and *SMAD4* mutations in 14 familial colon cancer kindreds, 11 of these displaying at least one MSS tumour. The microsatellite analysis data derived typically from one single tumour per family suggest that these kindreds do not segregate DNA mismatch repair gene mutations, but does not exclude this possibility. Previous studies had evaluated *MLH1* and

MSH2 mutations in the series with negative results.[37] [38] *SMAD* gene mutation analysis was performed by automated sequencing covering the translated region of the genes. Genetic alterations were not detected in *SMAD2* or *SMAD4* in any of these patients. In *SMAD3*, three discrepancies were detected between GenBank sequence (U76622) and sequences from our patients, firstly the A to G change at the third position of codon 103 (exon 2). Homozygous A to G change was seen in 11 of our 14 patients and in three of them the substitution was heterozygous. This discrepancy has been reported earlier and the variant does not cause any amino acid change.[9] [11] A second, silent change detected was a C to T transition at nucleotide 907 (exon 6). This change was homozygous and it was present in one of our 14 HNPCC patients (in family 31). The frequency of these variants in the normal population was not analysed, as the changes were silent. The third change was an adenine to guanine transition at nucleotide 545, which is predicted to convert isoleucine to valine at amino acid 170 (fig 1). This change was detected in two patients (in families 65 and 75). For this variant, 110 Finnish controls were analysed by restriction enzyme digestion (*Hga*I). Seven out of 110 controls displayed the change (6.4%). To compare further the frequency of this polymorphism in colon cancer patients and controls, 132 patients were included in the analysis. Taken together, in the 14 HNPCC patients and 132 colon cancer patients the frequency of this polymorphism was 8.9% (13/146). From those 13 cancer patients who had valine instead of isoleucine at codon 170, four turned out to be familial. Segregation of the polymorphism was analysed in two of these families where DNA from multiple family members was available and the polymorphism did not segregate with cancer in these families.

SMAD2, *SMAD3*, or *SMAD4* mutations were not found in any of our patients using a cDNA based mutation analysis. It should be noted that like all other mutation detection methods, this method may miss a subset of mutations. Also, the potential existence of founder mutations in the Finnish population may have hampered our efforts to detect *SMAD* gene defects in HNPCC. However, it is likely that defects of *SMAD2*, *SMAD3*, or *SMAD4* are not a common cause of familial colon cancer. Further work is necessary to unravel the molecular background of MSS HNPCC.

S ROTH*
M JOHANSSON*
A LOUKOLA*
P PELTOMÄKI†
H JÄRVINEN‡
J-P MECKLIN§
L A AALTONEN*

*Department of Medical Genetics, Haartman Institute, PO Box 21, (Haartmaninkatu 3), 00014 University of Helsinki, Finland
†Human Cancer Genetics Program, Comprehensive Cancer Centre, Ohio State University, Columbus, Ohio, USA
‡Second Department of Surgery, Helsinki University Central Hospital, Helsinki, Finland
§Department of Surgery, Jyväskylä Central Hospital, Jyväskylä, Finland

