Hereditary and somatic DNA mismatch repair gene mutations in sporadic endometrial carcinoma

Robert B Chadwick, Robert E Pyatt, Theodore H Niemann, Samuel K Richards, Cheryl K Johnson, Michael W Stevens, Julie E Meek, Heather Hampel, Thomas W Prior, Albert de la Chapelle

EDITOR—Endometrial cancer (EC) is the second most common malignancy in the hereditary non-polyposis colorectal cancer (HNPCC) syndrome.¹ In a recent large study, cumulative cancer incidences by the age of 70 in HNPCC mutation carriers were: colorectal 82%, endometrial 60%, gastric 13%, and ovarian 12%.² Interestingly, in female mutation carriers the incidence of endometrial cancer (60%) exceeded that of colorectal cancer (CRC) (54%), as had been suggested earlier.^{2,3}

Predisposition to HNPCC is the result of germline mutations in the mismatch repair genes.⁴ Detectable mutations in the two major genes, *MLH1* and *MSH2*, account for some 3% of all colorectal cancers.⁵ One might therefore assume that a similar proportion of all endometrial cancer patients would have such mutations; however, in a number of studies addressing this question, extremely few germline mutations have been found. Summarising the studies by Katabuchi *et al.*,⁶ Kobayashi *et al.*,⁷ Lim *et al.*,⁸ Gurin *et al.*,⁹ and Kowalski *et al.*,¹⁰ only one germline mutation (in *MLH1*) was found in a total of 352 EC patients (0.3%). In these studies, mutations were sought in all patients whose tumours were microsatellite instability (MSI) positive.

Recent reports have suggested that *MSH6* might account for many endometrial cancers and that families with these mutations show atypical features of HNPCC with endometrial and ovarian cancers outnumbering colorectal cancers.^{11,12} Additionally, MSI, a hallmark of HNPCC, was low in most tumours associated with *MSH6* mutations or was preferentially shown by mononucleotide repeats rather than dinucleotide repeats.¹²⁻¹⁴ Previous studies have reported that 9-25% of sporadic endometrial cancers display microsatellite instability.^{7,9,15-18} In the majority of cases, this instability arises through hypermethylation of the *MLH1* promoter region.^{9,19-21} This epigenetic change results in reduced (or no) expression of the *MLH1* transcript.²²

This study was undertaken to revisit the issue of microsatellite instability and mismatch repair gene mutations in sporadic endometrial cancer. By initially studying tumour tissue, both germline and somatic mutations were evaluated in the *MSH2*, *MLH1*, and *MSH6* genes in a retrospective series of microsatellite stable and microsatellite unstable endometrial cancers.

Material and methods

All patients diagnosed with endometrial adenocarcinoma between October 1996 and

February 1998 at the Ohio State University Hospital were considered retrospectively. Among these 85 patients, archival tissue was available from 74.

After appropriate investigational review board (IRB) approval, these 74 charts were reviewed and the tissue blocks recovered. Histological sections were made, stained with haematoxylin and eosin, and the histological diagnosis critically re-evaluated. Sections 50 µm thick were cut from regions of the tumour containing as high a proportion of tumour cells as possible (typically >50%). To obtain non-malignant tissue, sections were obtained either from tissue emanating from other organs, primarily lymph nodes, that were histologically cancer free, or alternatively sections were made from parts of the endometrial tissue that had no cancer cells. All materials were unlinked from their identifiers before being subjected to DNA extraction and genetic analyses.

Tissue sections were deparaffinised with two xylene washes. Rehydration was accomplished through 20 minute incubations in decreasing concentrations of alcohol (100%, 80%, 50%) at room temperature followed by an overnight incubation in double distilled water at 4°C. DNA was extracted by lysis of the tissue for 18 hours at 55°C with 1 mg/ml proteinase K in 400 µl of buffer (10 mmol/l Tris, 400 mmol/l NaCl, 2 mmol/l EDTA, and 0.7% sodium dodecyl sulphate, pH 8.2). Degraded proteins were precipitated with 2.5 volumes of saturated NaCl after centrifugation. DNA was precipitated with 2.5 volumes of 100% ethanol at -20°C, washed in 70% cold ethanol, then dissolved in 50 µl of TE buffer (10 mmol/l Tris and 1 mmol/l EDTA, pH 8.0).

