LETTERS TO JMG

Association between markers in chromosomal region 17q23 and young onset hypertension: a TDT study

S-Y Wu, C S J Fann, Y-S Jou, J-W Chen, W-H Pan

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Hypertension is one of the most important risk factors for cardiovascular diseases. Despite extensive research examining the causes of blood pressure variation, a significant proportion of blood pressure variation is yet to be explained. Studies of families and twins suggest that 20-40% of blood pressure variation can be attributed to genetic factors.[1] Evidence shows that the genetic contribution is even greater for young onset hypertension.[2] We feel that genetic approaches focusing on young onset hypertension will provide new insight into the pathogenesis of hypertension.

In our previous report, the affected sib pairs (25 independent, affected sib pairs) method showed positive signs of linkage for markers of the atrial natriuretic peptide gene (*NPPA*) (D1S1612, p=0.0162), angiotensinogen gene (*AGT*) (D1S547, p=0.0263), lipoprotein lipase gene (*LPL*) (D8S1145, p=0.0284), and angiotensin converting enzyme gene *(DCP1)* (D17S2193, p=0.0256),[3] indicating that multiple pathogenic pathways may be involved in the aetiology of young onset hypertension. Owing to this aetiological complexity, in the current study we focus on high resolution mapping of *AGT* (located on 1q42-43) and *DCP1* (located on 17q23), genes of the renin angiotensin system (RAS). Renin catalyses the first step of the activation pathway of angiotensinogen to angiotensin I, which is then cleaved to angiotensin II by angiotensin I converting enzyme. This cascade can lead to aldosterone release, vasoconstriction, and increased blood pressure. Although the RAS has been extensively studied, it remains unclear how and to what extent RAS gene variants contribute to the blood pressure variations in various human populations.

MATERIALS AND METHODS

We have recruited 59 nuclear families (a total of 214 subjects) from a hypertension clinic at Taipei Veterans General Hospital, Taiwan. Our study group included 81 young onset hypertensive patients (59 probands and 22 affected sibs, mean age 30.4 (SD 0.95)), 39 normotensive sibs (mean age 32.2 (SD 1.6)), and 94 parents. Our previous study included 25 affected sib pairs from 18 families for affected sib pair analysis. This transmission disequilibrium test (TDT) study used information from all 59 families with probands. Therefore, the former is a subset of the latter. The protocol of this study was approved by the Human Investigation Committee of the Institute of Biomedical Sciences, Academia Sinica.

Polymorphic microsatellite markers located on 1q42-43 and 17q23 were selected based on GeneMap'99 and comprehensive human genetic maps from the Marshfield Medical Research Foundation, and obtained from Multi-Colored Fluorescent Human MapPairs Markers of Research Genetics (Huntsville, AL). Nine markers on 1q42-43 were selected: D1S2805 (245.05 cM), D1S3462 (247.23 cM), D1S459 (247.23 cM), D1S1540 (252.12 cM), D1S235 (254.64 cM), D1S517 (262.96 cM), D1S1149 (262.96 cM), D1S1594 (265.49 cM), and D1S547 (267.51 cM). The six markers on 17q23 were D17S1297 (83.40 cM), D17S1295 (83.40 cM), D17S942 (85.94 cM), ATA108a05 (88.76 cM), D17S789 (89.32 cM), and D17S2193 (89.32 cM). The polymerase chain reaction protocol for microsatellite markers was performed as previously reported.[3] Fragment analysis was performed using an ABI 377 DNA sequencer and analysed by GeneScan version 3.0 and GenotypeR version 3.0. The allele calling was conducted independently by two readers and cross checked.

In addition to conventional TDT that only used data from heterozygous parents, an extended TDT (S-TDT and combined Z score) developed by Spielman *et al*[4] was also carried out. Information from 42 young hypertensive patients and both of their parents (representing 35 families) were used for conventional TDT. In the sibship transmission disequilibrium test (S-TDT), information from 25 hypertensive patients and 21 normotensive sibs from 17 families were used. Then a Z score was obtained by combining information from the conventional TDT and the S-TDT. For comparisons, we also carried out Horvath's SDT,[5] which uses an exact p value to test the difference between affected and unaffected sibs. In this test, only information from 44 young hypertensive patients and their normotensive sibs (39) were used. Since these markers were close to each other, we performed haplotype TDT using the "TRANSMIT" program.[6] In this analysis, data from all 59 families were used. Information from the 24 single parent families was incorporated into the analysis by use of expectation maximisation algorithms. Because of our limited sample size, only haplotypes created by two markers were included.

Because we tested 15 markers with multiple alleles, a Bonferroni procedure was carried out to adjust for multiple comparisons, as suggested by Spielman *et al*.[4]

RESULTS AND DISCUSSION

No association with any marker in the region of 1q42-43 was found (data not shown). The *AGT* was the first gene linked to hypertension,[7] but results showing linkage of *AGT* and hypertension has not been consistent across various populations. A

Abbreviations: TDT, transmission disequilibrium test; S-TDT, sibship transmission disequilibrium test; RAS, renin angiotensin system

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Table 1 Results of Spielman's S-TDT and Horvath's SDT

	Allele	TDT χ^2	S-TDT Z score	Combined Z score	p value with Bonferroni adjustment*
Spielman's TDT and S-TDT marker					
ATA108a05†	3	23.684	3.009	5.783	$<10^{-5}$
D17S789‡	4	3.920	2.419	3.135	0.015
Horvath's SDT					
ATA108a05†	3				0.027
D17S789‡	4				0.197

*Adjustment includes multiple markers and alleles.
†Allele 3=160 bp.
‡Allele 4=159 bp.

Table 2 Results of haplotype TDT using "TRANSMIT" program

Haplotype	D17S942†	ATA108a05‡	D17S789§	Obs	Exp	χ^2
2 markers	2	3		18.96	12.47	9.10*
	3	3		2.00	1.54	0.28
	4	3		20.04	13.48	6.70*
		3	4	32.92	21.07	16.20*
		3	5	14.08	8.38	8.80*
		3	6	0.00	0.52	1.10

D17S942 (85.90 cM) - ATA108a05 (88.76 cM) - D17S789 (89.32 cM).
*p<0.05 (p value with Bonferroni adjustment).
†Allele 2=168 bp, 3=170 bp, 4=172 bp.
‡Allele 3=160 bp.
§Allele 4=159 bp, 5=161 bp, 6=163 bp.
Obs: observed. Exp: expected.

meta-analysis concluded that *AGT* contributes significantly but moderately to human blood pressure variance.[8] Our result is consistent with the negative findings of a study carried out in central China.[9] However, a case-control study carried out by Chiang *et al*[10] in Taiwan showed a positive association between the M235T polymorphism and hypertension in adults aged 60 and above.[10] These divergent results may be the result of a different definition of hypertension and different research designs. Further studies are required to examine the role of the *AGT* gene in the pathogenesis of hypertension in Chinese as well as in other ethnic groups.

