Incidence of germline *hMLH1* and *hMSH2* mutations (HNPCC patients) among newly diagnosed colorectal cancers in a Slovenian population

**EDITOR**—Hereditary non-polyposis colorectal cancer (HNPCC) syndrome is a common autosomal dominant predisposition to colorectal cancer. Clinical diagnostic features of sporadic and HNPCC associated colorectal cancer do not differ significantly and until recently the identification of HNPCC patients was based mainly on their family history. Because of the importance for relatives of HNPCC patients to be clinically examined frequently in order to detect the disease at an early, curable stage, the International Collaborative Group (ICG) on HNPCC proposed criteria for identification of HNPCC families. According to the guidelines agreed by the ICG in Amsterdam in 1990, an HNPCC family has to fulfil the following criteria: there should be at least three relatives with colorectal cancer (one of whom is a first degree relative to the other two), at least two successive generations should be affected, and one relative should be diagnosed under the age of 50. CRC patients with HNPCC syndrome can also develop cancer of the endometrium, stomach, ovary, and urinary and hepatobiliary tracts. In several epidemiological studies, the incidence of HNPCC has been estimated to be between 0.5% and 15% of all colorectal cancers. The identification of mismatch repair genes (MMR), of which at least five (*hMLH1*, *hMSH2*, *PMS1*, *PMS2*, and *hMSH6 (GTBP)*) are associated with HNPCC, has enabled mutational analysis in families fulfilling complete or partial Amsterdam criteria. Carriers of germline MMR mutations have a higher than 80% risk for cancer by the age of 75. The great majority of germline mutations were found in approximately equal proportions in *hMLH1* and *hMSH2*, while mutations in the other three MMR genes have been reported only in a limited number of cases. Germline *hMLH1* and *hMSH2* mutations were also found in a considerable proportion of colorectal cancer patients from families not fulfilling the Amsterdam criteria, especially if they were young, indicating that some HNPCC families might be missed if they are preselected before mutational analysis.

Mutations in MMR genes result in microsatellite instability (MSI), which is characteristic of more than 90% of tumours in HNPCC patients but was also found in 12-15% of sporadic colorectal tumours. In the absence of other diagnostic criteria, MSI analysis of tumours could be valuable markers in HNPCC identification. Here we report an effective MSI analysis of CRC and subsequent mutational analysis of the *hMLH1* and *hMSH2* genes in tumours with considerable MSI for identification of HNPCC among randomly collected, newly diagnosed colorectal cancers. This approach allowed us to identify HNPCC families and to estimate the minimal incidence of HNPCC in a Slovenian population based solely on molecular genetic analysis.

Primary colorectal adenocarcinomas and corresponding normal tissue samples were collected from patients who gave consent for testing of their DNA. Between 1996 and 1998, 300 newly diagnosed CRC patients from clinics all over Slovenia participated in this study. The sample of CRC is thus representative for the Slovenian population. All samples were gathered in a central institution where two physicians histologically evaluated each resection for a high proportion of tumour tissue. We also confirmed a high proportion of cancer cells versus normal cells in tumour tissue in many samples that exhibited LOH during MSI analysis. Normal colorectal mucosa taken from a site distant from the tumour was used as a normal control in the study.

We isolated DNA after tissue digestion using standard phenol/chloroform extraction and ethanol precipitation from frozen colorectal tumours and corresponding normal tissue samples. For MSI analysis of tumour and control
conformational analysis. We observed 17 di
hMLH1 coding regions as well as all exon/intron boundaries of
of germline mutations in two MMR genes. The whole
instability tumours were further analysed for the presence
positive for BAT26 or BAT25.

only this marker we would have missed one of 29 high MSI
mutation (8-7).

tissue pairs, we tested the majority of samples with the fol-
lowing markers: mononucleotides BAT26,11 BAT25,11 and
BAT40,20 dinucleotides D2S123,21 D5S346,22 TP53,23
D11S1294,24 D112179,24 D17S250,25 D18S58,26 and
D18S69,27 and tetranucleotide MYCLY.28 Tumours were
scored as high MSI if more than 40% of tested markers were
positive and were scored as low MSI if less than 20% of
tested markers were positive. During the analysis, it
turned out that for determination of high MSI status four
selected markers were sufficient, BAT26, D2123, BAT25,
and D5S346. Therefore, we analysed additional samples in
multiplex PCR reactions for microsatellite markers BAT26
d and D2123, and BAT25 and D5S346, respectively. In this
case, tumour tissue was scored as high MSI if at least two
of four markers were altered.

MSI analysis was performed as previously described.30
Briefly, after PCR amplification products were run on a
thin (0.4 mm) denaturing polyacrylamide gel matrix fixed
on one of the glass plates followed by an optimised silver
staining protocol that markedly improved the resolution.
MSI was detected as some additional bands in tumour
DNA compared to control DNA (fig 1A).

For mutational analysis of hMLH1 and hMSH2
mismatch repair genes (MMR), we used PCR/non-isotopic
conformation analysis (article in preparation). The basic
principle of this method is a combination of three analyses
which are all based on changes in three dimensional DNA
structures, that is, single strand conformation analysis
(SSCA), heteroduplex analysis (HA), and double strand
conformational polymorphisms, one sample also
showed altered double stranded conformation, and in two
samples heteroduplexes were also formed. Eleven altera-
tions were in hMLH1 and six in hMSH2. Sequencing
showed four presumably pathogenic mutations, three in
hMLH1 and one in hMSH2 (table 1). A new polymor-
phism IVS9+10A>G in hMLH1 (allele frequency 0.02)
has also been detected (fig 1B, C).

Mutations G67R and I655T in hMLH1 have been pre-
viously reported11 32 in connection with hereditary bowel
cancer, while Q562X has been detected for the first time
among Slovenian patients with high MSI CRC tumours.
We also detected a germline deletion of three consecutive
amino acids from 188-190 in hMSH2 in a person with a
high MSI tumour (OG/97-2426-T). The same mutation
was previously found in a Slovenian patient (HNPCC-8-
2B) with a family history of CRC.33 Although these two
patients live in different parts of Slovenia and have different
family names, a retrospective pedigree search showed
common roots two generations back and resulted in the
identification of an extensive Slovenian HNPCC pedigree
(fig 2). Similarly, other patients with germline mutations
were contacted for their family history. All patients with
germline mutations accessed via MSI analysis had at least
one first degree relative with HNPCC associated cancer.

In this study, we used a molecular genetic approach for
identification of HNPCC families. Our approach was
based firstly on microsatellite instability (MSI) analysis of
newly diagnosed randomly collected CRC and subse-
quently on mutational analysis of mismatch repair genes
(MMR) in MSI positive tumours. We used a solely
molecular genetic approach for detecting HNPCC families
because (1) a considerable proportion of germline MMR
mutations were found in patients from families not fulfill-
ing the Amsterdam criteria,13–15 and (2) access to well
organised cancer registries is not available in many
countries. In Slovenia, a cancer registry exists but it does
not contain family data. No specialised registries for
hereditary forms of cancer are available. In our first
attempt to identify Slovenian HNPCC families, 600 colo-
rectal cancer patients were questioned for their family his-
tory data. A total of 44 patients reported at least one first
degree relative with CRC and 16 families agreed to
contribute blood samples for DNA analysis. Germline
mutation was detected in one family only.33 This approach

<table>
<thead>
<tr>
<th>Gene and patient</th>
<th>Exon</th>
<th>Nucleotide change</th>
<th>Consequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>hMLH1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OG/98-2989</td>
<td>2</td>
<td>199 G→A</td>
<td>G67R</td>
<td>31</td>
</tr>
<tr>
<td>OG/97-4266</td>
<td>15</td>
<td>1684 C→T</td>
<td>Q562X</td>
<td>This study</td>
</tr>
<tr>
<td>OG/97-3941</td>
<td>17</td>
<td>1964 T→G</td>
<td>I655T</td>
<td>32</td>
</tr>
<tr>
<td>hMSH2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OG/97-2426</td>
<td>3</td>
<td>561 del TGAGGCTCT</td>
<td>In frame del of codons 188-190</td>
<td>33</td>
</tr>
</tbody>
</table>

Table 1 Pathogenic germline hMLH1 and hMSH2 mutations identified in high MSI tumours

Figure 2  Extended Slovenian HNPCC family with deletion of three amino acids (188-190) in hMSH2. We found this germline deletion in a subject with high MSI tumour (OG/97-2426). We also previously detected the same mutation in a Slovenian patient (HNPCC-8-2B) with a family history of CRC.33 A retrospective pedigree search showed common roots of both patients’ families two generations back. The resulting extended HNPCC family spans six generations and has 162 family members. Affected members are shaded. Presymptomatic DNA testing identified five non-carriers and one carrier of the mutation (8-7).
showed some inaccuracy of data obtained directly from patients and suggested that epidemiological studies of HNPCC incidence based on patients’ answers should be treated with caution.

With a molecular genetic approach, we found germine mutations in four out of 300 newly diagnosed colorectal cancers. These four alterations are unambiguously pathogenic mutations; one is a deletion, one is nonsense, and two are missense mutations. Two mutations are so far specific for Slovenian families: a deletion of codons 188-190 in exon 3 of hMLH1 (fig 2) and a C to T substitution in exon 15 of hMLH1 resulting in a stop codon at 562. A substitution of Ile to Thr at codon 655 detected in a patient OG/97-3941 was previously reported in a patient with gastric carcinoma.32 Interestingly, also, Slovenian patient OG/97-3941 had a metachronic gastric cancer two years after colon cancer. The Gln to Arg substitution at codon 67 was previously described in a Swedish HNPCC family33 and its pathogenicity was proved in a functional assay in yeast.34

It is possible that we missed some tumours with MMR gene mutation, because we analysed only MSI positive tumours. Cases with MMR mutations without MSI were also reported.35 However, 1.3% (4/300) tumours with germine MMR gene mutations in the Slovenian population is lower than the 2% observed in a similar study in a Finnish population. If the common ancestral Finnish specific mutation, which accounts for a half of all Finnish HNPCC families,36 is excluded the percentage in our country is even higher than in Finland. No comparison with other populations using a similar approach is currently available. Finnish and Slovenian molecular genetic based estimations of HNPCC incidence are considerably lower than the 5–10% estimated in the majority of epidemiological studies.36 However, Evans et al37 also reported the incidence of HNPCC to be 1.4%. Their population based study of 1137 consecutive cases of colorectal cancer showed a lower frequency of familial bowel cancer than previous studies and may reflect a lower incidence of inherited mutations in the HNPCC MMR genes than is currently accepted.38 Since germine mutation in MMR genes is a reliable indicator of HNPCC syndrome, the estimation of incidence of this hereditary disorder in a Slovenian population could be calculated. If we consider 19 000 newborns annually and 850 newly diagnosed colorectal cancer cases annually (of which 1.3% have germine MMR gene mutation), the HNPCC incidence in the Slovenian population is approximately 1 in 1700. However, this estimate represents the absolute minimum. It is possible that we missed some mutations because of technical limitations and because we did not analyse three minor genes (hPMS1, hPMS2, and hMSH6), which are also predisposing factors for HNPCC. Another reason for underestimation of the disease frequency might be that we only searched for mutations in patients with colorectal cancer, but this cancer accounts for only approximately two thirds of cancers in HNPCC affected families.39

The proportion of patients younger than 50 years in our study was the same as it is for colorectal cancer patients in the complete National Cancer Registry indicating that there was no bias in overestimation according to age.

With molecular genetic analysis of colorectal cancers, we were able to identify Slovenian families with a hereditary form of CRC. Only patients with germline MMR mutations who wished to be acquainted with the results of DNA testing were further contacted by both a gastroenterologist and a geneticist for future medical surveillance and family history data. Some extended HNPCC pedigrees have thus been detected. Through this study, we succeeded in providing more information about the role of molecular genetic analysis to general physicians and HNPCC family members. Presymptomatic DNA testing was offered to well informed and consenting non-symptomatic relatives. A prevention programme in the sense of periodic clinical examination of relatives with a constitutional mutation has been initiated. In particular, records of young CRC patients have been re-examined for family and clinical data and additional potential HNPCC families have been identified. This enabled us to initiate building of a national HNPCC registry.

We conclude that a molecular genetic approach with evaluated genetic markers for efficient MSI analysis and subsequent MMR gene analysis of patients and subsequent MMR positive tumours is valuable for identification and surveillance of HNPCC families and may serve also as a model for detection of familial cases of CRC in other populations.