Microsatellite sequences were amplified using the Bethesda panel.¹³ Owing to limited availability of normal tissue, tumour DNA was used for MSI determination without its corresponding normal DNA pair. Amplifications were done in 15 µl PCR reaction volumes using 1 µl of each 8 µmol/l primer (the 5' primer is fluorescently labelled), 10 ng of genomic DNA, and 8 µl of Qiagen's HotStarTaq Master Mix. The thermal cycling profile was one cycle at 95°C for 12 minutes, followed by 45 cycles at 95°C for 10 seconds, 55°C for 15 seconds, and 72°C for 30 seconds, followed by one cycle at 72°C for 30 minutes, followed by a soak at 4°C. Respective PCR reactions for each marker were pooled together and loaded on to the PE3700 automated sequencer. Allele sizing and calling was done using Genotyper software (Applied Biosystems). For polymorphic markers D2S123, D5S346, and D17S250 samples were scored as MSI positive if more than two alleles

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Division of Human Cancer Genetics, The Ohio State University, Department of Molecular Virology, Immunology, and Medical Genetics, and the Comprehensive Cancer Center, The Ohio State University, 420 West 12th Avenue, Columbus, Ohio 43210, USA

R B Chadwick
S K Richards
C K Johnson
M W Stevens
J E Meek
H Hampel
A de la Chapelle

Department of Pathology, The Ohio State University Medical Center, 121 Hamilton Hall, 1645 Neil Avenue, Columbus, Ohio 43210, USA

R E Pyatt
T H Niemann
T W Prior

Correspondence to:
Dr de la Chapelle,
delachapelle-1@
medctr.osu.edu

were present. Since normal DNA was not available for comparison from all samples, it is possible that instances of MSI where the normal sample was homozygous and the tumour sample had only two alleles were missed. However, this limitation does not apply to the two mononucleotide repeat markers, suggesting that this did not lead to any serious underestimate of MSI, as these markers are more sensitive to MSI than the dinucleotide repeats.^{23 24} For homozygous markers BAT25

and BAT26, samples were scored as MSI positive if the pattern deviated from the normal homozygous pattern. The BAT markers are polymorphic in African Americans.^{25 26} However, the ethnicity of the subjects in this study was known and all three African American subjects (Nos 31, 65, and 70) were screened for mutations in the mismatch repair genes. Out of the 74 tumours tested, 17 (or 23%) were found to be MSI positive, 14 MSI high and 3 MSI low (table 1).

Table 1 Summary of results in those 42 patients in whom mutational analyses were carried out