Association between allele 3 of ATA108a05 and young onset hypertension was shown by Spielman's ($p<10^{-5}$) and Horvath's (p=0.027) TDTs (table 1). A weaker but similar association was observed between allele 4 of D17S789 and young onset hypertension using Spielman's (p=0.015) but not Horvath's method (p=0.197) (table 1). Because allele 3 of ATA108a05 shows strong association by both the Spielman and Horvath tests, our haplotype analysis was carried out focusing on this particular allele. In the two marker haplotype TDT analysis, four out of six estimated haplotypes that contain allele 3 of ATA108a05 and markers proximal or distal to it showed significant association after Bonferroni's correction (table 2). The insignificant associations were with the lower frequency alleles: allele 3 (4.4%) of D17S942 and allele 6 (1.6%) of D17S789. The results in tables 1 and 2 suggest that allele 3 of ATA108a05 warrants further investigation.

The insertion or deletion of a 287 bp Alu repeat element in intron 16 of the *DCP1* gene has also been associated with coronary artery disease,[11] but direct linkage between hypertension and *DCP1* is preliminary.[12] Many studies have shown that insertion/deletion (I/D) polymorphism of *DCP1* is associated with hypertension,[13 14] but none has shown linkage between hypertension and I/D polymorphism and other markers of *DCP1*. Two studies, using the quantitative trait locus approach, showed linkage between I/D polymorphism and blood pressure in a sex and age specific manner.[14 15] A recent genome scan quantitative locus approach study by Levy *et al*[16] found a linkage between systolic blood pressure and two peaks located on chromosome 17 at 67 cM and at 94 cM, and a linkage between diastolic blood pressure and a peak located on chromosome 17 at 74 cM. The *DCP1* gene is located in the region from 85 cM to 90 cM. In our study, the region from 85.9 cM to 89.32 cM was strongly associated with hypertension. Our findings suggest that the marker (as close as 3 cM distal to *DCP1*) may be linked to young onset hypertension in Han Chinese.

Further studies are required to examine whether variation in *DCP1* may contribute to young onset hypertension, considering that there are many functional genes and expressed sequence tags (ESTs) in the region near the *DCP1* locus. Examples include growth hormone, voltage gated sodium channel type IV (*SCN4A*), and regulator of G protein signalling 9 (*RGS9*). Ascertainment of more probands with young onset hypertension in many clinics is in progress to replicate our finding.

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Paternal contribution to the risk for pre-eclampsia

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Pre-eclampsia is a major cause of fetal and maternal morbidity and mortality with a still obscure aetiology. A major feature in pre-eclampsia is placental maladaptation, probably because of inadequate invasion of fetal trophoblast cells in the myometrium and spiral arteries that might be related to local oxidative stress. Reactive oxygen species (ROS), lipid peroxides, and other toxic compounds are metabolised by biotransformation enzymes in scavenging and detoxifying processes. Increasing evidence suggests an important function of antioxidants and detoxification enzymes in pre-eclampsia. We recently proposed that the 105Ile→Val polymorphisms in the glutathione S-transferase P1 gene (*GSTP1*), associated with lower enzyme detoxification capacity, enhanced maternal susceptibility to pre-eclampsia.[1] Glutathione S-transferase P1-1 (GSTP1-1) is an important detoxification enzyme and is the main GST isoform in placenta and decidua.[2] The GSTP1-1 level was found to be lower in placental and decidual tissue of pre-eclamptic women as compared with corresponding tissues of normal pregnant women.[2] Since placenta is of fetal origin and therefore characterised by both maternal and paternal contribution, the risk for pre-eclampsia might be modified by maternal as well as paternal genetic variations in detoxification activities. We therefore studied *GSTP1* polymorphisms in a cohort of 113 pre-eclampsia trios (mother, father, baby) in which the mothers had suffered pre-eclampsia and 317 Dutch, population based, healthy controls, recruited by advertisement (149 men, 168 women).

METHODS

The mothers in the study group consisted of Dutch women who had previously been admitted to the antenatal wards of the University Medical Centre, Nijmegen for pre-eclampsia. Pre-eclampsia was defined as the occurrence after 20 weeks' gestation of a diastolic blood pressure greater than 90 mm Hg and concordant proteinuria (urinary protein greater than 0.3 g/l in a 24 hour collection period or a protein/creatinine ratio greater than 0.3 g/10 mmol). The local ethical committee on human experimentation approved the study protocol. Genomic DNA from mothers and fathers was extracted from blood samples collected by venepuncture using the Wizard™ genomic DNA purification kit, according to the instructions of the manufacturer (Promega, Madison, WI, USA). DNA from offspring was collected from buccal cell samples collected on sterile swabs as described by Richards *et al*.[3] After extraction, DNA was further purified by phenol/chloroform extraction. Subsequently, the 105 Ile→Val polymorphism in *GSTP1* was assessed by polymerase chain reaction as described previously.[1] Chi-square analyses and relative risks approximated by odds ratios were used for statistical evaluation of differences in polymorphic rates and allele frequencies. A p value of 0.05 represents statistical significance. Association analysis was also performed using the transmission disequilibrium test (TDT) described by Spielman *et al*.[4] The TDT test evaluates the observed number of parent-offspring transmissions of alleles, compared with the number of transmissions expected by chance. Only parents heterozygous for the polymorphism tested are informative for the test. Association was tested using chi-square statistics. A p value <0.05 was considered significant.

RESULTS

No significant sex differences in the frequency of the Val^{105} allele could be shown in the population based control group. The distribution of polymorphic variants in the *GSTP1* gene in pre-eclampsia trios and controls is shown in table 1. The

Key points

- An imbalance between toxic compounds, such as lipid peroxides and reactive oxygen species, and detoxifying or scavenging substances may contribute to the initiation and progression of pre-eclampsia, a serious disorder in human pregnancy.
- Glutathione S-transferase P1-1 is a major biotransformation enzyme in placenta and decidua. The 105Ile→Val polymorphism in the glutathione S-transferase P1 gene is associated with lower enzyme detoxification capacity.
- Such polymorphisms in the genome of both mother and offspring, as well as the father appear to be associated with pre-eclampsia.

GSTP1 Val^{105}/Val^{105} genotype was found significantly more often in mothers, fathers, and offspring of pre-eclamptic pregnancies than in controls ($p=0.005$, $p<0.0001$, and $p=0.0006$, respectively). Also, the Val^{105} allele was present significantly more often in mothers (0.32), fathers (0.37), and offspring (0.38) of pre-eclamptic pregnancies compared with the frequency in controls (0.22). Comparisons of *GSTP1* genotypes and allele frequencies between pre-eclampsia mothers, fathers, and offspring showed no statistically significant differences.