The Ministry of Science and Technology of the Republic of Slovenia supported this study (Project J3-7/919-0381). The scope of the study was agreed by the Medical Ethical Commission of the Republic of Slovenia in October 1995. We are grateful to the patients who participated in the study. We thank Rastko Golouh and Anton Cesar for patients’ tissue samples. We also thank Tomaz Kralovec and Acel Novk for excellent technical assistance.

METKA RAVNIK-GLAVAC*†
UROS POTOCNIK*†
DAMJAN GLAVAC*†

*University of Ljubljana, Medical Faculty, Institute of Pathology, Laboratory of Molecular Genetics, Korytkova 2, 1000 Ljubljana, Slovenia
†Institute of Biochemistry, Medical Faculty, Ljubljana, Slovenia

Correspondence to: Dr Ravnik-Gradac, ravnik@ibmi.mf.uni-lj.si


Does the survival motor neuron protein (SMN) interact with Bcl-2?

Editor—Spinal muscular atrophy (SMA) is an autosomal recessive disease resulting from mutations in the telomeric copy of the survival motor neuron gene (SNMN), which results in reduced expression of the survival of motor neurons (SMN) protein.1 2 The SMN protein is ubiquitously expressed but is found at high levels in motor neurons.3 7 The SMN protein associates with Sm proteins,8 9 S1-p protein,9 and itself.10 SMN is found in structures termed gem8 that are associated with coiled bodies in the nucleus. The SMN protein is involved in RNA biogenesis10 and is important for the maturation of a functional snRNP complex that performs splicing.11 The complete loss of SMN is lethal12 whereas the low levels of SMN found in SMA cause loss of the motor neurons.13 14 The mechanism by which the reduction of SMN protein results in the loss of motor neurons is unknown. Some groups have suggested it occurs by apoptosis.15 16 Apoptosis is a conserved, highly regulated mechanism of non-chronic cell death for the removal of surplus, aged, or damaged cells.17 Apoptosis is regulated by the interactions of apoptosis agonists and antagonist with the Bcl-2 protein being one of the key inhibitors of apoptosis.18

Recently Iwahashi et al19 have suggested a direct interaction between SMN and Bcl-2 using transfected constructs. In an effort to confirm and extend their results, we have attempted to coimmunoprecipitate SMN and Bcl-2 both in a native environment and using transfected cells. The SMN protein and Bcl-2 are expressed in Jurkat cells and in spinal cord. Jurkat cells are a human lymphoblast T cell line that has been previously shown to express Bcl-2 and can be induced to undergo apoptosis.17

Immunoprecipitation experiments were performed using two different methods.17 24 Fig 1A shows a western blot of the immunoprecipitation reactions using the method of Iwahashi et al.17 Jurkat cell lysate was precipitated with anti-SMN (MANSMA2), anti-Bcl-2 (Bcl-2(100)), and anti-dystrophin (MANDYS-1) monoclonal antibodies. The precipitated proteins were western blotted and probed with anti-SMN or anti-Bcl-2 polyclonal antibodies. The precipitated proteins were western blotted and probed with anti-SMN or anti-Bcl-2 polyclonal antibodies. The reciprocal immunoprecipitation reactions using anti-SMN antibodies precipitating Sm proteins. Similar results were obtained using Jurkat cell lysate and in spinal cord. The blots were stripped and repriovided with anti-Bag-1 (fig 1A–3) and anti-Sm antibodies Y12 (fig 1A–4). Sm proteins and Bag-1 have been previously shown to interact with SMN and Bcl-2 respectively and act as controls for the immunoprecipitation procedure.19 40 The expected results were obtained with Bcl-2 antibodies immunoprecipitating Bag-1 and Sm antibodies precipitating Sm proteins. Similar results were obtained using Jurkat cells undergoing apoptosis after induction with phytohaemagglutinin (data not shown).

In normal spinal cord (fig 1B), SMN-C3 detects a 38 kDa band in the immunoprecipitation reaction using MANSMA2 but not in the immunoprecipitation reactions using Bcl-2(100) and MANDYS-1. Similarly, in the reciprocal experiment, the anti-Bcl-2 rabbit polyclonal antibody Bcl-2 (AC21) detects a 29 kDa band only in the sample immunoprecipitated with Bcl-2(100) and not in the other immunoprecipitation reactions. As immunoprecipitation experiments failed to show a direct interaction of native SMN and Bcl-2 in proliferating and apoptotic Jurkat cells and in spinal cord, we investigated cells transfected with SMN/Bcl-2 expression constructs. We have prepared various SMN expression constructs with or without the HA epitope tag in the amino-terminus. Immunoprecipitations were performed...
on the transfected cells using an HA epitope monocolonal and the Bcl-2 antibodies described above. The HA antibodies resulted in immunoprecipitation of SMN and not Bcl-2 whereas Bcl-2 antibodies immunoprecipitated Bcl-2 alone (fig 2A). The transfection studies provided no evidence for interaction of SMN and Bcl-2.

Dual labelling of COS-7 cells cotransfected with SMN and HA tagged SIP-1 expression constructs shows SMN and SIP-1 are colocalised as previously reported (fig 2B). Dual labelling of SMN and Bcl-2 using cotransfection of a HA tagged SMN expression construct and a Bcl-2 expression construct failed to show convincing colocalisation of SMN and Bcl-2 in either COS-7 (fig 2C) or HeLa cells (not shown). As seen in fig 2C, in the cotransfected cell Bcl-2 is expressed abundantly throughout the cytoplasm. Similarly, overexpressed SMN is located ingems, cytoplasmic particles, and throughout the cytoplasm. Even under these conditions of extreme overexpression, there is no clear example of colocalisation, as would be expected if they associate. The apparent association of diffuse staining of SMN and Bcl-2 in the cytoplasm most likely arises from overlapping expression distributions as supported by the immunoprecipitation results.

Although we cannot exclude the possibility that SMN and Bcl-2 interact indirectly with each other in cell death pathways, these results strongly indicate that SMN and Bcl-2 do not directly interact in vivo. It is most likely that the high expression levels in the experiments of Iwahashi et al. resulted in artefactual aggregation of Bcl-2 and SMN and that this interaction does not exist in vivo. In order to show clearly a synergistic effect of Bcl-2 and SMN either by direct or indirect mechanisms, it is important to eliminate the possibility that transfection of SMN increases Bcl-2 levels. It appears most likely that the reduction in SMN levels results in inefficient splicing which leads to a reduction or accumulation of a critical product that causes death of the motor neurons.

Figure 1 (A). Endogenous SMN and Bcl-2 are not coimmunoprecipitated from Jurkat cells. Shown is a western blot of proteins immunoprecipitated with anti-Bcl-2 (Bcl-2(100)), anti-SMN (MANSMA2), and control (MANDYS-1) monoclonal antibodies. The western blot was serially probed with the antibodies shown below the blot as previously described. 1 SMN protein (arrow), as detected with anti-SMN chicken polyclonal antibody C3, is immunoprecipitated with MANSMA2 and not communoprecipitated with Bcl-2. 2 Bcl-2 protein (arrow), as detected with the anti-Bcl-2 polyclonal antibody Bcl-2(C21), is immunoprecipitated with the Bcl-2(100), but is not communoprecipitated with SMN. The 30 kDa band present in all three lanes is non-specific and is probably the IgG light chain, as previously reported. 3 Bag-1 protein, which is known to associate with Bcl-2, is communoprecipitated only with Bcl-2. This indicates communoprecipitation conditions were suitable to precipitate associated proteins. 4 SM proteins coprecipitate with SMN as detected with anti-SM polyclonal antibody Y12 as previously reported and are not communoprecipitated with Bcl-2. This also shows conditions were suitable to precipitate associated proteins. (B) Endogenous SMN and Bcl-2 are not communoprecipitated from non-SMA spinal cord. Shown is a western blot of spinal cord proteins immunoprecipitated with anti-Bcl-2 (Bcl-2(100)) (lane 2), anti-SMN (MANSMA2), and control (MANDYS-1) monoclonal antibodies. The western blot was serially probed with the antibodies shown below the blot. 1 SMN is detected only in the sample precipitated with MANSMA2 and is not communoprecipitated with Bcl-2. 2 The reciprocal experiment shows Bcl-2 is precipitated only with Bcl-2(100) and is not communoprecipitated with SMN. (C) Western blot of unprecipitated Jurkat cell lysate and immunoprecipitations probed with anti-Bcl-2 (Bcl-2(100)) and anti-SMN (SMN C3) antibodies. Methods. Approximately 1.5 x 10^6 cells from subconfluent cultures were lysed with 1 ml of ice cold lysis buffer. The lysate was preclariated by incubating the lysate with washed Ommersb cells (Calbiochem) for 30 minutes on ice. The Ommersb cells were removed by centrifugation, and the preclariated lysate was divided into 3 aliquots of 300 µl each. Approximately 1 µg of anti-SMN (MANSMA2) was added to the first aliquot, 1 µg of Bcl-2 (100) monoclonal (Santa Cruz Biotechnolgy, Santa Cruz, CA) was added to the second aliquot, and 1 µg of the anti-bag1 monoclonal antibody Bcl-2(C21) was added to the last aliquot. MANDYS-1 was made from the same myeloma cell line used to make MANSMA2. The antibodies were rocked on ice with the lysate for one hour to overnight. Immune complexes were captured with protein G sepharose (Sigma). Briefly, 40 µl of a 50% suspension of protein G sepharose was washed with 1 ml of lysis buffer. The protein G sepharose was washed with 1 ml of lysis buffer five times for five minutes on ice. The residual wash buffer was removed by aspiration and sepharose was boiled in 30 µl of SDS buffer for two minutes. The samples were resolved by SDS-PAGE on 12% gels as described previosly. Spinal cord collected from a type I SMA and a non-SMA subject was dissected and ground into a fine powder in liquid nitrogen using a mortar and pestle. The powder was dissolved in 1 ml of Empigen BB (1% Empigen BB, 5 mmol/l EDTA, 1 mmol/l PMSF, 5 mmol/l DTT, and 100 mmol/l Tris, pH 8, 10 µmol/l aprotinin, 1 µmol/l leupeptin, 1 µmol/l pepstatin A) or the Iwahashi lysis buffer. The insoluble material was removed by centrifugation and immunoprecipitations carried out on the supernatant as described above.
**Figure 2** (A) Immunoprecipitation of transiently transfected HA tagged SMN and Bcl-2 from COS-7 cells. COS-7 cells, transiently cotransfected with Bcl-2 and HA tagged SMN expression constructs, were immunoprecipitated using rabbit anti-Bcl-2 polyclonal (lanes 1-3) and rabbit anti-HA (lanes 5-7) polyclonal antibody as indicated above the blot. As indicated before the blot, lanes 1 and 7 were probed with anti-HA monoclonal antibody. Lanes 2 and 6 were probed with anti-SMN monoclonal antibody MANSMA2. Lanes 3 and 5 were probed with anti-Bcl-2 (100) monoclonal antibody. As seen in lanes 1 and 2, HA-SMN is not coprecipitated when using the anti-Bcl-2 polyclonal antibody. In the reciprocal experiment (lane 5), Bcl-2 is not communoprecipitated with HA-SMN. The middle and lower panels show western blots of the proteins remaining in the supernatants following the immunoprecipitation reactions. These are included to indicate the relative levels of Bcl-2, SMN, and HA-SMN expression in the transfected cells. Additionally, neither SMN nor HA-SMN are depleted from the supernatant incubated with anti-Bcl-2 polyclonal antibody (middle panel), but Bcl-2 is depleted (bottom panel). The reciprocal experiment shows only HA-SMN is depleted by the anti-HA monoclonal antibody (middle panel), but neither SMN nor Bcl-2 (bottom panel) is depleted. (B) Immunofluorescent detection of SMN and HA tagged SIP-1 in transiently cotransfected COS-7 cells. SMN (left panel), indirectly labelled with an FITC conjugated antibody, is located in gems, cytoplasmic particles, and diffusely in the cytoplasm. HA tagged SIP-1 (middle panel), indirectly labelled with TRITC conjugated antibodies, shows a similar distribution pattern. Merging the left and middle images (right panel) clearly shows the association of SMN and SIP1 as previously reported. (C) Dual labelling of HA tagged SMN and Bcl-2 detected with anti-HA polyclonal and anti-Bcl-2 monoclonal antibodies. SMN (left panel), indirectly labelled with an FITC conjugated antibody, is localised in gems, cytoplasmic particles, and faint diffusely staining (green indicated by arrows). Bcl-2 (middle panel), indirectly labelled with a TRITC conjugated secondary antibody, is expressed throughout the cytoplasm (red, diffuse fluorescence). In contrast to the SIP1-SMN merged image above, the merged image (right panel) indicates a lack of colocalisation of SMN and Bcl-2. Methods. COS-7 cells (∼10^6) were seeded onto coverslips and prepared as described previously. Bcl-2(100) and Bcl-2(C21) (Santa Cruz Biotechnology Inc) and anti-HA polyclonal antibody (BabCO) were used at 1/500 dilution. Lanes 3 and 5 were probed with anti-Bcl-2(100) monoclonal antibody. As seen in lanes 1 and 2, Bcl-2 is not coimmunoprecipitated with HA-SMN. The middle and lower panels show western blots of the proteins remaining in the supernatants following the immunoprecipitation reactions. These are included to indicate the relative levels of Bcl-2, SMN, and HA-SMN expression in the transfected cells. Additionally, neither SMN nor HA-SMN are depleted from the supernatant incubated with anti-Bcl-2 polyclonal antibody (middle panel), but Bcl-2 is depleted (bottom panel). The reciprocal experiment shows only HA-SMN is depleted by the anti-HA monoclonal antibody (middle panel), but neither SMN nor Bcl-2 (bottom panel) is depleted. (B) Immunofluorescent detection of SMN and HA tagged SIP-1 in transiently cotransfected COS-7 cells. SMN (left panel), indirectly labelled with an FITC conjugated antibody, is located in gems, cytoplasmic particles, and diffusely in the cytoplasm. HA tagged SIP-1 (middle panel), indirectly labelled with TRITC conjugated antibodies, shows a similar distribution pattern. Merging the left and middle images (right panel) clearly shows the association of SMN and SIP1 as previously reported. (C) Dual labelling of HA tagged SMN and Bcl-2 detected with anti-HA polyclonal and anti-Bcl-2 monoclonal antibodies. SMN (left panel), indirectly labelled with an FITC conjugated antibody, is localised in gems, cytoplasmic particles, and faint diffusely staining (green indicated by arrows). Bcl-2 (middle panel), indirectly labelled with a TRITC conjugated secondary antibody, is expressed throughout the cytoplasm (red, diffuse fluorescence). In contrast to the SIP1-SMN merged image above, the merged image (right panel) indicates a lack of colocalisation of SMN and Bcl-2. Methods. COS-7 cells (∼10^6) were seeded onto coverslips and prepared as described previously. Bcl-2(100) and Bcl-2(C21) (Santa Cruz Biotechnology Inc) and anti-HA polyclonal antibody (BabCO) were used at 1/500 dilution. The secondary antibodies, rabbit anti-haemagglutinin antibody conjugated to TRITC (Sigma) and donkey anti-chicken F(ab)2 antibody conjugated to FITC (Jackson Immunoresearch) were used at 1/400 dilution. SIP-1 cDNA was ligated to haemagglutinin (HA) tag and subcloned into the pcDNA3 vector. Bcl-2 was subcloned into pcDNA3 vector. DNA plasmids were prepared for transfection using Endo Free Plasmid Kit (Qiagen). COS-7 cells were transfected with an Optronics colour digital camera using FITC and TRITC/DAPI double pass filters. Total magnification of images is 945×.