Correspondence to: Dr Aaltonen, lauri.aaltonen@helsinki.fi

1 Heldin CH, Miyazono K, ten Dijke P. TGF-β signalling from cell membrane to nucleus through SMAD proteins. *Nature* 1997;**390**:465-71.
2 Hata A, Shi Y, Massague J. TGF-β signalling and cancer: structural and functional consequences of mutations in SMADs. *Mol Med Today* 1998;**6**:257-62.
3 Eppert K, Scherer SW, Ozcelik H, *et al*. MADR2 maps to 18q21 and encodes a TGFbeta-regulated MAD-related protein that is functionally mutated in colorectal carcinoma. *Cell* 1996;**86**:543-52.
4 Thiagalingam S, Lengauer C, Leach FS, *et al*. Evaluation of candidate tumour suppressor genes on chromosome 18 in colorectal cancers. *Nat Genet* 1996;**13**:343-6.
5 Takagi Y, Kohmura H, Futamura M, *et al*. Somatic alterations of the DPC4 gene in human colorectal cancers in vivo. *Gastroenterology* 1996;**111**:1369-72.
6 Miyaki M, Iijima T, Konishi M, *et al*. Higher frequency of Smad4 gene mutation in human colorectal cancer with distant metastasis. *Oncogene* 1999;**20**:3098-103.
7 Howe JR, Roth S, Ringold JC, *et al*. Mutations in the SMAD4/DPC4 gene in juvenile polyposis. *Science* 1998;**280**:1086-8.
8 Houlston R, Bevan S, Williams A, *et al*. Mutations in DPC4 (SMAD4) cause juvenile polyposis syndrome, but only account for a minority of cases. *Hum Mol Genet* 1998;**7**:1907-12.
9 Roth S, Sistonen P, Salovaara R, *et al*. SMAD genes in juvenile polyposis. *Genes Chrom Cancer* 1999;**26**:54-61.
10 Friedl W, Kruse R, Uhlhaas S, *et al*. Frequent 4-bp deletion in exon 9 of the SMAD4/MADH4 gene in familial juvenile polyposis patients. *Genes Chrom Cancer* 1999;**25**:403-6.
11 Arai T, Akiyama S, Okabe S, Ando M, Endo M, Yuasa Y. Genomic structure of the human SMAD3 gene and its infrequent alterations in colorectal cancers. *Cancer Lett* 1998;**122**:157-63.
12 Zhu Y, Richardson JA, Parda LF, Graff JM. SMAD3 mutant mice develop metastatic colorectal cancer. *Cell* 1998;**94**:703-14.
13 Yang X, Letterio JJ, Lechleider RJ, *et al*. Targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-beta. *EMBO J* 1999;**18**:1280-91.
14 Datto MB, Frederick JP, Pan L, Borton AJ, Zhuang Y, Wang XF. Targeted disruption of Smad3 reveals an essential role in transforming growth factor beta-mediated signal transduction. *Mol Cell Biol* 1999;**19**:2495-504.
15 Leach FS, Nicolaides NC, Papadopoulos N, *et al*. Mutations of mutS homolog in hereditary nonpolyposis colorecral cancer. *Cell* 1993;**75**:1215-25.
16 Bronner CE, Baker SM, Morrison PT, *et al*. Mutation in the DNA mismatch repair gene homologue hMLH1 is associated with hereditary non-polyposis colon cancer. *Nature* 1994;**368**:258-61.
17 Papadopoulos N, Nicolaides NC, Wei YF, *et al*. Mutation of mutL homolog in hereditary colon cancer. *Science* 1994;**263**:1625-9.
18 Nicolaides NC, Papadopoulos N, Liu B, *et al*. Mutations of two PMS homologues in hereditary nonpolyposis colon cancer. *Nature* 1994;**371**:75-80.
19 Miyaki M, Konishi M, Tanaka K, *et al*. Germline mutation of MSH6 as the cause of hereditary nonpolyposis colorectal cancer. *Nat Genet* 1997;**17**:271-2.
20 Aaltonen LA, Peltomäki P, Leach FS, *et al*. Clues to the pathogenesis of familial colorectal cancer. *Science* 1993;**260**:812-16.
21 Ionov Y, Peinando MA, Malkhosyan S, Shibata D, Perucho M. Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. *Nature* 1993;**363**:558-61.
22 Thibodeau SN, Bren G, Schaid D. Microsatellite instability in cancer of the proximal colon. *Science* 1993;**260**:816-19.
23 Hemminki A, Peltomäki P, Mecklin JP, *et al*. Loss of the wild type MLH1 gene is a feature of hereditary nonpolyposis colorectal cancer. *Nat Genet* 1994;**8**:405-10.
24 Markowitz S, Wang J, Myeroff L, *et al*. Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. *Science* 1995;**268**:1336-8.
25 Lu SL, Kawabata M, Imamura T, *et al*. HNPCC associated with germline mutation in the TGF-β type II receptor gene. *Nat Genet* 1998;**19**:17-18.
26 Grady WM, Myeroff LL, Swinler SF, *et al*. Mutational inactivation of transforming growth factor β receptor type II in microsatellite stable colon cancers. *Cancer Res* 1999;**59**:320-4.
27 Knudson AG. Antioncogenes and human cancer. *Proc Natl Acad Sci USA* 1993;**90**:10914-21.
28 Vogelstein B, Kinzler KW. *The genetic basis of human cancer*. New York: McGraw-Hill, 1998.
29 Vogelstein B, Fearon ER, Hamilton SR, *et al*. Genetic alterations during colorectal-tumor development. *N Engl J Med* 1988;**319**:525-32.
30 Korn WM, Yasutake T, Kuo WL, *et al*. Chromosome arm 20q gains and other genomic alterations in colorectal cancer metastatic to liver, as analyzed by comparative genomic hybridization and fluorescence in situ hybridization. *Genes Chrom Cancer* 1999;**25**:82-90.
31 Ried T, Knutzen R, Steinbeck R, *et al*. Comparative genomic hybridization reveals a specific pattern of chromosomal gains and losses during the genesis of colorectal tumors. *Genes Chrom Cancer* 1996;**15**:234-45.
32 Meijer GA, Hermsen MA, Baak JP, *et al*. Progression from colorectal adenoma to carcinoma is associated with non-random chromosomal gains as detected by comparative genomic hybridisation. *J Clin Pathol* 1998;**51**:901-9.
33 Fearon ER, Cho KR, Nigro JM, *et al*. Identification of a chromosome 18q gene that is altered in colorectal cancers. *Science* 1990;**247**:49-56.
34 Cho KR, Oliner JD, Simons JW, *et al*. The DCC gene: structural analysis and mutations in colorectal carcinomas. *Genomics* 1994;**19**:525-31.
35 Hahn SA, Schutte M, Hoque AT, *et al*. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. *Science* 1996;**271**:350-3.
36 Vasen HF, Mecklin JP, Khan PM, Lynch HT. The International Collaborative Group on Hereditary Non-Polyposis Colorectal Cancer (ICG-HNPCC). *Dis Colon Rectum* 1991;**34**:424-5.
37 Nyström-Lahti M, Wu Y, Moisio AL, *et al*. DNA mismatch repair gene mutations in 55 kindreds with verified or putative hereditary non-polyposis colorectal cancer. *Hum Mol Genet* 1996;**5**:763-9.
38 Holmberg M, Kristo P, Chadwicks RB, *et al*. Mutation sharing, predominant involvement of the MLH1 gene and description of four novel mutations in hereditary nonpolyposis colorectal cancer. *Hum Mutat* 1998;**11**:482.