The TDT test applied to the parents heterozygous for the Val^{105} allele was found to be positive (χ^2 TDT=4.24, $p< 0.05$), confirming linkage disequilibrium of pre-eclampsia with this allele.

DISCUSSION

Epidemiological studies show that pre-eclampsia has hereditary characteristics. Both the mother and the fetus may contribute to the risk of pre-eclampsia, the contribution of the fetus and trophoblastic factors also being affected by paternal genes. Strong support for a paternal role in pre-eclampsia comes from a study showing an association between pre-eclampsia and changing paternity.[5]

The polymorphism in *GSTP1* resulting in an isoleucine to valine substitution at amino acid 105 was associated with concomitant reduction of enzyme activity in heterozygotes and even more in homozygotes.[6] We now show that this polymorphism in *GSTP1* in paternal and fetal genes, in addition to the earlier reported association between pre-eclampsia and this polymorphism in the maternal genome, may contribute to the risk for pre-eclampsia.[1] Furthermore, we were able to confirm linkage disequilibrium of pre-eclampsia with the Val^{105} allele, again strongly suggesting that the *GSTP1* polymorphism is indeed associated with the disease. This is also in accordance with the recent findings of Esplin *et al*,[7] who reported that both men and women who were the product of a pregnancy complicated by pre-eclampsia were significantly more likely than control men and women to have a child who was also the product of a pregnancy complicated by pre-eclampsia.

To our knowledge, this is the first polymorphism in the paternal genome related to pre-eclampsia. Higher frequencies of the Val^{105} allele in pre-eclampsia might result in a lower detoxification capacity in the trophoblast and inadequate coping with local oxidative stress, resulting in a higher susceptibility to pre-eclampsia.

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Table 1 Distribution of the *GSTP1* 105Ile→Val polymorphism in pre-eclampsia mother-father-baby trios

	PE mothers (n=113)	PE fathers (n=113)	PE offspring (n=111)	Controls (n=317)
Genotype				
Ile^{105}/Ile^{105}	51 (45)	47 (42)	40 (36)	187 (59)
Ile^{105}/Val^{105}	51 (45)	49 (43)	58 (52)	120 (38)
Val^{105}/Val^{105}	11 (10)	17 (15)	13 (12)	10 (3)
OR (95% CI)	3.3 (1.3-8.7)	5.4 (2.3-13.3)	4.1 (1.6-10.4)	1

Percentages are given in parentheses. *GSTP1* genotypes: Ile^{105}/Ile^{105}, wild type; Ile^{105}/Val^{105}, heterozygous; Val^{105}/Val^{105}, homozygous. PE, pre-eclampsia; OR (95% CI), odds ratio and 95% confidence interval calculated for the Val^{105}/Val^{105} genotype versus the other two genotypes in pre-eclampsia mothers, fathers, and offspring versus Dutch population based controls.
GSTP1 polymorphism could not be determined in two children.

Association of polymorphisms and allelic combinations in the tumour necrosis factor-α-complement MHC region with coronary artery disease

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It is a complicating factor in the search for disease associated genes in the human population that most diseases are very heterogeneous clinically and that certain genetic factors may not alone cause susceptibility to a disease, but in association with other genetic and environmental factors. It is especially true for coronary artery disease (CAD) and disease susceptibility genes in the human major histocompatibility complex (MHC). Given the conservation of whole haplotypes (polymorphic frozen blocks or extended haplotypes) and the *cis* acting genes within the MHC, it is highly likely that disease association is the result of a multiplicity of interactive genetic influences rather than a single gene.[1] By tradition, a disease is said to be MHC associated if the frequency of one or more alleles is increased or decreased significantly when a patient group is compared with a relevant control group. This approach cannot uncover the possible interactions of different alleles and may result in both false positive and false negative association. In our study on patients with CAD, we make an attempt to investigate not only the impact of single allelic variations within the MHC, but also the impact of a combination of these allelic variations on susceptibility to the disease.

The tumour necrosis factor-α (*TNF*α) gene is located on chromosome 6 between the class I and III clusters of the human MHC.[2] It has been suggested that TNFα plays a role in cardiovascular pathophysiology as it may affect lipid metabolism[3] and predispose to obesity related insulin resistance.[4] Several TNFα variants with polymorphisms in their promoter regions have been described.[5] Two of them (−308G-A and −238G-A) have been found to be associated with a variety of MHC linked diseases.[5–7]

Complement factor genes are located just a few hundred kilobases (kb) from the TNFα locus in class III clusters.[8] The fourth component of the classical complement pathway, C4 encoded by two adjacent genes (*C4A* and *C4B*), and a component of the alternative pathway, factor B (Bf), have a high degree of polymorphism. Several studies indicate that the complement cascade is involved in vascular inflammation, contributes to the development of atherosclerosis, and is a key event mediating the local inflammatory response occurring in the infarcted myocardium.[9 10] The non-expressed variants of the two C4 genes (C4A*Q0 and C4B*Q0) and the haemolytically inactive C4A*6 allele have previously been found to be associated with several immunological diseases.[11–14]

In the present study, we determined the frequency of six alleles in the MHC in a stretch of a few hundred kb (TNFα −308A, TNFα −238A, C4A*Q0, C4A*6, C4B*Q0, and Bf *F) in patients with severe CAD who underwent bypass surgery and in healthy control patients. To study the impact of the combinations of these alleles on the susceptibility to CAD, we investigated whether the distribution of the different combinations of alleles corresponded to the expected values. In addition, we have retrospectively studied the effect of the same genetic factors on the probability of developing myocardial infarction (MI) among patients with severe CAD.

METHODS

Patients

Patients (n=318, aged 35-73) with signs of severe coronary atherosclerosis tested by coronary angiography (>70% stenosis in one or more arteries, and clinical signs of stable or unstable angina pectoris, typical ECG abnormalities) were enrolled. All patients had received coronary artery bypass grafting (CABG) by open heart surgery at the National Institute of Cardiology in 1995 and 1996. Healthy controls (n=248, aged 35-73) were randomly recruited from the same areas as the cases, and stratification by age and sex was used to match approximately the age and sex distribution of the controls with that of cases. The control group represents asymptomatic and apparently clinically disease free subjects (no symptoms of CAD, normal ECG, normal blood pressure). All subjects completed a series of questionnaires including questions on family and personal history of CAD. In cases, an additional questionnaire was completed, including items on previous MI and occurrence of angina pectoris. Clinical and biological features of the cases and controls are shown in table 1.