This work was funded by MDA, Families of SMA, and the Preston Fund, and NIH grant NS 38650.

DANIEL D COOVERT‡†
THANH T LE‡
GLENN E MORRIS*
NGUYEN THI MANE
MARTINA KRALEWSKI*
MICHAEL SENDTNER*
ARTHUR H M BURGHESE

*Department of Medical Biochemistry, Ohio State University, College of Medicine, Columbus OH 43210, USA
‡MRIC Biochemistry Group, NE Wales Institute, Wrexham, UK
§Department of Neurology, University of Wurzburg, 97080 Wurzburg, Germany
*Department of Neurology, Ohio State University, College of Medicine and Dept of Molecular Genetics, College of Biological Sciences, Columbus, OH 43210, USA

Correspondence to: Dr Burghes, Department of Medical Biochemistry, 363 Hamilton Hall, 1645 Neil Avenue, Columbus, OH 43210, USA, burghes.1@osu.edu

†These authors contributed equally to this work.
Novel mutations in the homogentisate-1,2-dioxygenase gene identified in Slovak patients with alkaptonuria

Table 1 List and frequencies of the mutations identified in 32 AKU chromosomes from Slovak patients. Positions of nucleotide changes are related to the transcription start site as described in Granadino et al (Human HGO transcript: AF 045167; the ATG initiation codon is located at position c168)

<table>
<thead>
<tr>
<th>Mutation Type</th>
<th>Nucleotide change</th>
<th>No and % of chromosomes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVS 1+1 G→A</td>
<td>Splice site</td>
<td>c183G→A</td>
<td>1(3.125%)</td>
</tr>
<tr>
<td>S 47 L</td>
<td>Massequencene c307C→T</td>
<td>1(3.125%)</td>
<td>Present report</td>
</tr>
<tr>
<td>IVS 5+1 G→A</td>
<td>Splice site</td>
<td>c509G→A</td>
<td>4 (12.5%)</td>
</tr>
<tr>
<td>G 152 fs</td>
<td>Frameshift</td>
<td>c621insG</td>
<td>2 (6.25%)</td>
</tr>
<tr>
<td>G 161 R</td>
<td>Massequencene c648G→A</td>
<td>1 (3.125%)</td>
<td>12</td>
</tr>
<tr>
<td>P 230 S</td>
<td>Massequencene c855C→T</td>
<td>1 (3.125%)</td>
<td>13</td>
</tr>
<tr>
<td>G 270 R</td>
<td>Massequencene c975G→A</td>
<td>1 (3.125%)</td>
<td>14</td>
</tr>
<tr>
<td>V 300 G</td>
<td>Massequencene c1066T→G</td>
<td>1 (3.125%)</td>
<td>15</td>
</tr>
<tr>
<td>P 370 fs</td>
<td>Frameshift</td>
<td>c1273insC</td>
<td>5 (15.625%)</td>
</tr>
</tbody>
</table>

Note: Table 1 continues with additional mutations.

silver staining essentially as described by Budowle et al.14 Fragments showing SSCP shifts were sequenced directly using the dye terminator cycle sequencing kit (Perkin Elmer) with Taq FS DNA polymerase. Sequences were resolved on an ABI-310 Automatic Analyser.

In our patients, we identified nine different mutations (tables 1 and 2). Four of them were novel mutations, two missense (S47L, G270R), a frameshift (P370fs), and a splice site mutation (IVS5+1G→A), increasing the total number of known AKU causing nucleotide changes within the HGO gene to 28. The remaining five mutations have been described previously: G161R and G152fs,6 P230S and V300G,7,8 and IVS1-1G→A.9

Novel mutation S47L is caused by a transition C→T at the second position of codon 47 (fig 1A). This transition abolishes a restriction site for Mbol in exon 3 PCR fragments. The presence of the S47L mutation in our patient was confirmed by Mbol digestion (fig 2A).

Mutation G270R is caused by transition G→A at the first position of codon 270, which creates a novel EcoN restriction site (fig 1C). Therefore, its presence in our patients was confirmed by restriction digestion of exon 11 PCR fragments with EcoN (fig 2B). Glycine at position 270, affected by this mutation, is conserved in man, mouse, and Aspergillus nidulans (fig 3).

Splice site mutation IVS5+1G→A affects the donor splice sites of intron 5 (fig 1B). Interestingly, Beltran-Valero de Bernabé et al identified in one patient from Holland a transversion G→T affecting the same position of intron 5 as our mutation IVS5+1G→A. This mutation, however, was not identified in our patients.

Mutation IVS1-1G→A abolishes restriction sites for Rsal, so the presence of this mutation on one AKU chromosome was confirmed by restriction analysis of the corresponding PCR fragment with this enzyme (fig 2A).

A novel P370fs frameshift mutation, caused by a single base insertion c1273insC (fig 1D), brings about a premature translation stop four codons downstream and subsequent

Table 2 Genotypes of all analysed AKU patients from 15 families indicating identified disease causing mutations within the HGO gene

<table>
<thead>
<tr>
<th>Patient No</th>
<th>Family code</th>
<th>HGO mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allele 1</td>
<td>Allele 2</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>ALK1</td>
<td>G152fs</td>
</tr>
<tr>
<td>2</td>
<td>ALK2</td>
<td>IVS5+1G→A</td>
</tr>
<tr>
<td>3</td>
<td>ALK2</td>
<td>IVS5+1G→A</td>
</tr>
<tr>
<td>4</td>
<td>ALK3</td>
<td>G161R</td>
</tr>
<tr>
<td>5</td>
<td>ALK4</td>
<td>G270R</td>
</tr>
<tr>
<td>6</td>
<td>ALK5</td>
<td>G270R</td>
</tr>
<tr>
<td>7</td>
<td>ALK6</td>
<td>IVS1-1G→A</td>
</tr>
<tr>
<td>8</td>
<td>ALK7</td>
<td>G152fs</td>
</tr>
<tr>
<td>9</td>
<td>ALK8</td>
<td>G161R</td>
</tr>
<tr>
<td>10</td>
<td>ALK9</td>
<td>S47L</td>
</tr>
<tr>
<td>11</td>
<td>ALK10</td>
<td>G161R</td>
</tr>
<tr>
<td>12</td>
<td>ALK11</td>
<td>G152fs</td>
</tr>
<tr>
<td>13</td>
<td>ALK12</td>
<td>G152fs</td>
</tr>
<tr>
<td>14</td>
<td>ALK13</td>
<td>G161R</td>
</tr>
<tr>
<td>15</td>
<td>ALK14</td>
<td>G161R</td>
</tr>
<tr>
<td>16</td>
<td>ALK15</td>
<td>G161R</td>
</tr>
<tr>
<td>17</td>
<td>ALK16</td>
<td>P230S</td>
</tr>
</tbody>
</table>
shortening of translated HGO protein from 445 to 373 amino acids.

The novel mutations were not identified in any of the 50 healthy controls, supporting the evidence that they are disease causing mutations, rather than polymorphisms.

Segregation of all mutations with AKU was confirmed in all families, except for the S47L mutation, where no DNA from family members was available (fig 4). However, serine at position 47 of the HGO protein molecule is conserved in man and mouse (fig 3). In *Aspergillus nidulans*, threonine is found at this site, which, as well as serine, belongs to the group of hydrophilic amino acids with uncharged polar side chains that are usually on the outside of the protein. Conversely, leucine, which is introduced into the HGO protein by transition c307C→T, is an amino acid with non-polar side chains that tend to cluster together on the inside of proteins. This indicates that substitution S47L may influence the HGO protein conformation and therefore also affect its function.

Recently, Beltran-Valero de Bernabé et al. provided the evidence that the CCC triplet or its inverted complement (GGG) are mutational hotspots in the HGO gene, because 34.5% (10/29) of HGO nucleotide changes identified so far involve these sequence motifs. Data shown in our report further support their finding, since 55.5% (5/9) of the mutations identified in our patients lie within or are adjacent to these triplets. Taking into account the novel mutations found in Slovak patients and one identified by Felbor et al., the total number of HGO nucleotide variations involving the CCC/GGG motif identified so far can be increased to 38.2% (13/34).

In all 17 analysed Slovak AKU patients, both disease causing mutations were found (table 2). The identification of nine different mutations in this sample was not expected.

---

**Figure 1** Part of the direct sequencing of exons 3 (A), 5 (B), 11 (C), and 13 (D) in patients heterozygous for mutations S47L, IVS5+1G→A, G270R, and P370fs, respectively (ABI 310, Perkin Elmer). In the case of exons 3, 5, and 11, reverse primers were used.