Patient No	Age at diagnosis	Family history and site	MSI markers positive	MSI classification	MSH2	MLH1	MSH6
2	39	Father prostate, paternal grandmother CSU, patient had second primary colon cancer, Hodgkin's lymphoma	0/5	N	No	No	No
3	74	No	4/5	H	No	Germline GAG(Glu) to GGG(Gly) at codon 578	No
5	73	Yes, CSU	0/5	N	No	No	No
7	40	Mother ovarian	0/5	N	No	No	No
9	67	Mother breast	4/5	H	No	No	No
10	77	Mother GI & sister pancreatic	0/5	N	No	No	No
13	50	Brother testicular	0/5	N	No	Somatic GAA(Glu) to AAA(Lys) at codon 668	Somatic GAT(Asp) to TAT(Tyr) at codon 575 Somatic GAA(Glu) to TAA(stop) at codon 1359
14	66	Mother & father CSU	0/5	N	No	No	No
15	51	Maternal aunt, mother, sister lymphoma	4/5	H	No	No	No
16	38	Aunt breast	0/5	N	No	No	No
17	58	Father lung, mother breast, maternal grandmother gallbladder	0/5	N	No	No	No
19	79	Sister breast, daughter colon	1/5	L	No	No	No
21	45	Sister melanoma, grandmother breast	0/5	N	No	No	No
23	65	Mother endometrial	0/5	N	No	No	No
26	64	Father, sister, brother colon	0/5	N	No	No	No
30	57	Sister breast, grandson brain	0/5	N	No	No	No
31	78	Maternal grandmother rectal	0/5	N	No	No	No
34	78	Half sister breast	2/5	H	Somatic A deletion in (A)7 repeat of exon 4	No	No
37	76	Aunt & sister breast	0/5	N	No	No	No
38	54	Family history of stomach cancer	0/5	N	No	Somatic GAG(Glu) to AAG(Lys) at codon 515	No
41	52	Father CSU	0/5	N	No	No	No
42	50	Mother leukaemia and colon	0/5	N	No	No	No
45	85	No	1/5	L	No	No	No
48	57	Mother pancreatic	0/5	N	No	No	No
50	67	Mother colon, father lung	4/5	H	No	No	No
51	74	Maternal aunt bone	3/5	H	No	No	Somatic C deletion in (C)8 repeat of exon 5
52	49	No	2/5	H	No	No	No
55	78	Family history of breast, bladder and colon	3/5	H	Germline GGC(Gly) to GAC(Asp) at codon 322	Somatic GAT (Asp) to AAT(Asn) at codon 203	No
56	77	Also family history of hematological malignancies	0/5	N	Somatic A deletion in (A)7 repeat of exon 4	No	No
57	68	Brother bladder	3/5	H	No	No	No
60	52	Mother & sister pancreatic, maternal uncle CSU	3/5	H	No	No	Somatic C insertion in (C)8 repeat of exon 5
61	56	Mother and two sisters colon	3/5	H	No	No	No
64	85	Mother breast & colon, two sisters breast	0/5	N	No	No	No
65	60	Mother breast, ovarian, colon, sister colon, grandmother endometrial	0/5	N	No	No	No
66	56	Patient had second primary colon cancer	0/5	N	No	No	No
67	77	Father, two aunts & uncle leukaemia, sister breast, uncle stomach	3/5	H	No	No	No
68	49	Mother colon, uncle prostate	0/5	N	No	No	No
70	57	Sister CSU	0/5	N	No	No	No
78	59	Maternal grandmother & aunt gastric, maternal aunt breast, father lung cancer	5/5	H	No	No	No
79	80	Mother breast	1/5	L	No	No	No
83	57	Mother gastric	5/5	H	No	No	No
84	72	Sister colon, sister ovarian	0/5	N	No	No	No
		Niece breast	0/5	N	No	No	No
		Sister breast	2/5	H	No	No	No

CSU=cancer site unspecified, H=high, L=low, N=negative.

Table 2 Primers used to amplify all exons of the *MSH6* gene. In order to facilitate subsequent direct sequencing, 5' primers were tailed with M13 forward (TGTAACACGACGGCCAGT) and M13 reverse (CAGGAAACAGCTATGACC) sequences

<i>MSH6</i>	Primer sequences
Exon 1	Fw: TGTAACACGACGGCCAGTTCGGTCCGACAGAACGGTTG Rv: CAGGAAACAGCTATGACCCCAAAATGCTCCAGACTCG
Exon 2	Fw: TGTAACACGACGGCCAGTGCCAGAAGACTTGGAAATTGTTTATTTG Rv: CAGGAAACAGCTATGACCCACACAAACACACACATGGCAGTAG
Exon 3	Fw: TGTAACACGACGGCCAGTCGTGAGCCTCTGCACCCGGCCC Rv: CAGGAAACAGCTATGACCCCATCACCCTAACATAAA
Exon 4A	Fw: TGTAACACGACGGCCAGTGTCTTACATTATGGTTTTC Rv: CAGGAAACAGCTATGACCCACATCAGAGCCACCAATG
Exon 4B	Fw: TGTAACACGACGGCCAGTCGAAGGGTCATATCAGATTTC Rv: CAGGAAACAGCTATGACCATACCAACAGTAGGGCGAC
Exon 4C	Fw: TGTAACACGACGGCCAGTCGTTAGTGAGGTGGTGATG Rv: CAGGAAACAGCTATGACCCAGTGAATACCAAGCCAGTTC
Exon 4D	Fw: TGTAACACGACGGCCAGTCTGTACCATGGATGCTCT Rv: CAGGAAACAGCTATGACCTTCCTCTTTTCTTTGAGG
Exon 4E	Fw: TGTAACACGACGGCCAGTAAAGTAGCAGAGTGAACAGACTGAG Rv: CAGGAAACAGCTATGACCAACATCACCCTAATGCCATCAC
Exon 4F	Fw: TGTAACACGACGGCCAGTCTGTCTCTCAGGAAGTTC Rv: CAGGAAACAGCTATGACCCAGCAATGCTTTAGGAGCCG
Exon 4G	Fw: TGTAACACGACGGCCAGTACTTGCCATCTCCTTTTGG Rv: CAGGAAACAGCTATGACCCCTGCTTTGGGAGTAATAAG
Exon 4H	Fw: TGTAACACGACGGCCAGTGAAAGGGCTCGAAAGACTGG Rv: CAGGAAACAGCTATGACCCCAAGGGCTACTAAGATAAAGCGAG
Exon 5	Fw: TGTAACACGACGGCCAGTGGGGAGATCGTTGGACTGTAATTG Rv: CAGGAAACAGCTATGACCTTGCTTCTTAAAGTCAGTGGCTG
Exon 6	Fw: TGTAACACGACGGCCAGTGTATGAACTGTTACTACC Rv: CAGGAAACAGCTATGACCCGAAATATCTTTTATACAT
Exon 7	Fw: TGTAACACGACGGCCAGTGCCAAATTTGCATAGTCTCTTAATG Rv: CAGGAAACAGCTATGACCCGCAATGTTGAGTGGCGTG
Exon 8	Fw: TGTAACACGACGGCCAGTCTTTTGTGTTTAAATTCCT Rv: CAGGAAACAGCTATGACCCAAACAGAAGTGCCCTCTCAA
Exon 9	Fw: TGTAACACGACGGCCAGTGATTTATCTCAAAATGTTGCTGTGCG Rv: CAGGAAACAGCTATGACCTTCACTAGCCAGGCAAACTTCCC
Exon 10	Fw: TGTAACACGACGGCCAGTATTTAAGGGAAGTTTGCC Rv: CAGGAAACAGCTATGACCGTTTATTAGATCATAATGTT