The study was approved by an institutional review committee and the subjects gave informed consent. The investigation conforms to the principles outlined in the Declaration of Helsinki.

Laboratory analysis

Total genomic DNA was extracted from white blood cells using the method of Miller *et al*.[15] The TNFα −238 and −308 polymorphisms were determined by DNA amplification by PCR using the primers suggested by Day et *al*.[1] The PCR products were digested at 37°C with *Msp*I to detect the −238 polymorphism and *Nco*I to detect the −308 polymorphism. The products were separated on a 4% agarose gel and stained with ethidium bromide. Bf allotypes were determined as described elsewhere.[17] C4 typing was performed according to Sim and Cross.[18]

Serum lipid parameters (quantified by standard enzymatic procedure) and blood glucose levels were measured after overnight fasting. In patients, the blood was drawn six months after the operations.

Statistical methods

Allele frequencies were calculated by allele counting and given with an estimate of the standard error (SE). Data were analysed using MedCalc and Arlequin software.[19] Distributions of the simultaneous occurrence of the alleles were tested using a likelihood ratio test. In the case of linkage disequilibrium (LD) between a pair of loci (significance level 0.05), coefficients were computed. LD coefficients (|D'|) are the ratio of the unstandardised coefficients to the maximum value they can take.[20] Maximum likelihood frequencies were computed using an expectation maximisation algorithm.[21] Hardy-Weinberg equilibrium was tested by using a χ^2 goodness of fit test. Fisher's exact test was used to test for differences in distributions of alleles and allelic combinations between the groups. Confidence intervals were calculated at the 95% level.

Table 1 Clinical and biological features of CAD patients undergoing CABG

Clinical and biological characteristic	Value	
	Cases	Controls
Age, years	57.6 (8.2)	57.2 (5.8)
Gender, male/female	242/76	189/59
Angina pectoris:		
No, rarely/frequently on effort/resting	51/96/171	248/0/0*
NIDDM in case history, yes/no	69/249	0/248*
Smoking, ever smoked/never smoked	210/108	144/104
Hypertension		
Yes/no	169/149	0/248*
Family history of myocardial infarction		
Yes/no	179/137	71/177*
Myocardial infarction in case history		
Yes/no	158/162	0/248*
Stenosis		
In one artery/more than one artery	81/237	0/0*
Body mass index, kg/m^2	28.2 (3.8)	28.0 (2.7)
Serum cholesterol, mmol/l†	6.25 (0.83)†	6.29 (0.78)
HDL cholesterol, mmol/l†	1.27 (0.17)†	1.31 (0.21)
LDL cholesterol, mmol/l†	4.04 (0.93)†	4.11 (0.89)
Triglycerides, mmol/l†	2.04 (0.7)†	1.96 (0.81)

*p<0.00001 cases *v* controls.
†Six months after the surgery, cases were treated by lipid lowering drugs.

ANOVA test was used to estimate the impact of the polymorphisms on the quantitative traits.

RESULTS

Frequencies of the genotypes and alleles

The frequencies of the genotypes and alleles in question in CAD patients and controls are presented in table 2. Significant differences were observed in the frequencies of C4B*Q0 between CAD patients and controls. The results were overall in Hardy-Weinberg equilibrium, with the exception of the C4B*Q0 allele distribution in CAD patients, where there was deviation (χ^2=12.41, p=0.0004) from the equilibrium. Analysis of the genotype distribution of this polymorphism showed that the deviation from the HWE was exclusively because of an increased frequency of the C4B*Q0/C4B*Q0 homozygotes among patients (p=0.02, odds ratio (OR)=3.8, 95% confidence interval 1.2-13.2), suggesting a recessive mode of action of this polymorphism on CAD.

Study of association between alleles tested and known risk factors for CAD

The mean values of several quantitative variables were compared between the different genotypes in both groups. No significant association with the lipid parameters (total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides) and blood pressure was observed (data not shown). The mean body mass index (BMI) in carriers of TNFα −308A was also compared with that of non-carriers, as was done by Herrmann *et al*.[5] In contrast to their results, we have not found significant differences between carriers and non-carriers and no association was found between obesity and the TNFα −308A allele (data not shown).

The mean blood glucose levels of carriers of TNFα −238A were lower than that of non-carriers, 5.52 (SD 0.57) mmol/l *v* 6.38 (SD 2.01) mmol/l (p=0.05), but the TNFα −238A allele frequency in subjects with diabetes did not differ significantly from that of subjects without diabetes (4.3% and 4.5% in subjects with and without diabetes, respectively).

Investigation of allelic combinations

We investigated with the exact p test whether the distribution of the different combinations of alleles corresponded to the expected values. Deviation from the equilibrium was found in patients with CAD in TNFα −308A and C4A*Q0 alleles (exact p<0.001, linkage disequilibrium (LD) coefficient (±|D′|) was 0.61, that is, the observed disequilibrium is 61% of the

Table 2 Genotype and allele frequencies in patients with CAD undergoing CABG (n=318) and in controls (n=248)

	Status	Genotype frequency 11/12/22* (%)	Allele 2 frequency (% (SE))
TNFα -238A	Cases	90.2/9.8/0.0	4.9 (1.7)
	Controls	90.7/9.3/0.0	4.6 (1.8)
TNFα -308A	Cases	72.0/25.2/2.8	15.4 (2.9)
	Controls	72.9/26.2/0.9	13.9 (3.1)
Bf*F	Cases	64.2/31.4/4.4	20.4 (3.2)
	Control	69.5/27.3/3.2	17.0 (3.3)
C4A*Q0	Cases	73.9/24.5/1.6	13.8 (2.7)
	Controls	70.9/27.4/1.7	15.6 (3.3)
C4A*6	Cases	91.5/7.9/0.6	4.6 (1.7)
	Controls	95.6/4.4/0.0	2.2 (1.3)
C4B*Q0	Cases	76.1/19.5/4.4†	14.2 (2.8)†
	Controls	81.5/17.3/1.2	9.9 (2.7)

*2 represents the allele under consideration, 1 codes for all other alleles.
†Deviation from Hardy-Weinberg equilibrium (χ^2=12.41, p=0.0004). The frequency of the homozygous carriers of the C4B*Q0 in CAD patients differs significantly from that of controls (p=0.02, odds ratio=3.8 (95% confidence interval 1.2–13.2). The frequency of the C4B*Q0 allele is different in cases and in controls (p=0.02, OR=1.5 (1.0–2.1)).