Novel mutation in the *MYOC* gene in primary open angle glaucoma patients

EDITOR—Glaucoma is the world's leading cause of irreversible blindness[1] and is characterised by progressive optic disc cupping with corresponding visual field loss. Both intraocular pressure (IOP) and positive family history are risk factors for the development of the disease.[2] Juvenile open angle glaucoma (JOAG) is a subtype of open angle glaucoma characterised by an early onset (10 to 35 years of age) and autosomal dominant inheritance with high penetrance,[3] a characteristic which has led several authors to investigate affected families in an attempt to identify a gene or genes associated with this condition.[4–10] With the use of genetic linkage analysis in families with JOAG, a genetic locus (GLC1A) was recognised on chromosome 1q21-q31.[4] The gene associated with GLC1A has been identified and it codifies a 57 kDa protein named trabecular meshwork induced glucocorticoid response protein (TIGR),[10] also known as myocilin (MYOC).[11] The *MYOC* gene is composed of three exons of 604, 126, and 785 bp, respectively.[12] During screening for mutations in the *MYOC* gene in 25 unrelated Brazilian patients with JOAG, an unreported mutation (Cys433Arg) was detected, present in seven of them.

Patients were followed at the Glaucoma Service of the State University of Campinas, Brazil. They underwent an ocular examination, including gonioscopy by Posner lens, applanation tonometry, slit lamp biomicroscopy, optic nerve evaluation, and automated perimetry (Humphrey 630, program 30-2). JOAG was defined as the presence of characteristic bilateral optic nerve damage and visual field loss in the presence of an open angle in subjects younger than 36 years of age. Each patient included in this study came from different families according to interview data. The study was approved by the Ethics Committee of the State University of Campinas. At the time of the ocular examination, the mean age of JOAG patients was 25.52 years (SD 6.99), ranging from 10 to 35 years, and the mean IOP was 29.96 mm Hg (SD 13.00). Thirteen patients (52%) were male and 12 patients (48%) were female; 13 (52%) were white, 11 (44%) were black, and one (4%) was Asian. Some of the patients had been taking antiglaucomatous medication or had undergone surgical procedures for IOP control and showed IOP levels lower than 22 mm Hg at the time of examination. Fourteen patients (56%) had a positive family history.