All exons of the *MSH2*, *MLH1*, and *MSH6* genes were screened by direct sequencing of genomic PCR products. In order to facilitate direct sequencing of PCR products for mutational analysis, all 5' and 3' PCR primers were tailed with M13 forward (TGTAACACGACGGCCAGT) and M13 reverse (CAGGAAACAGCTATGACC) sequences (table 2 and supplemental data).²⁷ PCR reactions were done in 25 µl volumes with 100 nmol/l of each of the respective PCR primers, 25 ng of genomic DNA, 100 µmol/l of each dNTP, 1.0 U AmpliTaq Gold DNA polymerase (Perkin-Elmer), 10 mmol/l pH 8.3 Tris-HCl, 50 mmol/l KCl, and 2 mmol/l MgCl₂. PCR fragments were purified using the Exonuclease I/Shrimp Alkaline Phosphatase PCR Product Presequencing Kit (USB). After purification according to the manufacturer's protocol, 2 µl of the PCR products were sequenced using the BigDye Terminator AmpliTaq FS Cycle Sequencing Kit (Applied Biosystems). Determination of somatic or hereditary mutation status for all mutations was done by comparing tumour chromatograms to normal DNA chromatograms amplified from archival lymph node tissue DNA from the respective patients.

Results

Out of the 17 MSI positive endometrial tumours and an additional 25 that were MSI negative, only two germline changes were found (table 1 and supplemental data). These were a GAG(Glu) to GGG(Gly) mutation at codon 578 of the *MLH1* gene in patient 3 and a GGC(Gly) to GAC(Asp) at codon 322 of the *MSH2* gene in patient 52. The *MLH1* mutation has been reported to be pathogenic

previously and the tumour from this person was MSI high.²⁸ Additionally, a functional assay indicates that this mutation is pathogenic.²⁹ Also, MLH1 immunohistochemistry showed virtually no expression of MLH1 in this patient's tumour (supplemental data). The patient is a 74 year old white female with a history of hypertension, diabetes mellitus type II since the age of 54, and chronic renal failure. It was noted that her family history was positive for diabetes mellitus and hypertension, but there was no mention of a family history of cancer on chart review.