Table 3 Maximum likelihood frequencies of allelic combinations. Comparisons of ratios between the frequency of TNFα -308A+C4A*Q0 allelic combinations with that of TNFα -308A without C4A*Q0 alleles, and with that of TNFα -308G + C4A*Q0 alleles in cases and controls. The same comparisons with the TNFα-238A, C4A*6, and TNFα-238G allelic combinations

	Frequency in cases	Ratio in cases	Frequency in controls	Ratio in controls	p	Odds ratio (95%CI)
(A) TNFα-308A + C4A*Q0	8%		3.8%		0.005	2.2 (1.3–3.8)
(B) TNFα-308A + no C4A*Q0	6.8%		10.5%		0.04	0.6 (0.4–0.9)
(C) TNFα-308G + C4A*Q0	7.1%		8.5%		0.5	0.8 (0.5–1.3)
(A/B)		1.18		0.36	0.0005	3.2 (1.7–6.3)
(A/C)		1.13		0.45	0.008	2.5 (1.3–4.9)
(D) TNFα-238A + C4A*6	3.3%		0.8%		0.009	4.2 (1.4–12.3)
(E) TNFα-238A + no C4A*6	1.6%		3.2%		0.1	0.5 (0.2–1.1)
(F) TNFα-238G+ C4A*6	1.3%		1.4%		1.0	0.9 (0.3–2.5)
(D/E)		2.1		0.25	0.001	8.4 (2.2–31.7)
(D/F)		2.5		0.6	0.06	4.6 (1.0–20.1)

theoretical maximum disequilibrium value) and in TNFα −238A and C4A*6 alleles ($p<0.001$, $\pm|D'|=0.71$) and in both patients and controls in C4B*Q0 and Bf*F alleles ($p<0.001$, $\pm|D'|=0.3$ in cases; $p<0.05$, $\pm|D'|=0.2$ in controls). The linkage between TNFα −308A and C4A*Q0 alleles was supported by the findings that five patients were homozygous for the C4A*Q0 allele and three of them had AA (60%) and one GA genotype at the TNFα −308 position. Complete negative LD ($\pm|D'|=-1$; the two alleles are never present on the same haplotype) was found in patients between TNFα −308A and C4A*6 and between TNFα −238A and C4AQ*0 and in both patients and controls between C4A*Q0 and C4B*Q0.

We examined the impact of these allelic combinations on the susceptibility to CAD. We computed the maximum likelihood frequencies of the different allelic combinations with an expectation maximisation algorithm and compared them between cases and controls. The estimated frequency of the TNFα −308A + C4A*Q0 allelic combinations and that of the TNFα −238A + C4A*6 (table 3) were significantly higher in CAD patients than in controls (8% *v* 3.8%, and 3.3% *v* 0.8%, respectively). In contrast, the frequency of the TNFα −308A without C4A*Q0 (pooled all C4A alleles, which is not C4A*Q0) was higher in controls (6.8% *v* 10.5%). The prevalence of other allelic combinations did not differ between the two groups (data not shown). If the ratios between the frequency of patients with TNFα −308A + C4A*Q0 alleles and with TNFα −308A without C4A*Q0 were compared in cases and in controls, the p value was 0.0005 and the OR 3.2 (95%CI 1.7-6.3), indicating that the risk of having CAD was 3.2 times higher in patients with TNFα −308A+C4A*Q0 alleles than with TNFα −308A without C4A*Q0. When the same ratio between patients with TNFα −308A + C4A*Q0 alleles and with TNFα −308G + C4A*Q0 were compared in cases and in controls, the p value was 0.008 and the odds ratio 2.5 (95%CI 1.3-4.9), indicating that the risk of having CAD was 2.5 times higher in patients with TNFα −308A + C4A*Q0 than in patients with TNFα −308G + C4A*Q0 allelic combination. Similar trends were observed in respect of TNFα −238A+C4A*6 (table 3), although because of the low prevalence of C4A*6, the comparison of the ratio between patients with TNFα −238A + C4A*Q0 alleles and with TNFα −238G + C4A*6 did not show a significant difference ($p=0.06$). These results suggest that the two allelic combinations (TNFα −308A + C4A*Q0 and TNFα −238A+C4A*6) give higher susceptibility to CAD than either allele alone.

Differences in the frequencies of alleles and allelic combinations between CAD patients with or without myocardial infarction in their case history

To investigate the role of these alleles and allelic association in CAD, we analysed the clinical status and case histories of the patients. The CAD patients were divided into two groups based on the occurrence of myocardial infarction in their case history before the bypass surgery. The frequencies of TNFα −308A and the C4A*Q0 alleles were significantly higher in patients with MI than without MI (table 4). The TNFα −308A and the C4A*Q0 alleles occurred together significantly more frequently in patients with preoperative MI than without preoperative MI, while there was no such association in the case of TNFα −238A + C4A*6 allelic combination.

There was no association between other symptoms (extent of stenosis, pre- and postoperative angina pectoris, thrombosis, left or right heart failure, hypertension, embolism, syncope, diabetes) and these alleles or allelic combinations (not shown).

DISCUSSION

In this study we investigated the distribution and association of six alleles in the MHC in CAD patients undergoing CABG and in controls. There is some evidence that complement plays

Table 4 Frequencies of alleles and allelic combinations in CAD patients undergoing CABG with or without myocardial infarction in their preoperative case history

	Preoperative MI	No preoperative MI	p	Odds ratio (95%CI)
No	158	162	0.8	—
TNFα -308A	19.9 (4.5)	12.0 (3.6)	0.004	1.8 (1.2–2.8)
C4A*Q0	15.7 (4.1)	9.0 (3.1)	0.005	1.9 (1.2–3.1)
TNFα-308A + C4A*Q0	10.8%	4.9%	0.02	2.2 (1.2–4.1)
TNFα -238A + C4A*6	3.5%	3.1%	1.0	1.1 (0.5–2.7)

MI=myocardial infarction.

an important role in the establishment of atherosclerosis. Complement C4 is a precursor of a subunit of the enzyme complex C3 convertase and is encoded by two closely related genes. The protein products of these loci are called C4A and C4B. Both genes are highly polymorphic and there is a relatively high frequency of the non-expressed variants, termed C4A*Q0 and C4B*Q0.[11] These alleles are associated with several autoimmune diseases.[12 13] The rare, haemolytically inactive C4A*6 allotype was reported to be associated with rheumatic heart disease.[14]

Our present findings indicate that the susceptibility of homozygous carriers of the C4B*Q0 allele to severe CAD is higher than that of non-carriers. Previously we found the prevalence of C4B*Q0 to be markedly lower in healthy, elderly, Hungarian people, particularly in men, as compared to healthy, young subjects.[22] We explained this observation by an increased morbidity and mortality from some diseases in middle aged carriers of the C4B*Q0 allele. This assumption was supported by our more recent observation indicating an increased frequency of the C4B*Q0 allele in 60-79 year old myocardial infarction patients as compared with age matched, healthy controls.[23]

Little is known about the mechanism of this greater susceptibility to cardiovascular disease associated with the C4B*Q0 allele. Here we looked for a possible link between the C4B*Q0 allele and some characteristics and symptoms of CAD patients undergoing CABG. We found, however, no differences in these factors between carriers and non-carriers of this allele.