---

**Figure 2** Detection of the (A) IVS1-1G→A, S47L (c307C→T) and (B) G270R (c975G→A) mutations by RsaI, MboI, and EcoNI restriction analysis, respectively. Mutations IVS1-1G→A and S47L abolish restriction sites for corresponding enzymes, while G270R creates a novel site for EcoNI. The figure also shows segregation of mutations IVS1-1G→A in family ALK6 (A) and G270R in family ALK4 (B). In both families, the fathers and affected daughters are heterozygous for screened mutations ((A) lanes 1 and 4, (B) lanes 2 and 4, as well as the patient in (A) lane 6). In (A) lanes 2, 3, 7, and 8 and (B) lanes 3 and 5 are subjects without corresponding mutations. In (A) lane 5 and (B) lane 1 is a 100 bp ladder.
because the founder effect had been considered to be the main reason responsible for an increased incidence of AKU in Slovakia. The most frequent mutations, G161R and G152fs (previously identified in two Slovak families by Gehrig et al6), were present on 50% of 32 screened AKU chromosomes (table 1). So far, these mutations have not been identified in any other screened population. This indicates that they might be specific for Slovakia. The high proportion of these two mutations can be explained by founder effect and subsequent genetic isolation. In addition, however, there must have been at least four other founders contributing to the gene pool of the Slovak AKU population (table 1). Three further mutations were each found on only one AKU chromosome, thus indicating that this mechanism is not the only one responsible for the high incidence of this disease in Slovakia (1:19 000).

Possible common origins of chromosomes carrying the same AKU mutations can be further traced by the analysis of DNA polymorphisms in the HGO gene and construction of haplotypes. This work is now in progress.

Figure 3  Comparison of primary structure of homogentisate-1,2-dioxygenase protein from man (HGO, AF000573), mouse (MHGO, U58988), and Aspergillus nidulans (HMGA, U30797) using ClustalX 1.3b. Positions conserved in all three organisms are indicated by (*). Arrows mark sites of identified missense mutations, novel mutations are shown in bold.

Figure 4  Non-radioactive SSCP analysis of exon 13 indicating segregation of mutation P370fs in family ALK5. Arrows mark the SSCP shifts corresponding to this mutation. The presence of the mutation in heterozygous state is indicated by (*). Patients were also heterozygous for mutation G270R (exon 11) (table 2).
Clinical and molecular correlates of somatic mosaicism in neurofibromatosis 2

Editor—Neurofibromatosis 2 (NF2) is an autosomal dominant disorder that is characterised by benign nervous system tumours (such as vestibular schwannomas (VSs), intracranial meningiomas, and spinal tumours) and other abnormalities.1 Somatic mosaicism (the presence of a mutation, deletion, or chromosomal abnormality in a subpopulation of somatic cells) is thought to be relatively common in NF2, affecting perhaps 15% of sporadic cases.2 3

There can be considerable clinical variability in mosaics because somatic mutation can occur at different stages of the postzygotic cell lineage. Evans et al reported the degree of mosaicism for five NF2 patients. Two patients with an estimated <10% of peripheral lymphocytes with NF2 mutations had ages of onset of symptoms of 41–48 years, while three patients with an estimated 21–44% of affected cells had ages of onset of symptoms of 21–28 years. This is consistent with a relationship between degree of mosaicism and disease severity, although there are too few patients to draw firm conclusions.

Few NF2 somatic mosaics have been reported, and clinical and molecular differences between mosaics and sporadic non-mosaic NF2 patients have not been quantified. To examine this question, we compared somatic mosaic and sporadic non-mosaic NF2 patients selected from NF2 populations in the United Kingdom (341 patients)1 3 and Germany (118 patients).7 8 The study groups included 13 previously identified somatic mosaics3 3 and 86 sporadic non-mosaic NF2 patients, all of whom had head and spine gadolinium enhanced magnetic resonance imaging. Sporadic NF2 patients were defined as non-mosaic if they had identified germline NF2 mutations with normal strength gel bands. In theory, somatic mosaic patients could have near normal band strength, but in practice band strength will be reduced when <50% of peripheral lymphocytes have NF2 mutations.2 Clinical data were not available in sufficient detail to determine if the distribution of lesions was non-uniform, which could result from somatic mosaicism.

The covariates that were examined were age at onset of symptoms, age at diagnosis, number of VSs (nose or unilateral versus bilateral), presence and number of intracranial meningiomas, presence of spinal tumours, and germ-line NF2 mutation types (frameshift or nonsense versus other identified mutations). In univariate analyses, the two tailed test was used for continuous variables and the two tailed Fisher exact test for binary variables. Multiple logistic regression was then used to examine the association between somatic mosaic status and covariates that differed between groups; interaction terms of age with number of tumours were also considered.

The characteristics of somatic mosaics and sporadic non-mosaiscs are compared in table 1. Age at onset of
Many ΔF508 heterozygote neonates with transient hypertrypsinaeemia have a second, mild CFTR mutation

EDITOR—Mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) gene have an extremely wide phenotypic spectrum. The “classical” severe form of cystic fibrosis (CF) is characterised by pancreatic insufficiency and chronic endobronchial infection. Milder forms may show fibrosis (CF) is characterised by pancreatic insufficiency. Missense mutations, particularly in the transmembrane domains, result in a milder, more variable disease. The sweat test, long regarded as the gold standard diagnostic test for cystic fibrosis, may give normal results in these milder forms.

Neonatal screening for CF relies on an increased immunoreactive trypsinogen (IRT) concentration in blood of affected babies during the first two months of life. However, this method has low specificity, particularly with samples taken in the first week of life, so mutation analysis is increasingly being used as a second tier. In the Trent region of the United Kingdom (UK), where ΔF508 accounts for over 80% of CF mutations, we currently use a three stage IRT-DNA-IRT protocol, which has a low requirement for second blood samples and for sweat testing. Any initial blood sample with IRT readings above the threshold is analysed for the ΔF508 CFTR mutation. Subjects who are heterozygous for ΔF508 are resampled at 27 days; if the IRT level is again above the threshold the child is referred for sweat testing. The families of heterozygous neonates

result in no CFTR protein, such as truncating mutations, or those, such as ΔF508, which result in mislocalisation of the protein, result in a severe phenotype with pancreatic insufficiency. Missense mutations, particularly in the transmembrane domains, result in a milder, more variable disease. The sweat test, long regarded as the gold standard diagnostic test for cystic fibrosis, may give normal results in these milder forms.

Neonatal screening for CF relies on an increased immunoreactive trypsinogen (IRT) concentration in blood of affected babies during the first two months of life. However, this method has low specificity, particularly with samples taken in the first week of life, so mutation analysis is increasingly being used as a second tier. In the Trent region of the United Kingdom (UK), where ΔF508 accounts for over 80% of CF mutations, we currently use a three stage IRT-DNA-IRT protocol, which has a low requirement for second blood samples and for sweat testing. Any initial blood sample with IRT readings above the threshold is analysed for the ΔF508 CFTR mutation. Subjects who are heterozygous for ΔF508 are resampled at 27 days; if the IRT level is again above the threshold the child is referred for sweat testing. The families of heterozygous neonates
than in non-F508 heterozygous neonates with transient hypertrypsinaemia born between April 1991 and November 1996 were available for testing. All had exceeded the IRT cut off value for the 6th day blood sample (usually 90 ng/ml but adjusted periodically to select approximately 0.5% of tested babies) and had an IRT concentration below 80 ng/ml in the 27th day sample. Cases with meconium ileus were excluded. There was a sample available from every subject fulfilling the selection criteria with meconium ileus were excluded. There was a sample available from every subject fulfilling the selection criteria.

It has previously been shown that neonates with transient hypertrypsinaemia carry the ΔF508 mutation at a higher frequency than the general population. Additionally, IRT remains on average higher in the repeat blood sample in babies who are heterozygous for AF508 than in non-AF508 babies. Thus, it is possible that a proportion of infants with transient hypertrypsinaemia and ΔF508 may have a second (mild) CF mutation. We have examined this hypothesis in a cohort of infants assessed through the Trent (UK) neonatal screening programme.

A cohort of 88 AF508 heterozygous neonates with transient hypertrypsinaemia born between April 1991 and November 1996 were available for testing. All had exceeded the IRT cut off value for the 6th day blood sample (usually 90 ng/ml but adjusted periodically to select approximately 0.5% of tested babies) and had an IRT concentration below 80 ng/ml in the 27th day sample. Cases with meconium ileus were excluded. There was a sample available from every subject fulfilling the selection criteria with meconium ileus were excluded. There was a sample available from every subject fulfilling the selection criteria.

Table 1 CFTR primers, PCR and SSCP analysis conditions

<table>
<thead>
<tr>
<th>Region amplified</th>
<th>Primer sequence</th>
<th>Anneal temp (°C)</th>
<th>Product size (bp)</th>
<th>Enzyme digest</th>
<th>SSCP conditions % glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 1</td>
<td>F 5' - GCC AAA TGA CAT CAC AGC AGG - 3'</td>
<td>61</td>
<td>211</td>
<td>None</td>
<td>3</td>
</tr>
<tr>
<td>Exon 2</td>
<td>F 5' - TGT CTT TCC GAA GCT CGG TGG - 3'</td>
<td>56</td>
<td>203</td>
<td>None</td>
<td>3</td>
</tr>
<tr>
<td>Exon 3</td>
<td>F 5' - CAG TCC TGG TAC TCG CTA AGG - 3'</td>
<td>55</td>
<td>309</td>
<td>MboI</td>
<td>2</td>
</tr>
<tr>
<td>Exon 4</td>
<td>F 5' - CCT TGG TTA ATC TCC TGG GA - 3'</td>
<td>56</td>
<td>438</td>
<td>BstUI</td>
<td>2</td>
</tr>
<tr>
<td>Exon 5</td>
<td>F 5' - ATT CAC CAG ATT TGC TAG TC - 3'</td>
<td>56</td>
<td>212</td>
<td>None</td>
<td>3</td>
</tr>
<tr>
<td>Exon 6</td>
<td>F 5' - CAT ATC TCA TCA AAT TTT TCG TTT - 3'</td>
<td>58</td>
<td>275</td>
<td>None</td>
<td>2</td>
</tr>
<tr>
<td>Exon 7</td>
<td>F 5' - TAC TAA AGT GGG CTT TTT GAA AAC - 3'</td>
<td>56</td>
<td>285</td>
<td>None</td>
<td>2</td>
</tr>
<tr>
<td>Exon 8</td>
<td>F 5' - GAG AAG ATA GTA AGC TAG ATG AAT - 3'</td>
<td>58</td>
<td>230</td>
<td>None</td>
<td>2</td>
</tr>
<tr>
<td>Exon 9 (1)</td>
<td>F 5' - TAA TGG ATC ATG GGC CAT GT - 3'</td>
<td>56</td>
<td>223</td>
<td>None</td>
<td>3</td>
</tr>
<tr>
<td>Exon 9 (2)</td>
<td>F 5' - AGA GTG CTA CCA TGA AAA AAC TAT - 3'</td>
<td>55</td>
<td>560</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Exon 10</td>
<td>F 5' - GAG GAA TTA TTT GAG AAA GC - 3'</td>
<td>55</td>
<td>258</td>
<td>None</td>
<td>3</td>
</tr>
<tr>
<td>Exon 11</td>
<td>F 5' - CAT TCA CAG TAG CCT ACC CA - 3'</td>
<td>58</td>
<td>491</td>
<td>Nol/HinFI</td>
<td>2</td>
</tr>
<tr>
<td>Exon 12</td>
<td>F 5' - GTG AAT CGA TGT GGT GAC GA - 3'</td>
<td>56</td>
<td>426</td>
<td>BstNI</td>
<td>2</td>
</tr>
<tr>
<td>Exon 13</td>
<td>F 5' - CTG GTT TAG CAT GAG GGG GT - 3'</td>
<td>53</td>
<td>280</td>
<td>None</td>
<td>2</td>
</tr>
<tr>
<td>Exon 14</td>
<td>F 5' - AGT TAG GAT TGA ATT TCT TCT TT - 3'</td>
<td>56</td>
<td>309</td>
<td>None</td>
<td>2</td>
</tr>
<tr>
<td>Exon 15</td>
<td>F 5' - AGA ATC TGG TAC TAA GGA CAG - 3'</td>
<td>55</td>
<td>444</td>
<td>HinFI</td>
<td>2</td>
</tr>
<tr>
<td>Exon 16</td>
<td>F 5' - CAT TCT ACT CAA TTG GAG ATG CA - 3'</td>
<td>58</td>
<td>239</td>
<td>None</td>
<td>2</td>
</tr>
<tr>
<td>Exon 17</td>
<td>F 5' - TGG GAG TGG ATT AAA CTA CAG TAG TGT TTT CTT TCT - 3'</td>
<td>60</td>
<td>250</td>
<td>None</td>
<td>2</td>
</tr>
<tr>
<td>Exon 18</td>
<td>F 5' - GCA GAC TAC CTG ACG ATG CAT AAT - 3'</td>
<td>60</td>
<td>208</td>
<td>None</td>
<td>2</td>
</tr>
<tr>
<td>Exon 19</td>
<td>F 5' - ATT CAG TAA ACT TGG CTT GGC ACC - 3'</td>
<td>60</td>
<td>413</td>
<td>HinFI</td>
<td>4</td>
</tr>
<tr>
<td>Exon 20</td>
<td>F 5' - GAG CCA CAC CAC TGA GGT GTT AGG - 3'</td>
<td>62</td>
<td>289</td>
<td>Rsal</td>
<td>3</td>
</tr>
<tr>
<td>Exon 21</td>
<td>F 5' - TAG ACA GGA CTT CAA CCC TCA ATC - 3'</td>
<td>56</td>
<td>254</td>
<td>None</td>
<td>2</td>
</tr>
<tr>
<td>Exon 22</td>
<td>F 5' - AGA TGA TTT TTT CTT TAA ACC AAC TGT - 3'</td>
<td>57</td>
<td>463</td>
<td>HinFI</td>
<td>2</td>
</tr>
<tr>
<td>Exon 23</td>
<td>F 5' - ACA TGA TGT CTA ATC GTG AGC - 3'</td>
<td>56</td>
<td>201</td>
<td>None</td>
<td>3</td>
</tr>
<tr>
<td>Exon 24</td>
<td>F 5' - ACA TCA TCG CAC CAA TGG AGG CAG - 3'</td>
<td>56</td>
<td>201</td>
<td>None</td>
<td>3</td>
</tr>
</tbody>
</table>