The GGC(Gly) to GAC(Asp) at codon 322 mutation of *MSH2* found in patient 52 is listed in the ICG-HNPCC database (<http://www.nfdht.nl/>) as both a pathogenic mutation and a polymorphism.³⁰⁻³² In the first paper describing this change it was considered a clinically insignificant polymorphism because it was found in one out of 30 unrelated controls.³⁰ Moreover, in another study the same change was reported not to segregate with the cancer predisposition.³³ To investigate its incidence further, we tested 50 grandparents from the families collected by the Centre d'Etude du Polymorphisme Humain and found it in one person. The glycine is conserved among human, mouse, rat, and yeast. *MSH2* immunohistochemistry of this tumour showed reduced expression of *MSH2* suggesting that this amino acid change may potentially contribute to pathogenicity (supplemental data). Notably, this tumour was MSI high and had an additional somatic truncating mutation in exon 4 of *MSH2* (fig 1). The *MSH2* antibody is specific for the carboxy terminus of *MSH2* and thus less protein would be expected to be detected in a tumour with a truncating mutation in the amino terminus. Additionally, the tumour had a somatic mutation of GAT(Asp) to AAT(Asn) at codon 203 of *MLH1*. Immunohistochemistry using anti-human MLH1 antibody also showed reduced expression of MLH1 protein (supplemental data). The patient is a 49 year old white woman with a history of ulcerative colitis since the age of 20. The family history is significant for her mother with breast cancer alive at the age of 71, her father with bladder cancer who died at the age of 79, and her paternal grandfather who died from colon cancer in his late 60s. Thus, in summary, the evidence regarding the *MSH2* germline amino acid substitution is inconclusive in that it may be either an innocuous polymorphism or a low penetrant pathogenic mutation. For these reasons we do not count it as a pathogenic germline mutation in this study. No pathogenic germline mutations were found in the *MSH6* gene in the 42 endometrial cancers studied.

Somatic truncating frameshift mutations were found in the coding repeat of seven adenosines in exon 4 of *MSH2* (fig 1). These 1 bp deletions lead to a predicted truncation at amino acid 245 of the *MSH2* protein and both tumours were MSI high. This region of *MSH2* has not been reported previously to be hypermutable. Mutations in this repeat were confined to MSI positive cancers, indicating

Figure 1 Somatic truncating frameshift mutations in exon 4 of *MSH2*. MSI positive endometrial tumours 31 and 35 have 1 bp deletions in the coding (A), repeat. This creates a termination codon at amino acid 245 of *MSH2*. Panels 1 and 3 are forward direction sequences of samples 31 and 52. Panels 2 and 4 are reverse direction sequences of these samples that have been reverse complemented. Owing to contaminating normal DNA, the frameshift is more evident in the reverse direction sequenced, particularly in sample 31 (panel 2).

that *MSH2* is also a target in the microsatellite instability model of carcinogenesis similar to the *TGFBR1*, *BAX*, *IGF2R*, *MSH3*, *MSH6*, *TCF4*, *MBD4*, and *RIZ* genes.³⁴⁻⁴¹ We are unable to evaluate the clinical and pathogenic significance of mutations that arise in mismatch repair genes in tumours that are already mismatch repair deficient. A case in point is tumour 52 that showed immunohistochemical deficiency of both *MLH1* and *MSH2* protein, no *MLH1* germline mutation, but two genetic changes in *MSH2*. Owing to the absence of high quality tissue in this retrospective study we were unable to determine the methylation status of the *MLH1* promoter and whether or not the two changes in *MSH2* affected different alleles. Such studies need to be done in order to determine if somatic mismatch repair gene mutations in mismatch repair deficient cells confer additional functional properties. In this context it is important to note that coding mononucleotide tracts that are vulnerable to frameshifts exist not only in *MSH6*, but also in *MSH2* as shown here.¹³ Somatic mutation of the mismatch repair genes (not including *MLH1* promoter methylation) occurred at a rate of 5-10% in endometrial cancers, 2/42, 3/42, and 4/42 for *MSH2*, *MLH1*, and *MSH6* respectively (table 1).

Discussion

In an unselected consecutive retrospective series of 74 endometrial cancers, microsatellite instability was found in 17 (23%). These 17 patients plus 25 other patients whose tumours were MSI

negative were studied for *MSH2*, *MLH1*, and *MSH6* somatic and germline mutations. Germline mutation in *MLH1* was found in one endometrial cancer patient. Thus, hereditary mutations in these genes contribute to sporadic endometrial cancer at a rate of at least 1.4% (1/74). If the germline missense change in *MSH2* is causally related to the cancer in one patient then the contribution of hereditary mutations would be 2/74. This incidence is higher than in several previous studies.⁶⁻¹⁰