Several studies have shown association between atherosclerosis and certain bacterial and viral pathogens. The most compelling evidence for an infectious factor in atherosclerosis is related to *Chlamydia pneumoniae*.[24] The complement system plays a principal role in the defence against bacterial infection. Therefore, it can be assumed that homozygous carriers of the silent C4B*Q0 allele have an impaired capacity to eliminate or mitigate *Chlamydia pneumoniae* infection. Finally, it cannot be ruled out that C4B*QO is a marker of a known or unknown gene in the MHC with linkage disequilibrium, or there are still unknown interactions with products of genes at other linked loci, which increase the susceptibility to the disease.

TNFα is an inducible cytokine with a wide range of proinflammatory and immunoregulatory actions. Through its effect on lipid metabolism,[25] obesity,[5] insulin resistance,[16] and endothelial function,[26] and stimulation of growth factors and adhesion molecules,[27] it could be involved in cardiovascular pathophysiology. The large and stable interpersonal differences in TNFα production indicate a genetic background. Wilson *et al*[28] raised considerable interest with their report that the −308A allele in the promoter region is transcribed in vitro at seven times the rate of the −308G allele. Moreover, the −308A allele has also been found to correlate with enhanced spontaneous and stimulated TNFα production in vivo.[29] Several studies have investigated TNFα polymorphisms in diseases in which dysregulation of TNFα production might have played a role and several of them found association between TNFα −238 and −308 promoter polymorphisms and some diseases.[5 6 16] Herrmann *et al*[5] investigated patients with coronary heart disease and found no association between polymorphisms in TNFα and susceptibility to the disease. The frequencies of TNFα −308A and −238A in patients with CAD undergoing CABG did not differ significantly from those of in controls in our present study either.

Investigation of the distributions of the different combinations of alleles showed some deviation from the calculated values, which was not totally unexpected, since all alleles are in the MHC within a stretch of a few hundred kilobases and linkage disequilibrium is one of the characteristic features of the MHC. However, it must be noted that the C4A genotypes were obtained by protein analysis, and it is possible that the quantitative null alleles were caused by multiple nucleotide changes resulting in the pooling of several different alleles as a single null allele. Therefore, the finding that there is linkage disequilibrium in CAD patients between TNFα −308A and C4AQ*0 does not necessarily mean that the two variants are in one haplotype, but together they are over-represented in these patients, suggesting that there might be a connection between the coincidence of the simultaneous occurrence of these variants and the development of CAD. Haplotype analysis would be needed to clarify whether there is functional importance that the alleles are in *cis* or *trans* positions.

At present it is not possible to explain the higher simultaneous occurrence of these two allelic combinations in patients with severe CAD, since apart from the higher occurrence of preoperative MI in patients with TNFα −308A + C4A*Q0 alleles, there were no clinical or laboratory parameters that differed in patients with or without these haplotypes. However, it can be hypothesised that since each allele was found to be involved in immunological disturbances, which play important roles in CAD, the simultaneous occurrence of the alleles increases the susceptibility to the development of the disease. It is also possible that genes linked with these alleles are also involved, since there are other candidate genes in the close vicinity,[8 30] including lymphotoxin α and β (related to TNFα), heat shock protein 70 (putative role in autoimmune inflammation), allograft inflammatory factor (allograft rejection), leucocyte specific transcript-1 (involved in macrophage activation), and several other genes with uncertain or unknown functions.

The TNFα −308A and C4A*Q0 alleles separately and together occurred at higher frequency in CAD patients with preoperative MI in their case history than in patients without MI. It is well known that the TNFα and the complements are involved in local inflammatory reactions in myocardial infarction.[10 31] Several studies reported raised TNFα levels in patients after MI, expressed by cardiac myocytes and macrophages migrated into the myocardium.[31] Complement activation is a key event mediating the deleterious effects of the local inflammatory response occurring in the infarcted myocardium. A partial C4 deficiency, which may include defective handling of immune complexes, can also be an additional risk factor in MI. In addition, inflammation is known to increase the probability of rupture of the vulnerable plaques.[32] Therefore it is reasonable to assume that the inflammation associated TNFα −308A and C4A*Q0 alleles may facilitate plaque rupture in MI. Besides, the connection between these alleles and high relative risk of CAD and MI correlates well with the recent findings that the imbalance of inflammatory processes (CRP, IL6, and IL1β) increases the risk of future cardiovascular disease significantly.[33]

Our study shows the importance of investigation of allelic association in the search for disease susceptibility genes. Several studies have investigated the TNFα polymorphisms in diseases in which dysregulation of TNFα production might have played a role and several of them found no association between TNFα −308A and −238A alleles and the suspected diseases.[5 34] It is possible that these negative results would change drastically if the C4A alleles were also considered.

Retrospective case-control studies may suffer from several biases, which may lead to false positive and false negative results. We have matched our patient and control groups for age, sex, and ethnicity to reduce this possibility. On the other hand, a survival bias cannot be avoided in a disease association study and prospective studies will be necessary to confirm the role of these alleles in CAD. Moreover, in this study only those CAD patients were analysed who were sent for CABG. Because only the most severe CAD patients undergo CABG, this is a selected population; thus it is possible that the conclusions of this study may not be extended to CAD patients in general, but only to the most severe cases. Furthermore, it is also possible that patients referred for CABG with MI in their case histories differ from patients with MI in general. This could be an

Key points

- There is an increased susceptibility to CAD in homozygous carriers of the C4B*Q0 allele.
- Subjects simultaneously carrying the TNFα –308A and C4A*Q0 or the TNFα –238A and the C4A*6 alleles have an increased risk for developing severe CAD.
- Among CAD patients, carriers of the TNFα –308A and the C4A*Q0 alleles have a higher risk of myocardial infarction.

explanation of the differences between the results of this study and those of a recent report by Nityanand *et al*,[35] where no association of C4A*Q0 and MI was found.