Letters
Polyacrylamide gels in 1% TBE except exon 11, which used 14% (37:1 acryl:bis) polyacrylamide gel. Intron 19 used non-denaturing electrophoresis on 8% gels. The IVS8-5T variant is an alternate splice site.

Table 2 Compound heterozygotes detected

<table>
<thead>
<tr>
<th>Domain and mutation type</th>
<th>Genotype</th>
<th>Exon</th>
<th>1st IRT</th>
<th>2nd IRT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transmembrane, missense</td>
<td>ΔF508/P67L</td>
<td>3</td>
<td>129</td>
<td>34*</td>
</tr>
<tr>
<td>ΔF508/R117H</td>
<td>4</td>
<td>110</td>
<td>21*</td>
<td></td>
</tr>
<tr>
<td>ΔF508/R117H</td>
<td>4</td>
<td>84</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>ΔF508/R117H</td>
<td>4</td>
<td>95</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>ΔF508/R117H</td>
<td>4</td>
<td>104</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>ΔF508/R117H</td>
<td>4</td>
<td>146</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>ΔF508/R117H</td>
<td>4</td>
<td>104</td>
<td>48*</td>
<td></td>
</tr>
<tr>
<td>ΔF508/R117H</td>
<td>4</td>
<td>129</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>ΔF508/R117H</td>
<td>4</td>
<td>111</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>ΔF508/R117H</td>
<td>4</td>
<td>175</td>
<td>72*</td>
<td></td>
</tr>
<tr>
<td>ΔF508/R117H</td>
<td>4</td>
<td>129</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>ΔF508/R117H</td>
<td>15</td>
<td>122</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>ΔF508/F5052V</td>
<td>17b</td>
<td>189</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>ΔF508/R1066H</td>
<td>17b</td>
<td>94</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Transmembrane, nonsense</td>
<td>ΔF508/R75X</td>
<td>3</td>
<td>86</td>
<td>26</td>
</tr>
<tr>
<td>ΔF508/R75X</td>
<td>3</td>
<td>171</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>ΔF508/R851X</td>
<td>14a</td>
<td>112</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>Regulatory, missense</td>
<td>ΔF508/F693L</td>
<td>13</td>
<td>109</td>
<td>29</td>
</tr>
<tr>
<td>Alternate splice site</td>
<td>ΔF508/3849+10KB C→T</td>
<td>i19</td>
<td>99</td>
<td>26*</td>
</tr>
<tr>
<td>ΔF508/3849+10KB C→T</td>
<td>i19</td>
<td>112</td>
<td>36*</td>
<td></td>
</tr>
</tbody>
</table>

None of these samples had the IVS8-5T variant sequence. *These patients have presented with symptoms of lung involvement.

Amplified fragments were screened for sequence alterations by single stranded conformational polymorphism analysis (SSCP)/heteroduplex analysis (HA) using standard conditions and procedures as in table 1 with 14% w/v (59:1 acrylamide: bisacrylamide) polyacrylamide gels and electrophoresis at 12°C in 0.5 × TBE except exon 11, which used a 14% (37:1 acryl:bis) polyacrylamide gel. Exon 9 was amplified using the IVS8-nT protocol and the primer sets 9 (1) followed by 9 (2). This excluded the two highly polymorphic regions before the exon (TG(n) + IVS8-nT) from amplification and greatly simplified mutation analysis. Full details of primer sequences and annealing temperatures can be found in table 1.

Amplified fragments were screened for sequence alterations by single stranded conformational polymorphism analysis (SSCP)/heteroduplex analysis (HA) using standard conditions and procedures as in table 1 with 14% w/v (59:1 acrylamide: bisacrylamide) polyacrylamide gels and electrophoresis at 12°C in 0.5 × TBE except exon 11, which used a 14% (37:1 acryl:bis) polyacrylamide gel. Intron 19 used non-denaturing electrophoresis on 8% (29.1 acryl:bis) polyacrylamide gels in 1 × TBE. Any samples displaying shifts (indicative of a sequence alteration) were investigated further by direct DNA sequencing (as per manufacturer’s instructions). This methodology identifies >95% of all mutations in our laboratory.

Second mutations were identified in 20 subjects (table 2) giving a compound heterozygote frequency of 22%. R117H was the most common second mutation found, constituting 45% of the compound heterozygotes identified. Fig 1 shows the range of second IRT reading displayed by non-ΔF508 babies, the putative ΔF508 heterozygotes, and the compound heterozygotes. The IRT distributions of the putative ΔF508 heterozygotes (19) and particularly the compound heterozygotes are shifted to the right. Forty one percent of ΔF508 heterozygote neonates with >25 ng IRT/ml in the 27th day blood sample possessed a second mutation compared to ~6% for those with <25 ng/ml, an enrichment factor of 2.35.

Recent work has suggested that “polyvariant mutant CFTR genes” may, when combined, result in less functional or pathologically insufficient CFTR. In the light of this, we determined the incidence of the intronic poly-T tract, IVS8-nT, which interacts with the R117H mutation. The IVS8-5T allele was present in three of the 91 neonates, none of whom were identified as being compound heterozygotes for a second CF mutation. The 71 remaining subjects are likely to be true heterozygotes with transient hypertrypsinæmia arising from causes other than CF.

In this study, we have confirmed that the combination of heterozygosity for ΔF508 and persisting mild hypertrypsinæmia in the newborn period carries a substantial risk of that infant having a second CF mutation. In addition, the concentration of IRT in whole blood at 27 days is a biochemical marker to refine this risk further.

All the mutations found in our cohort have been reported previously and for the more common ones there are data on phenotypic presentation. In general, the severity of lung disease is less predictable than the degree of pancreatic involvement. Some females with this genotype are completely asymptomatic. Three ΔF508/R117H compound heterozygotes with the 7T variant from the cohort have already presented with symptoms of lung disease. An increased frequency of IVS8-5T in non-CF transient neonatal hypertrypsinæmia, with or without heterozygosity for ΔF508, has been reported in two studies. However, in our cohort, IVS8-5T was not present in any of the compound heterozygotes detected and its overall frequency was not significantly different from that reported in the general population.

Five other neonates screened had missense mutations in transmembrane domains. These have been reported in patients with presenting phenotypes ranging from “cystic fibrosis” to oligosperma, but there have been too few cases.
described to define genotype-phenotype correlations. The missense mutation F693L is located in the regulatory domain of CFTR and was first identified in a young girl with AF508 on her other allele who was diagnosed with pancreatic insufficient cystic fibrosis.

Three subjects had nonsense mutations which are normally associated with severe disease as they introduce a stop codon, leading to truncated, usually inactive, CFTR protein being transcribed. The 3849+10 kb C→T mutation, identified in two subjects in our cohort, activates a partially active, cryptic splice site within the intron that causes an 84 bp “exon” to be inserted, in frame, within exon 19. Homozygotes for this mutation usually have relatively mild lung disease, while compound heterozygotes may show pancreatic insufficiency and may have more severe lung disease.

Compared to other neonatal metabolic screening tests, that for CF has a relatively low sensitivity; the more successful programmes detect approximately 90% of severe, “classical” CF. With the increasing recognition of milder forms of CFTR deficiency, and the tendency to use DNA analysis rather than the sweat test as the ultimate diagnostic arbiter, the perceived effectiveness of neonatal screening is being further eroded. Nevertheless, provided that the prime aim of the programme remains the detection of classical cystic fibrosis, in the way the term was understood 10 years ago, performance using either a two stage IRT method or one of the newer IRT-DNA protocols may be regarded as acceptable.

The difficulties with DNA based protocols arise from the need to provide explicit counselling to families where the baby has been found to be heterozygous for AF508 but has given a normal sweat test or had a normal IRT concentration in a second blood sample. In most screening programmes more babies will fall into this group than will be diagnosed with classical cystic fibrosis (representative figures are given in reference 15). The present study shows that such babies, particularly the subgroup with IRT in the second blood sample >25 ng/ml, have a high likelihood of a second CFTR mutation. Should further testing of AF508 heterozygotes be restricted to mutations associated with a severe phenotype or should extended mutation analysis, at least in the higher risk group with second IRT >25 ng/ml, become part of routine investigation? Would such investigation improve the accuracy of counselling of such families?

The majority of compound heterozygotes detected in this study have mutations usually associated with milder forms of CFTR disease. However, some of our hypertyrpsinaemic cohort have genotypes previously reported in severe forms of CF and may be regarded as having given false negative screening test results. Genotype-phenotype correlation is complicated by the existence of polyvariant CFTR genes and modifier polymorphisms, as well as possible modifiers at other loci. It is probably unsound to extrapolate data from clinically selected cases to a cohort which was selected on an entirely different basis. The clinical significance of compound heterozygosity detected through neonatal screening will only become apparent through systematic follow up. Preliminary indications are that some of the expected mild cases develop significant early symptoms, six of the compound heterozygotes in our cohort (indicated in table 2) were unblinded by being diagnosed with cystic fibrosis, enabling them to be followed forwards from their neonatal screening results. All have developed symptomatic respiratory infections associated with positive bacterial cultures. A prospective study is under way to determine the clinical spectrum present in such compound heterozygotes.

It is generally agreed that diagnosing adult onset disease during childhood is undesirable if there is no effective preventive treatment. One would not normally wish to predict fertility problems in an otherwise healthy male neonate. However, in so far as early active treatment can reduce the impact of lung disease in moderate CFTR deficiency, previous warning that a child is thus predisposed would be valuable. Thus, the increased possibility of mild CF spectrum disease should be raised during counselling for AF508 heterozygosity detected on newborn screening. There may be a case for more active clinical follow up, particularly in the persistent mild hypertyrpsinaemia group.

However, until we are more confident in predicting outcome from genotype, the prospective identification of “milder” mutations should not be included in routine neonatal screening protocols.

The support of the Sheffield Children’s Hospital Research and Development Fund and the Cystic Fibrosis Trust (UK) is gratefully acknowledged.

JIM BOYNE‡
STEVE EVANS‡
RODNEY J POLLITT†
CHRISTOPHER J TAYLOR‡
ANN DALTON‡

8 http://www.genet.sickkids.on.ca/cftr/
Further evidence for genetic heterogeneity of autosomal dominant disorders with accumulation of multiple deletions of mitochondrial DNA

Editor—Disorders of oxidative phosphorylation are highly heterogeneous from both a clinical and a genetic point of view. The nuclear as well as the mitochondrial genomes contain genes that are necessary for respiratory chain function. Consequently, different modes of inheritance are encountered in disorders of oxidative phosphorylation.