In all, the previous authors studied 352 EC tumours for microsatellite instability and found 78 that were MSI positive (22%). These 78 patients were studied by various methods for germline mutations in *MLH1* and *MSH2* and only one was found, corresponding to a frequency of 1/352 or 0.3%. Because of small numbers and varying methodologies, it is not possible to assess which of the two estimates (this study of 1.4% and that by previous authors of 0.3%) is most likely to be correct. Intuitively, the high incidence of EC in HNPCC families would seem to suggest that germline mutation must occur with appreciable frequency in "sporadic" EC. The large study of Wijnen *et al*¹² disclosed as many as 10 different truncating germline mutations of *MSH6* among HNPCC or HNPCC-like families. These families had been ascertained clinically as being positive for the original or modified Amsterdam criteria. The families in which *MSH6* was mutated characteristically had many patients with EC. It therefore seems that

in addition to *MLH1* and *MSH2*, one would expect *MSH6* to be mutated in some "sporadic" EC patients. As can be seen in table 1, "sporadic" EC patients nevertheless often have some degree of a family history of cancer. This study is the first one that specifically searched for mutations in *MSH6* in sporadic EC; the absence of mutations is somewhat surprising in view of the findings of Wijnen *et al.*¹² It remains to be seen whether a larger series of patients might disclose mutations in *MSH6*. If not, one may need to consider whether truncating mutations of *MSH6* are somehow enriched in the Dutch population and rare elsewhere.

- To revisit the previously stated minimal role of hereditary nonpolyposis colorectal cancer (HNPCC) in "sporadic" endometrial cancer (EC), 74 unselected ECs were studied for microsatellite instability (MSI); 17/74 (23%) were MSI(+).
- Mutational analyses were performed for *MSH2*, *MLH1*, and *MSH6* in these 17 patients and an additional 25 MSI negative patients, most with a family history of cancer.
- One definite germline mutation was found in *MLH1*. A missense change in *MSH2* needs further study. Thus, the proportion of hereditary mutations was at least 1/74 (1.4%), but *MSH6* did not contribute.
- Frameshifts in a previously unreported hypermutable region of seven coding adenosines in exon 4 of *MSH2* were discovered in two MSI positive tumours.
- In conclusion, heritable mismatch repair deficiency accounts for a small but definite proportion of sporadic EC.

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website extra

The supplementary data (figs 2–7) can be found on the JMG website

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Absence of learning difficulties in a hyperactive boy with a terminal Xp deletion encompassing the *MRX49* locus

E S Tobias, G Bryce, G Farmer, J Barton, J Colgan, N Morrison, A Cooke, J L Tolmie

EDITOR—The genetic counselling of a pregnant woman who carries an Xp chromosomal deletion is far from straightforward. While the precise locations of the *CDPX1* (arylsulphatase E), steroid sulphatase (*STS*), and Kallman

(*KAL1*) genes are known and FISH probes are available for these well characterised genes, the positions of putative mental retardation genes in this region have not yet been determined. Clinical and molecular studies undertaken over

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**Duncan Guthrie
Institute of Medical
Genetics, Yorkhill,
Glasgow G3 8SJ, UK**
E S Tobias
J Colgan
N Morrison
A Cooke
J L Tolmie

**Department of Child
and Family Psychiatry,
Yorkhill NHS Trust,
Glasgow G3 8SJ, UK**
G Bryce
J Barton

**Department of
Paediatrics, Raigmore
Hospital, Old Perth
Road, Inverness
IV2 3UJ, UK**
G Farmer

Correspondence to:
Dr Tobias,
gbcv55@udcf.gla.ac.uk

Figure 1 (a) Case 6, (b) case 8, (c) case 9, (d) case 4, (e) case 12, and (f) case 13 of Ballabio et al,¹ (g)–(j) cases BA16, BA20, BA139, and BA75 of Schaefer et al,³ (k) boy with IQ of 46, short stature, generalised ichthyosis, hypogonadotrophic hypogonadism, nystagmus, and photophobia,² (l) boy with aggressive and hyperactive behaviour, myoclonic epilepsy, developmental delay, and no speech aged 4 years 8 months,⁴ (m) monozygous male twins with X linked ichthyosis, learning difficulties (LD), and epilepsy,¹⁰ (n) our patient, with short stature, Binder syndrome, and ichthyosis (consistent with the loss of the *SHOX*, *CDPX1*, and *STS* genes, respectively) but no significant learning difficulties. The presence (+) or absence (-) of LD is indicated for each case. A broken line indicates the chromosomal region within which the breakpoint is assumed to lie, while a solid line indicates a retained region.