Another important concern might be that the gene pool of the cases and the controls differs, which could account for the associations observed. In the quest of alleles contributing to the susceptibility to the disease, several other polymorphisms were investigated in these populations on other chromosomes as well[36–38] (Szalai *et al*, unpublished data). The distributions of the vast majority of these alleles did not differ between cases and controls, for example, ACE D, 53.1%, 54.2%; apoE4, 10.1%, 9.2%; factor V Leiden mutation of the blood coagulation system, 4.8%, 4.1%; PlA2 allele of the platelet glycoprotein IIb/IIIa receptor, 11.3%, 13.5%; F allele of the C3 component of complement, 16.5%, 16.7%; chemokine receptor 5 CCR5Δ32, 10.5%, 11.7%; CCR264I, 12.3%, 11.3%; stromal derived factor 1-3'A, 19.1%, 20.4%; RANTES-28G, 4.2%, 3.3%; RANTES-403A, 20.8%, 17.8%; methylenetetrahydrofolate reductase 677T, 34.8%, 37.2%; apo(a) $(TTTTA)_n$ repeat polymorphism, mean n= 8.6 (SD 1.1) *v* 8.5 (SD 0.8), in cases and controls, respectively. Altogether, 32 polymorphisms were investigated and only two differences have been found between the two populations (MCP-1 –2518[36] and C4B*Q0 (this report)), both of which could contribute to the susceptibility to CAD. The careful selection of our patients and these results ensure that the differences in the gene pool between cases and controls are as minimal as possible.

In summary, according to this study, homozygous carriers of the C4B*Q0 allele have an increased risk of developing severe CAD. The simultaneous occurrences of the TNFα –308A + C4A*Q0 and the TNFα –238A + C4A*6 alleles are higher in CAD patients undergoing CABG than in healthy controls. Among CAD patients, carriers of the TNFα –308A + C4A*Q0 allelic combination have a higher risk of myocardial infarction.

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CTLA-4/CD28 gene region is associated with genetic susceptibility to coeliac disease in UK families

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Coeliac disease (CD) is a malabsorption disorder characterised by a small intestinal enteropathy that reverts to normal on removal of dietary gluten. Susceptibility to disease has a strong genetic component. Ninety percent of patients in northern Europe have the HLA class II alleles DQA1*0501 and DQB1*0201, which encode the cell surface molecule HLA-DQ2.[1] However, haplotype sharing probabilities across the HLA region in affected sib pairs suggest that genes within the MHC complex contribute no more than 40% of the sib familial risk of CD, making the non-HLA linked gene (or genes) the stronger determinant.[2]

Attempts have been made to identify these loci using genome wide linkage studies. Zhong *et al*[3] performed an autosomal screen in 45 affected sib pairs from the west coast of Ireland, using 328 microsatellite markers. They found evidence of linkage with lod scores of greater than 2.0 in five areas: 6p23 (separate from HLA), 7q31.3, 11p11, 15q26, and 22cen. A larger genome wide search involving 110 affected Italian sib pairs using 281 markers found no evidence of linkage in these five areas.[4] It did, however, propose a novel susceptibility locus at 5qter, important in both symptomatic and silent CD, and another at 11qter, which appeared to differentiate the two forms. In UK families an initial genome wide search,[5] followed by a study of 17 candidate regions[6] identified five areas with lod scores of greater than 2.0: 6p12, 11p11, 17q12, 18q23, and 22q13. Of these, 11p11 replicates one of the loci identified by Zhong *et al*[3] and it is likely that this area contains an important non-HLA susceptibility locus. However, in general the results of these studies are disappointingly inconsistent.

A number of candidate genes have been investigated in linkage and association studies. Of these, the only region with repeatedly positive results is the locus on chromosome 2q33 containing the cytotoxic T lymphocyte associated (*CTLA-4*) gene and the *CD28* gene. CD28 and CTLA-4 molecules are expressed by T lymphocytes and interact with their ligands B7-1 (CD80) and B7-2 (CD86) during antigenic stimulation of T cells via the T cell receptor. CD28 provides a co-stimulatory signal to T cell activation, while CTLA-4 provides a negative signal and thus is thought to be an important regulator of autoimmunity.[7] *CTLA-4* was investigated in a French study of coeliac patients versus controls and showed allelic association of disease with the A allele of the position +49 A/G dimorphism (+49*A/G).[8] The association has recently been replicated in a study of Swedish families,[9] which also showed some evidence of linkage and association with neighbouring microsatellite markers. A study of Finnish families also showed linkage and association in this region[10]; however, this was maximal at the marker locus D2S116, and association was not detected at +49*A/G. A study of *CTLA-4/CD28* in Italian and Tunisian families,[11] however, showed no evidence of linkage or association.

The *CTLA-4/CD28* gene region has shown linkage and/or association with a number of chronic inflammatory disorders, including type 1 diabetes.[12 13] These studies show linkage and association of *CTLA-4* polymorphisms with type 1 diabetes in Italian, Spanish, and French populations, but not in UK, Sardinian, or Chinese data sets.[13] We have studied this region in our own sample of UK coeliac families using the transmission/disequilibrium test (TDT). In contrast to conventional case-control studies, the TDT is not liable to produce false positive results owing to unrecognised population stratifications.

MATERIALS AND METHODS

Family sample

Affected subjects with both parents available for genotyping were selected from our established collection of multiply affected pedigrees.[6] In pedigrees where more than one subject fulfilled this criterion, only one was selected on a random basis. This was to ensure that the study would test for association rather than just linkage. Additional trios of affected subjects with their parents were recruited with the help of an article written for the UK Coeliac Society newsletter. A total of 166 families were studied; however, in 24 of these families only one parent was available for genotyping. All affected subjects were diagnosed according to the revised ESPGAN criteria.[14]

The study was the approved by the St Thomas's Hospital Ethics Committee and all subjects provided informed consent.

Microsatellite and SNP genotyping

Six finely spaced microsatellite markers and two single nucleotide polymorphisms (SNPs), within and surrounding the *CTLA-4* gene were genotyped (fig 1). Markers were chosen in

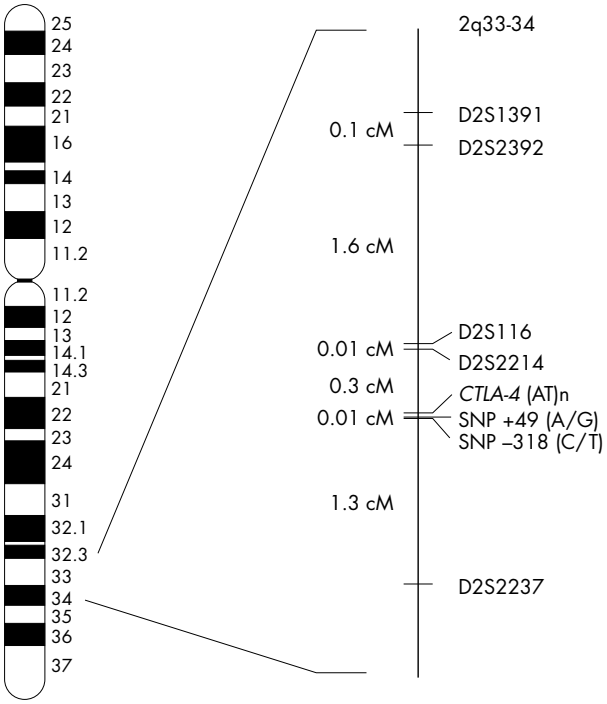

Figure 1 Map of chromosome 2q33-2q34 showing the position of microsatellite markers and SNPs used in the study.