Single large scale deletions of mitochondrial DNA (mtDNA) usually occur in sporadic cases. However, multiple deletions of mtDNA also occur in autosomal dominant disorders. These deletions are generated de novo as somatic mutations in each affected subject. The nuclear gene defects predisposing to secondary mtDNA deletions in these patients remain unknown.

The disorder discovered by Zeviani et al was later found in several families and was called autosomal dominant progressive external ophthalmoplegia (ADPEO), as ptosis and external ophthalmoplegia are the major clinical findings. More generalised weakness of the skeletal muscles and sudden unexpected death are also common clinical features. Additional clinical features vary among different families.

Linkage analysis provided direct evidence for genetic heterogeneity of ADPEO. One locus predisposing to ADPEO in a Finnish family was assigned to chromosome 10q23.3-q24.3. Another locus was assigned to chromosome 3p14.1-p21.2 in three Italian families. In another Italian ADPEO family the disorder is linked to chromosome 4q34-3q35.

We previously reported three unrelated Belgian families with progressive external ophthalmoplegia and multiple deletions of mtDNA. Only one of these families was of sufficient size to examine cosegregation of PEO with the known loci on chromosomes 10q, 3p, and 4q.

Fig 1 shows the updated pedigree of the Belgian PEO family. Several male to male transmissions indicate clear autosomal dominant inheritance. The diagnosis of ADPEO was based on the clinical symptoms and the presence of multiple mtDNA deletions on Southern blots of muscle biopsy specimens in two patients. The detailed clinical features and the muscle biopsy findings with mtDNA analysis have been described elsewhere.

Blood samples were obtained after informed consent. The primer sequences for the polymorphic DNA markers on chromosomes 3, 4, and 10 were obtained from the Genome Database (http://gdbwww.gdb.org). The linkage analysis was carried out by MLINK 5.1 using a disease gene frequency of 1/10 000. Since variable penetrance of the disease was shown in the 10q linked Finnish family, only affected subjects were included in our linkage analyses. Subjects were considered affected when clinical examination showed PEO and absent Achilles tendon reflexes (fig 1). Dead, but not clinically examined subjects were considered affected if there was a positive family history obtained from relatives and the appearance of PEO in photographs.

The average expected maximum lod score in computer simulation analysis was 1.5, assuming a 80% informative linked marker at 5% recombination distance from the PEO gene. In 500 simulated replicates, the maximum lod score obtained was 3.60. Linkage analysis excluded all three known loci (table 1). Few markers generated minor positive lod scores (Z) of 0.5 to 1 at large recombination distances (0), but flanking markers and haplotype analysis clearly excluded the candidate regions.

Our data provide further evidence for the genetic heterogeneity of autosomal dominant PEO, indicating that

![Figure 1](http://jmg.bmj.com/)

**Figure 1** Pedigree of the autosomal dominant Belgian PEO family. Filled symbols indicate affected subjects. Subjects III.5 and III.7 had multiple mtDNA deletions on Southern blots of DNA isolated from skeletal muscle biopsies. Open symbols indicate healthy subjects. A plus sign (+) denotes subjects from whom DNA samples were available for genetic analysis. The proband is indicated by an arrow.
at least four loci in the nuclear genome predispose to somatic mutations of mtDNA with accumulation of multiple mtDNA deletions. To localise the fourth disease locus, we will perform a random genome search in this Belgian family.

The different genes involved in autosomal dominant PE0 may encode different functional polypeptide subunits of a single multienzyme complex or may be independently functioning molecules. The gene products may play a role in one single or even in several pathogenetic mechanisms resulting in this complex human disease. Indeed, there are several hypotheses of different pathogenetic mechanisms such as defects in mtDNA replication, defects in repair of damaged mtDNA, or in effective elimination of free oxygen radicals and secondary mtDNA damage. Cloning of one of the ADPEO genes may improve our understanding of how the nuclear and the mitochondrial genome interact. In mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), an autosomal recessive disease, all associated with multiple deletions of skeletal muscle mtDNA deletions, functional mutations in the thymidine phosphorylase gene were recently reported. A similar demonstration of molecular defects in autosomal dominant PE0 genes might provide a rational basis for treatment or prevention of these disorders.

This work was supported in part by a grant of the Fund for Scientific Research-Flanders (PWO-6.3009.94.N).

The di®erent functional polypeptide subunits of a single multienzyme complex or may be independently functioning molecules. The gene products may play a role in one single or even in several pathogenetic mechanisms resulting in this complex human disease. Indeed, there are several hypotheses of different pathogenetic mechanisms such as defects in mtDNA replication, defects in repair of damaged mtDNA, or in effective elimination of free oxygen radicals and secondary mtDNA damage. Cloning of one of the ADPEO genes may improve our understanding of how the nuclear and the mitochondrial genome interact. In mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), an autosomal recessive disease, all associated with multiple deletions of skeletal muscle mtDNA deletions, functional mutations in the thymidine phosphorylase gene were recently reported. A similar demonstration of molecular defects in autosomal dominant PE0 genes might provide a rational basis for treatment or prevention of these disorders.
stones were normal. At 1 year he was able to sit alone and spoke his first words. There were no health problems. His joint anomalies were noticed at birth.

We examined the patient at the ages of 6 months and 1 year. At 1 year weight was 12 kg (>90th centile), height 80.6 cm (>97th centile), and OFC 47 cm (>50th centile). In the “anatomical position”, the elbow joints were slightly flexed and active extension from that position was not possible. Pronosupination was slightly limited. The wrist joints were radially deviated. Flexion, extension, abduction, and adduction at the wrists were somewhat stiff but almost normal in range. The fingers were short and bilateral camptodactyly of the fifth finger was present.

X rays of the arms, forearms, and hands showed enlarged and poorly modelled distal humeral, proximal radial, and proximal ulnar metaphyses, abnormally long radius and ulna, a very large triquetrum the size of that of a 3 1/2 year old child, and shortness of all the components of the digital rays, but especially marked in the distal phalanges (fig 2). No synostoses were noted. X rays of the whole skeleton showed no other relevant anomalies.

Patient III.4 was born at term. Pregnancy and delivery were uneventful. Birth weight was 3400 g, length 45 cm, and head circumference 35 cm. Development milestones were normal. He was able to sit alone at 9 months and to walk at 12 months. He spoke his first words at 12 months and the first sentences at 2 years. He had no health problems. His joint anomalies were noticed at birth.

We examined the patient at the ages of 2 1/2 and 3 years. At 3 years weight was 18.5 kg (>97th centile), height was 106 cm (>97th centile), and OFC was 49.6 (>25th centile). Flexion at the elbow joints, radial deviation of the wrists, and limitation in the range of movement at the elbow and wrist joints were slightly more marked than in his younger brother. Brachydactyly, bilateral camptodactyly of the fifth fingers and partial bilateral syndactyly between the second and third fingers were evident. The x ray features were very similar to those of III.5. The size of the triquetrum was that of an 8 year old child (fig 3). His symptoms improved after one year of physiotherapy.

The mother, II.2, was 33 years old at the time of examination. Weight was 60 kg (>50th centile), height was 165 cm (>50th centile), and OFC was 53 cm (>25th centile). She denied any health problems and was of normal intelligence. Her joint anomalies had been present since birth and she had not noticed any worsening of her symptoms. She had never received any rehabilitation therapy.

Figure 1 Pedigree of the patients. II.2 is 33 years old, III.4 is 3 years old, and III.5 is 1 year old.

Figure 2 X ray of the hand and forearm of III.5 at 6 months showing enlarged and poorly modelled bony components of the elbow joint, a very large triquetrum (arrow) of the size of that of a 3 1/2 year old child, and brachydactyly.

Figure 3 X ray of the hand and forearm of III.4 at 2 1/2 years showing enlarged and poorly modelled bony components of the elbow joint, a very large triquetrum (arrow) of the size of an 8 year old child, and brachydactyly.
In the anatomical position, flexion at the elbow joints was $10^\circ$ and active extension from that position was impossible (fig 4). Pronosupination was slightly limited. The wrist joints were radially deviated. At the wrist, flexion was limited to about $60^\circ$ while extension, abduction, and adduction were almost normal. The hands showed brachydactyly and bilateral camptodactyly of the fifth finger (fig 5).

X-rays of the arms, forearms, and hands showed enlarged and poorly modelled distal humeral, proximal radial, and proximal ulnar metaphyses, abnormally shaped carpal bones, scaphoid-lunate fusion, large triquetrum, and shortness of all the components of the digital rays, especially marked at the distal phalanges (figs 6 and 7). No synostoses were present. X-rays of the whole skeleton showed no other relevant anomalies.

The unusual association of brachydactyly, abnormally shaped carpal bones, and elbow dysplasia in our family and
that described by Liebenberg in 1973 could be ascribed to the same autosomal dominant clinical condition. Our patients showed only some slight differences from Liebenberg’s family.

In our family, the bony components of the elbow joints were consistently enlarged and poorly modelled. In Liebenberg’s patients the radial head had a “bulbous appearance” while the humeral condyles and the ulnar olecranon and coronoid processes were “underdeveloped”. The large triquetrum and camptodactyly of the fifth fingers, which were described only in the 7 year old child of the family of Liebenberg, were constant findings in our patients. Moreover, patient III.4 had bilateral partial syndactyly between the second and third fingers. Triquetro-pisiform fusion, which was present in all Liebenberg’s patients, was not found in our family. However, since the ossification centre of the pisiform appears at about 10 years, triquetro-pisiform fusion would not be expected to be seen in the children III.4 and III.5. Yet, in the mother, II.2, the fusion is between the scaphoid and lunate.

The differential diagnosis between Liebenberg syndrome and the other skeletal disorders involving elbow dysplasia/carpal synostoses and brachydactyly, such as the multiple synostoses or facioaudiosymphalangism syndrome (MIM 186500), the tarsal, carpal, and digital synostoses syndrome (MIM 186400), and the Banki syndrome (MIM 109300) is essential for prognosis and genetic counselling. Indeed, Liebenberg syndrome seems to cause no other relevant problem apart from limited flexion-extension of the elbows and wrists while the evolution of the other disorders is often more severe.

The presence of tarsal synostoses and symphalangism clearly differentiates both the multiple synostoses syndrome (SYNS1) (MIM 186500) and the tarsal, carpal, and digital synostoses syndrome (MIM 186400) from Liebenberg syndrome which features only carpal synostosis. Moreover, most SYNS1 patients have conductive deafness and a characteristic facies with a broad hemicylindrical appearance while the humeral condyles and the ulnar olecranon were “underdeveloped”. Liebenberg’s patients the radial head had a “bulbous appearance” while the humeral condyles and the ulnar olecranon and coronoid processes were “underdeveloped”.

The recent discovery that point mutations in the NOG gene in the 17q22 region are the cause of both proximal symphalangism (SYM1) and SYNS1 raises the question of whether Liebenberg syndrome is also caused by mutations in the same gene. The NOG gene codes for the polypeptide noggin that acts as an inhibitor of bone morphogenetic proteins (BMP), which are members of the transforming growth factor β superfamily of signalling proteins (TGF-β-FM). Thus, mutations in the NOG gene are likely to cause an increased BMP activity which leads to excess cartilage and failure of joint formation. Considering the wide variability of the phenotypic effects of NOG gene mutations, which range from simple symphalangism to the full SYNS1 syndrome, it would not be surprising if Liebenberg syndrome and some of the other syndromes featuring synostoses were also caused by NOG gene mutations.

G TIBERIO* M C DIGILIO* M GRAZIANI† F TESTA‡ A GIANNOTTI*  
*Servizio di Genetica Medica, IRCCS “Bambino Gesù”, Piazza S Onofrio 4, 00165 Roma, Italy  
†Servizio di Radiologia, IRCCS “Bambino Gesù”, 00165 Roma, Italy  
‡Reparto di Ortopedia, IRCCS “Bambino Gesù”, 00165 Roma, Italy

Correspondence to: Dr Giannotti, giannott@ospbg.net


Prenatal detection of trisomy for the entire long arm of chromosome 7

EDITOR—Duplication of the long arm of chromosome 7 is extremely rare; most of the reported cases are partial trisomies. The first and only previous case of complete 7q duplication was reported in 1978 by Wahrman et al, who described a proband at 3 years of age with a phenotype including a large face with sloping forehead, downward slanting palpebral fissures, bilateral epicanthic folds, low set, malformed ears, micrognathia, hypertelorism, and skeletal abnormalities. The extent of the duplication and verification of breakpoints were determined using FISH probes.