Genomic DNA from peripheral blood (withdrawn from the antecubital vein) was prepared using DNAzol™ Reagent according to the manufacturer's instructions (GIBCO/BRL, Gaithersburg, MD). Primers were designed to amplify the three exons of the *MYOC* gene, according to the reported sequence (GenBank accession numbers U85257 and Z98750). Exon 3 was the first to be analysed, since this is the region where the majority of the mutations have been found.[12] SSCP analysis was performed on a Pharmacia PhastSystem (Pharmacia, Uppsala, Sweden) using a 20% homogeneous polyacrylamide gel. Samples with abnormal mobility shifts were sequenced using Thermo Sequenase Cycle Sequencing Kit with γ-^{32}P ATP (Amersham Life Science Inc, Cleveland, Ohio), according to the manufacturer's instructions.

Seven patients (28%) showed a point mutation in exon 3, a heterozygous T to C transition at nucleotide position 1374 leading to a cysteine (Cys) 433 for arginine (Arg) substitution. The SSCP pattern shows two abnormal
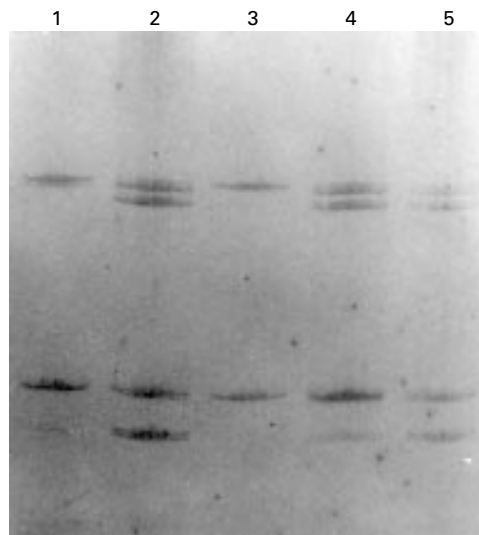

Figure 1 Silver stained SSCP gel showing the 195 bp PCR product encompassing amino acids 412 to 476 of the MYOC gene. Lane 1: control sample. Lanes 2-5: samples of JOAG patients. Samples 2, 4, and 5 show abnormal mobility shifts as compared with the control.

mobility shifts (figs 1 and 2). Analysis through SSCP and restriction digestion with *Fok*I was performed in 130 control samples of different ethnic origin in order to discard the possibility of the new substitution being a polymorphism. Cleavage of the approximately 195 bp amplified fragment with *Fok*I generates three fragments of 13, 50, and 132 bp in size in the absence of the mutation, and in its presence the 132 bp fragment is cleaved into two additional 33 and 99 bp fragments. The mutation was not present in any of the samples. The Cys433Arg mutation was present in five (71%) white patients and two (29%) black patients. Six patients (85%) of those with the mutation had a positive familial history of glaucoma. The mean age of patients harbouring this mutation was 27.00 years (SD 6.02), ranging from 15 to 35 years and the mean IOP was 39.13 mm Hg (SD 12.62). In contrast, JOAG patients without the mutation had a mean age of 24.02 (SD 7.46) and a mean IOP of 25.65 mm Hg (SD 11.68) (p>0.05). In another patient, a previously reported mutation (Pro370Leu) was identified.[13]

One of the patients who showed the Cys433Arg mutation had the family investigated for its presence. Nine subjects were studied and four harboured the Cys433Arg mutation (fig 3). Three of them had glaucoma and one had ocular hypertension without optic nerve or visual field damage. The other members who did not have the disease were not affected with the Cys433Arg mutation.

In order to discriminate between a founder effect and a de novo recurrence, haplotype analysis was performed in the six patients with a positive family history who had the new mutation and in one family using four microsatellite markers, mapped at band 1q21-25, closely linked to the GLC1A locus, D1S210, D1S2790, D1S1619, and NGA19. Polymerase chain reaction (PCR) was carried out following standard procedures and primer sequences were obtained from the Genome Data Bank. For allele scoring, PCR products were size fractionated on a 6% polyacrylamide-urea gel and autoradiographed. The analysis of four microsatellite markers showed that the Cys433Arg mutation is associated with a common haplotype, suggesting that these patients inherited their mutation from a common ancestor. A pedigree of the family analysed can be seen in fig 3, depicting a potential disease haplotype.