The patient was delivered by caesarean section at 34 weeks’ gestation to a G1, P0, 15 year old female. She was first seen at 33 weeks’ gestation when a sonogram showed multiple congenital anomalies including severe hydrocephalus, a two vessel umbilical cord, bowed femur, clubbed feet, and dilated kidneys.

At birth, the infant was cyanotic with Apgar scores of 3 and 4 at one and five minutes, respectively. She weighed 3000 g and was 41 cm long. Head circumference was 44 cm at 34 weeks’ gestation is 31.5 cm). Multiple congenital anomalies were noted in addition to those seen on sonogram and included contractures of both hands and feet, missing digits from the left hand, contracted right hand, bilateral hip dislocation, low set, malformed ears, micrognathia, small mouth, high arched palate, hypertelorism, wide, beak-like bridge of the nose, increased intermammary distance, and pulmonary hypoplasia (fig 1). Mild to moderate tricuspid regurgitation was present. The infant was placed on a ventilator with 100% oxygen, but saturation did not improve. The infant died six hours after birth from respiratory failure.
At necropsy, additional congenital anomalies were noted, including a dilated, tortuous right ureter with kinking (fig 2). The lungs were markedly hypoplastic with dilated airways and apical blebs. The hydrocephalic brain contained about 500 ml of CSF in markedly dilated ventricles. The mesencephalic aqueduct was markedly stenotic.

Cytogenetic studies on percutaneous umbilical blood sampling (PUBS) obtained at 33 weeks' gestation and cultured fibroblasts obtained from the umbilical cord at birth showed a duplication of the entire long arm of chromosome 7. This was confirmed by a FISH probe for the elastin gene at 7q11.2 (Oncor). Three copies of the FISH probes for the elastin gene and markers at 7q36 were present in each metaphase analysed, indicating that the patient was trisomic for chromosome 7q.

Dual labelled centromere probes for 7 (Oncor) and 15 (Vysis) identified two centromeres each, with the 15 centromere on the derivative chromosome, confirming 7q and 15p breakpoints. The patient’s karyotype was 46,XX,der(15)t(7;15)(q11.2;p11.2) (fig 3). The mother’s karyotype was normal, 46,XX; however, no sample was available from the father for analysis. A fibroblast cell line established from umbilical cord on the patient is available through Coriell Cell Repositories (Camden, NJ).

Of all previously reported cases of trisomy 7q, there has been only one reported case involving duplication of the entire long arm of chromosome 7; we report the second case. Trisomy 7q produces abnormalities affecting virtually every system in the body including the central nervous system, the face, the musculoskeletal system, the heart, and the genitourinary system, but the combination of specific malformations may vary among patients. Our case showed a number of features similar to the previously reported case of duplication of the whole of 7q by Wahrman et al, including frontal bossing, hypertelorism, low set, malformed ears, short neck, downward slanting palpebral fissures, and skeletal and kidney abnormalities. The features in our case not reported by Wahrman et al include hydrocephalus, bilateral clubbing...
Cockayne syndrome associated with low CSF 5-hydroxyindole acetic acid levels

EDITOR—Cockayne syndrome is a rare, clinically heterogeneous disorder, characterised by severe growth failure, cognitive impairment, characteristic facies, and photosensitivity. In the older patient the face has a characteristic aged appearance with sunken orbits, a relatively large, “beak-like” nose, and narrow mouth and chin. Both the central and peripheral nervous systems are involved in this neurodegenerative disorder with pigmented retinopathy, delayed nerve conduction velocities, sensorineural hearing loss, progressive spasticity, and cerebellar involvement with dysarthria, tremor, and ataxia. The cerebral histopathological changes most commonly seen are patchy demyelination of the subcortical white matter and microscopic calcifications throughout the central nervous system. Calcification of the basal ganglia may be visible on CT scan. The diagnosis of Cockayne syndrome is made on clinical grounds in association with the failure of RNA synthesis in cultured fibroblasts or lymphoblastoid cells to recover to normal rates after UV-C irradiation. Inheritance is presumed to be autosomal recessive. Here we report a patient with Cockayne syndrome in whom cerebrospinal fluid 5-hydroxyindole acetic acid was markedly reduced. To date there are no reports of abnormalities of the cerebrospinal fluid neurotransmitters in association with Cockayne syndrome. This finding may provide insight into the pathogenesis of the central nervous system abnormalities. Furthermore we have described the

### Table 1 Clinical characteristics of duplication 7q and trisomy 7

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>q71.2 qter</th>
<th>q71.2 qter</th>
<th>q71.2 qter</th>
<th>q71.2 qter</th>
<th>q72.2 qter</th>
<th>q72.2 qter</th>
<th>q72.2 qter</th>
<th>q72.2 qter</th>
<th>q73.1 qter</th>
<th>q73.1 qter</th>
<th>q73.1 qter</th>
<th>q73.1 qter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Developmental retardation</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hypertelorism</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Epicanthus</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Strabismus</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Low set ears</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Small palpebral fissures</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Frontal bossing</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hypotonia</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Low birth weight</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Wide open fontanelle</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Small nose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cleft palate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Microcephalopathy</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Skeletal abnormalities</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Neonatal death</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Kyphoscoliosis</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Large head</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>High arched palate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Short neck</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Widely spaced nipples</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Kidney abnormalities</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lung hypoplasia</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cardiac defect</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

We wish to thank Mrs Cindy Cuteness for her technical expertise.

Correspondence to: Dr Wenger, swenger@hsc.wvu.edu

References

patient’s body composition in terms of resting energy expenditure, total body protein, fat, and bone mineral density which are relevant to management of the progressive cachexia associated with this disorder.

The proband, a 16 year old male, is the third child of consanguineous Sri Lankan parents and was born at term with a birth weight of 2722 g. His early expressive speech development was delayed, but gross and fine motor development were within normal limits. He initially presented at 8 years of age with tremor and poor motor coordination. A cerebral CT scan at that time was normal. He was re-evaluated at the age of 14 years. At this time he had an obvious intention tremor, gait ataxia, mild cognitive impairment, bilateral high tone sensorineural hearing loss, and poor growth. His parents reported extreme sun sensitivity, dry skin, and poor sweating in hot conditions.

On physical examination at 14 years, the proband had slightly sunken eyes, thin hair, and dry skin. His speech was slow and mildly dysarthric. His height, 136 cm, and weight, 32 kg, were both below the 3rd centile (50th centile for 9 years) and head circumference, 52.5 cm, was on the 2nd centile (50th centile for 8 years). He had normal secondary sexual characteristics. Neurological examination showed cerebellar dysfunction with a marked, coarse, irregular intention tremor with overshooting, slow fine finger movements, and heel-shin incoordination. His gait was broad based and unsteady with flexed posture and toe walking. Dystonic posturing of his left arm and fingers were noted intermittently. There was minimal involuntary movement at rest. Muscle tone and power were within normal limits. Deep tendon reflexes were brisk, but his plantar reflexes were downward. There was no clinical evidence of peripheral neuropathy. Examination of his cranial nerves showed saccadic hypermetria, but no nystagmus. The pupils were poorly reactive to light and he was unable to converge. On neuropsychological testing there were specific deficits in
short term memory, abstract reasoning, skills of generativ-ity, and mental flexibility.

A repeat CT scan at 14 years of age showed generalised cerebral atrophy and calcification of the globus pallidus, which on MRI scan presented as decreased signal intensity of the globus pallidus (fig 1). In addition, there was atrophy of the cerebellum, temporal lobe structures, hippocampal formation, and brain stem. The pattern of myelination approximated that of a 12 month old child. Nerve conduction studies showed absent sensory action potentials and moderately slowed motor conduction (29 m/sec at the right arm). The hearing and visual acuity approximated that of a 12 month old child. Nerve conduction velocity measurement of sural nerve biopsy. The electrocardiogram showed a sinus rhythm with a heart rate of 70/minute. The ECG tracing showed a left bundle branch block pattern.

Cerebrospinal fluid amino acid levels including tryptophan were normal. On analysis of cerebrospinal fluid neurotransmitter levels, urinary 5-hydroxyindole acetic acid was found to be low (0.02 and 0.03 µmol/l). Cerebrospinal fluid and plasma lactates were normal. On analysis of cerebrospinal fluid neurotransmitter levels, urinary 5-hydroxyindole acetic acid was found to be low (0.02 and 0.03 µmol/l).

The clinical diagnosis of Cockayne syndrome was confirmed by survival studies of Epstein-Barr virus transformed B cells after irradiation with UVB (fig 3). These studies show that the proband’s cells were very sensitive to irradiation, with only 1% of cells surviving a dose of 100 J/sq m UVB. In comparison, the normal controls’ cells did not approach 1% survival until irradiated with doses of 250 J/sq m and over. To confirm the Cockayne phenotype further, the rate of RNA polymerase II transcriptions in the lymphoblastoid cell was compared with control lymphoblastoid cell. The constitutive incorporation of 14C-uridine over 90 minutes was found to be 64 ± 2% (n=4) of the control lymphoblastoid cells (after normalisation against thymidine incorporation). This is within the 40-70% range for Cockayne syndrome lines reported by Balaje et al., who also found that xeroderma pigmentosa cells had the same rate of RNA synthesis as controls.

Because of progressive weight loss, the proband was referred for nutritional assessment. The resting energy expenditure measured by indirect calorimetry was 75% of the predicted value, 3856 kJ/24 h, with a predicted resting energy expenditure based on age, sex, height, and weight for healthy children of 5135 kJ/24 h. The respiratory quotient was 0.89, indicative of appropriate nutritional intake.

Table 1 Clinical features previously reported in Cockayne syndrome and those seen in our patient

<table>
<thead>
<tr>
<th>Clinical feature</th>
<th>Reported in Cockayne syndrome*</th>
<th>Present in this patient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of onset</td>
<td>3–5 y</td>
<td>8 y</td>
</tr>
<tr>
<td>Psychomotor development</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Delayed</td>
<td>50/131 (38)</td>
<td>+</td>
</tr>
<tr>
<td>Central nervous system</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Movement disorder</td>
<td>4/131 (6)</td>
<td>+</td>
</tr>
<tr>
<td>Tremor</td>
<td>42/131 (32)</td>
<td>+</td>
</tr>
<tr>
<td>Speech abnormality</td>
<td>28/131 (21)</td>
<td>+</td>
</tr>
<tr>
<td>Gait abnormality</td>
<td>35/131 (27)</td>
<td>+</td>
</tr>
<tr>
<td>Basal ganglia calcification</td>
<td>25/27 (92)</td>
<td>+</td>
</tr>
<tr>
<td>Ophthalmological</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pigmentary retinopathy</td>
<td>70/128 (55)</td>
<td>+</td>
</tr>
<tr>
<td>Cataracts</td>
<td>13/24 (54)</td>
<td>+</td>
</tr>
<tr>
<td>Optic atrophy/hypoplasia</td>
<td>46/128 (36)</td>
<td>–</td>
</tr>
<tr>
<td>Nystagmus</td>
<td>9/23 (22)</td>
<td>–</td>
</tr>
<tr>
<td>Inability to converge</td>
<td>43/128 (34)</td>
<td>–</td>
</tr>
<tr>
<td>Hearing loss</td>
<td>27/128 (21)</td>
<td>–</td>
</tr>
<tr>
<td>Dental caries</td>
<td>16/27 (59)</td>
<td>–</td>
</tr>
<tr>
<td>Skin photosensitivity</td>
<td>9/14 (64)</td>
<td>–</td>
</tr>
<tr>
<td>Growth failure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biochemical investigations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Increased cerebrospinal fluid protein</td>
<td>19/30 (63)</td>
<td>+</td>
</tr>
<tr>
<td>Decreased cerebrospinal fluid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-hydroxyindole acetic acid</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Number reported and percentage in brackets with reference.
Total body protein measured by neutron capture analysis was low for age (65%) but above predicted for weight (112%), height (112%), and lean body mass (108%). Total body bone mineral density measured by dual energy x ray absorptiometry was on the 50th centile for age, 1.141 g/cm². Total body fat tissue measured by dual energy x ray absorptiometry was 31.2%.

Our patient’s clinical features are consistent with the diagnosis of Cockayne syndrome (table 1). Diagnostic criteria include growth failure after normal or slightly low birth weight, neurological dysfunction with cerebral white matter involvement plus at least three of the following features: cutaneous photosensitivity, progressive pigmentary retinopathy/cataracts, optic disc atrophy, miotic pupils or decreased lacrimation, sensorineural hearing loss, dental caries, short stature, and a characteristic physical appearance of “cachectic dwarfism”. Atypical features in our patient include later age of onset of neurological signs, absence of dental caries, and only mild cognitive impairment. The diagnosis of Cockayne syndrome was supported by finding hypersensitivity to UV light in Epstein-Barr virus transformed lymphoblasts. The pathogenesis of Cockayne syndrome is poorly understood. The cells of patients with Cockayne syndrome are hypersensitive to the lethal effects of UV light because they have a defective subpathway of nucleotide excision repair known as “transcription coupled repair”. Cockayne syndrome is genetically heterogeneous. Two disease genes have been identified, CSB on chromosome 10q11 and the CSA gene maps to chromosome 5. Both proteins play an essential role in preferential repair of transcription blocking lesions from active genes. The CSB protein is a member of the SW12/SNF2 family of ATPases whose function is thought to involve remodelling of protein-DNA interactions, such as chromatin structure in different situations. The CSA gene encodes a “WD repeat” protein.

A unique finding in our patient was very low cerebrospinal fluid 5-hydroxyindole acetic acid and normal homovanillic acid levels on three separate occasions. The peripheral serotonin levels were normal. The metabolic pathways of 5-hydroxyindole acetic acid and homovanillic acid are shown in fig 4. The concentrations of 5-hydroxyindole acetic acid and homovanillic acid in the cerebrospinal fluid are thought accurately to reflect the turnover of catecholamine neurotransmitters. The low 5-hydroxyindole acetic acid, in association with normal tryptophan and homovanillic acid in the cerebrospinal fluid and normal peripheral serotonin, is suggestive of low brain serotonin turnover with normal dopamine turnover and may suggest a primary central serotonin deficiency, a selective serotoninergic tract degeneration, or an isolated central nervous system tryptophan hydroxylase deficiency. This finding in our patient may provide some insight into the mechanism of the neurological complications and the initiation of Cockayne syndrome given the putative roles of serotonin in control of movement and appetite. We treated our patient with 5-hydroxytryptophan (5 mg/kg/day) in an attempt to increase his cerebrospinal fluid 5-hydroxyindole acetic acid levels, with no change in levels observed. However, from a clinical point of view, he has had no further deterioration in cognitive or neurological function.

The cause of death in the majority of cases of Cockayne syndrome has been secondary to inanition and thus attention to nutrition is essential to patient care and well being. Although our patient was always small for his age, he recently showed accelerated weight loss. In the setting of premature aging we predicted an increased metabolic rate, but, surprisingly, detailed energy expenditure studies indicated a lowered metabolic rate. This is similar in subjects with anorexia nervosa, whose resting energy expenditure is reduced when compared to healthy, aged matched controls. With re-feeding, however, the resting energy expenditure in anorexia nervosa subjects normalises, with concomitant increases in body weight. It would therefore appear possible to manage Cockayne syndrome patients with programmes that correct the energy deficits, documented by energy expenditure studies. Whether such strategies will prevent deterioration in nutritional status remains to be shown.

We would like to thank Dr P McVeagh, Department of Paediatrics, Royal Alexandra Hospital for Children, for referring this patient to the Neurogenetic Service. We would also like to thank Ms Jane Allen, Department of Nutrition and Dietetics, Royal Alexandra Hospital for Children, for performing the energy expenditure studies, Mr Nick Hayward for transforming our patient’s B cells, and Dr Adam Steinberg, Department of Radiology, Royal Alexandra Hospital for Children for interpreting the MRI findings.

Correspondence to: Dr North, Department of Paediatric and Child Health, Royal Alexandra Hospital for Children, PO Box 3515, Parramatta 2124, NSW, Australia, KathleenN@nch.edu.au
Unilateral lobar pulmonary agenesis in sibs

EDITOR—Pulmonary agenesis is an extremely rare congenital malformation which has been classified morphologically by the extent to which bronchopulmonary tissue is absent. Spencer1 divided pulmonary agenesis into (1) complete bilateral agenesis, (2) unilateral agenesis with (a) complete absence of bronchi, (b) rudimentary bronchus present but no pulmonary tissue, or (c) poorly developed main bronchi with poorly organised parenchyma, and (3) lobar agenesis. The incidence of this rare condition is not known. According to the calculations of Mardini and Nyhan2 it may range between 0.0034 and 0.0097%. Familiality is known. According to the calculations of Mardini and Nyhan2 it may range between 0.0034 and 0.0097%. Familiality is known. According to the calculations of Mardini and Nyhan2 it may range between 0.0034 and 0.0097%. Familiality is known. According to the calculations of Mardini and Nyhan2 it may range between 0.0034 and 0.0097%. Familiality is known.

The paucity of such observations prompts us to report another instance of pulmonary agenesis in a brother and a sister of healthy and non-consanguineous parents. The family history was unremarkable and both pregnancies were uneventful. The mother had no infections and denied any abuse of teratogenic substances. Chromosomes were not studied in either case.

The first child, a female, was delivered spontaneously at term with a birth weight of 3500 g. Birth length and head circumference were not recorded. The mother was 27 years old and the father 36 years old. At the age of 11 months she was admitted to hospital because of stridor and dyspnoea. The interpretation of the chest x ray was spontaneous pneumothorax on the left side with dextrocardia and compression atelectasis of the right upper lobe. Over the following years, she suffered from asthmatic bronchitis secondary to recurrent infections. Pulmonotomotor development was within the normal range. Pulmonary re-evaluation at the age of 12 years, including bronchoscopy, showed a suspicion of partial agenesis of the right lung. At the age of 24 years her bronchial asthma deteriorated. Ten years later she was admitted to hospital because of decompensated cor pulmonale. Since then she has had repeated episodes of acute cardiopulmonary decompensation. She died at the age of 42 years following a further episode of decompensated cor pulmonale. Necropsy disclosed dextrocardia, agenesis of the middle lobe, aplasia of the upper lobe, and hypoplasia of the lower lobe of the right lung with hypoplasia of the right pulmonary artery.

The younger brother was born at term six years after his sister. Birth weight was 2980 g and length 48 cm. From early on, tachypnoea, dyspnoea, and feeding difficulties were noticed. Physical examination showed diminished breath sounds on the right upper side with the cardiac impulse shifted to the right side and a systolic heart murmur. Chest x ray (fig 1) showed no heart on the left side but a hypertensive right lung with prominent vasculature. The carina projected at the level of T6 and the right main stem bronchus seemed sharply cut off at its lower end. The right side was reduced in volume and seemed almost completely opacified except for a median lucency from herniated left lung. No skeletal abnormalities were visible. At the age of 7 months, he was treated for right sided pneumonia. Two months later he was admitted to hospital because of severe asthmatic bronchitis. There was a rapid deterioration leading to respiratory arrest. Necropsy disclosed agenesis of the right upper lobe, partial atelectasis of the remaining right lung, severe hypoplasia of the right pulmonary artery, dextrocardia, hypertrophy of the right atrium and ventricle, a patent foramen ovale, and a defect in the upper part of the ventricular septum. Furthermore, there was bilateral meatal ureteral stenosis with hydroureters and hydronephrosis, a small accessory spleen, and mild hydrocephalus internus.

In the review on agenesis of the lung by Gilbert and Opitz,4 approximately 200 patients with unilateral involvement were found. In 70% of cases the left side was involved and males predominated over females. Associated malformations were present in 50% of the cases. Major anomalies included congenital heart defects, spinal anomalies, anal atresia, tracheo-oesophageal atresia, spleen anomalies, diaphragmatic hernia, renal anomalies, cleft palate, and limb and facial defects.5 Cunningham and Mann6 found that ipsilateral anomalies of the first and second branchial arch or radial ray defects or both are the most common overt malformations. In contrast to bilateral agenesis of the lung, unilateral absence is compatible with life, but has a high mortality, most likely because of the presence of associated malformations/infections of the remaining lung tissue. Infants with right sided lung agenesis have a higher mortality and die significantly earlier. This is probably related to a more severe mediastinal and cardiac displacement into the right chest.7 Survival to adult age is possible.
as shown in our patient 1 or in a case with agenesis of the left lung detected at necropsy in a 72 year old woman.²

Most cases present in the neonatal period with non-specific respiratory symptoms such as cyanosis, tachypnoea, dyspnoea, stridor, or feeding difficulties. Physical examination may be normal, but can show diminished breath sounds on the affected side with the heart and mediastinum shifted to the ipsilateral side. Chest x rays which show a homogeneous density in onehemithorax suggest the diagnosis, but endoscopic, bronchographic, and echocardiographic studies, as well as computerised tomography are necessary for confirmation.

The aetiology of lung agenesis remains unknown. According to the possible associated anomalies, this rare malformation has been discussed as part of the VACTERL association,¹⁰ and Goldenhar syndrome.¹¹ Warkany et al.¹² induced pulmonary agenesis of one lung, but also other major malformations of the eyes and cardiovascular and urogenital systems by maternal gestational vitamin A deficiency in rats. Chromosomes were not studied in the older reports. However, Say et al.¹³ described a patient with agenesis of the left lung who had a duplication of the region 2p21→p24 resulting in a partial trisomy 2p and Schober et al.¹⁴ reported a case with left pulmonary agenesis and associated malformations in partial trisomy 2p (p21→pter) and 21q (pter→q21) owing to an unbalanced segregation of a maternal reciprocal translocation t(2;21). The minimal overlapping region of duplication includes the segment 2p21→p24. Therefore, Say and Carpenter¹⁵ suggested studying patients with pulmonary agenesis for the presence of such a duplication. Unilateral agenesis has also been documented in trisomy 18¹⁶ and in trisomy of a C group chromosome.¹⁷

Pulmonary agenesis in twins was reviewed by Lehmann.¹⁸ In female monozygotic twins, one sister had agenesis of the left and the other agenesis of the right lung.¹⁹ Yount²⁰ documented agenesis of the right lung in concordantly affected monozygotic twins, while others found pulmonary agenesis in only one of monozygotic twins.²¹ Concordant occurrence of congenital malformations in monozygotic twins does not necessarily imply a genetic cause and vice versa.²²

The only clear indication of possible autosomal recessive inheritance of unilateral pulmonary agenesis so far was the family reported by Brimblecombe.¹ The healthy parents were first cousins and had two normal children and two affected girls with agenesis of the right upper and middle lobes. Our report is the second case of unilateral lobar pulmonary agenesis in sibs and supports the hypothesis of an autosomal recessively inherited disease. Alternatively, our observation could be explained by an extrinsic origin (infection during pregnancy, drugs, environmental substances, etc), for which, however, we do not have the slightest proof. Further support for the presence of a rare autosomal recessive gene for unilateral pulmonary agenesis comes from the observation of Mardini and Nyhan,² who documented parental consanguinity in four patients; two patients had right upper and middle lobe agenesis and two presented with left sided pulmonary agenesis. All had associated congenital heart defects and three infants presented with abnormalities of the thumb. Podlech et al.²³, who observed a family with one healthy and two affected children with bilateral agenesis of the lung, concluded that an autosomal recessive mode of inheritance could also be valid for bilateral pulmonary agenesis. Embryogenesis of human lung development is poorly understood, but in mice Fgf10 (fibroblast growth factor 10) is an important regulator of limb and lung formation.²⁴ Homozygous Fgf10 deficient mice die at birth owing to lack of lung development.

In conclusion, our report suggests an autosomal recessive gene or genes which cause pulmonary agenesis with optional heart, kidney, and other defects. In such cases, a 25% recurrence risk should be considered in genetic counselling.

We are grateful to Dr U Willi for interpretation of the chest x ray.

SIV FOKSTUEN

ALBERT SCHINZEL

Institut für Medizinische Genetik, Universität Zürich, Rämistrasse 74, 8001 Zürich, Switzerland

Correspondence to: Professor Schinzel, schinzel@medgen.unizh.ch

Correction

In the paper by Carroll et al in the February 1999 issue of the journal (J Med Genet 1999;36:94-6), the intronic polymorphism in PTEN was reported as IVS4+109InsACTAA. The correct sequence should be IVS4+109InsATCTT. The authors would like to apologise for any confusion caused by this error.