Table 1 Results of allele-wise TDT analysis using ETDT

Marker	χ^2 df	p value
D2S1391	13.8, 6	0.032
D2S2392	14.4, 16	NS
D2S116	3.7, 10	NS
D2S2214	19.4, 7	0.007
CTLA-4	12.6, 15	NS
D2S2237	6.7, 10	NS
SNP+49	0.19, 1	NS
SNP-318	0.02, 1	NS

order to allow a direct comparison with previous studies, and at a density sufficient to realistically detect any locus across the region using association methods. Microsatellites were genotyped in all 166 families, but SNPs were genotyped only in the 142 families in which both parents were available for genotyping. Microsatellite markers were selected from the Genethon and CHLC maps: D2S116, D2S2392, D2S2214, D2S2237, D2S1391, and CTLA4(AT) (fig 1). Fluorescently labelled polymerase chain reaction (PCR) products were analysed using the ABI377XL Genetic Analyser (Applied Biosystems). Products were sized using the Genescan version 3.1 program, and scored using the Genotyper version 2.5 program (Applied Biosystems, Foster City, CA USA; http:www.applied-biosystems.com).

The two known dimorphisms within the *CTLA-4* gene, +49*A/G and –318*C/T, were genotyped using the PCR-restriction fragment length polymorphism (PCR-RFLP) method. PCR reactions for +49*A/G were performed using forward and reverse primers previously described by Marron *et al*.[13] The 152 bp product was cleaved for 16 hours at 60°C using 10 units of *Bst*EII per reaction. The digested A allele yields a fragment of 130 bp and the G allele yields an intact 152 bp fragment. The T allele of –318*C/T also creates a restriction site,[15] allowing a similar technique to be used for genotyping. Primers were therefore designed around this site: forward primer 5′-TGGACTGGATGGTTAAGGATG-3′ and reverse primer 5′-AGAAGGCACTTGAATAGAAAGC-3′. The 275 bp PCR product was cleaved for 16 hours at 37°C using 4 U *Mse*I per reaction. The C allele produces a 262 bp fragment, whereas the T allele produces a 169 bp and a 95 bp fragment. All fragments were separated on a 2% agarose gel and visualised with ethidium bromide staining under UV fluorescence. Microsatellite and SNP data were checked for genotyping errors using the PEDCHECK program.[16]

Data analysis

Data for biallelic markers were analysed using the transmission disequilibrium test (TDT).[17] In order to avoid biases, only subjects with data available from both parents were included in the analysis. For multiallelic markers such biases do not arise, as long as the affected subject is heterozygous for the marker,[18] so all 166 families were analysed. Multiallelic data were analysed using the ETDT program,[19] which carries out a logistic regression analysis to determine whether different marker alleles vary in terms of their probability of being transmitted from a heterozygous parent to an affected offspring. When this overall allele-wise test was significantly positive for a marker, we examined the transmission counts for individual alleles, to see which one(s) made the major contribution to the observed effect.

RESULTS

The results of the allele-wise TDT analysis as performed by the ETDT program are shown in table 1. D2S2214 provides evidence for unequal transmission of different alleles to affected offspring (χ^2 =19.4, 7 df, p=0.007). Examining the individual allele transmissions (table 2), it seems that most of this effect is the result of preferential transmission of allele *278, which is transmitted from 92 heterozygous parents and not transmitted from 49 (χ^2=13.1, 1 df, p=0.0003). A lesser contribution (χ^2=9.333, 1 df, p=0.002) to the effect is made by allele *276. The only other somewhat positive result is with D2S1391 (χ^2 =13.8, 6 df, p=0.03), but given that eight markers were tested this result could easily have occurred by chance. For both *CTLA-4* single nucleotide polymorphisms, alleles were transmitted approximately equally, providing no evidence at all for the direct involvement of these polymorphisms in the susceptibility to CD.

Table 2 Transmission of individual alleles of D2S2214 to affected offspring from heterozygous parents

Allele	*274	*276	*278	*280	*282	*284	*286	*288
Passed	25	28	92	4	2	1	15	9
Not passed	33	56	49	7	6	2	18	5
Chi-squared (1 df)	1.103	9.333	13.113	0.818			0.273	1.143
p value	NS	0.002	0.0003	NS			NS	NS

DISCUSSION

This study of UK families provides further evidence that the *CTLA-4/CD28* gene region on chromosome 2q33 contains an important non-HLA susceptibility locus for coeliac disease. This evidence comes from a positive TDT result with the D2S2214 microsatellite (p=0.007), indicating that allelic association with the disease is present with this locus. The use of the TDT method rather than a case-control sample means that this positive result is not the result of unrecognised population stratifications. Although association has not previously been shown with this marker, it has been shown with two nearby markers, D2S116 and +49*A/G. D2S116 lies 0.01 cM centromeric of D2S2214 and showed association significant at p=0.0001 in one previous study.[10] The +49*A/G SNP lies 0.3 cM telomeric of D2S2214 and has been found to be associated with CD in two previous studies with significance p=0.0001[8] and p=0.007.[9] We have found no evidence for association of +49*A/G or D2S116 in the current study. The –318*C/T SNP lies within the *CTLA-4* gene promoter region and is thus a candidate to be an aetiological polymorphism, but it did not show any evidence of association in the current study. The third known polymorphism of the *CTLA-4* gene is the *CTLA-4* (AT) microsatellite positioned in the 3′ untranslated region of exon 3. Theoretically, this variation could affect gene expression by affecting mRNA stability; however, in keeping with two previous studies,[9 10] we failed to detect any evidence for association.

The finding that different polymorphisms within the same region are positive in different samples is consistent with the hypothesis that none of them influences susceptibility to CD directly but that there is another, as yet untested, susceptibility locus within the region. Different patterns of linkage disequilibrium between markers in different populations, along with chance variations, would then account for the different results obtained. Association of the *CTLA-4/CD28* region with genetic susceptibility to coeliac disease has now been reported in UK, French, Finnish, and Scandinavian populations. No association has yet been found in Italian, Tunisian, or Dutch[20] populations. These differences might result from chance factors, from different frequencies of the susceptibility locus among cases in different populations, or because of differences in the pattern of linkage disequilibrium across the region. It may be of some interest that the pattern observed across Europe seems different from that found in the type 1 diabetes studies, in particular in relation to UK families, in that association is found for coeliac disease but not for diabetes.

We have used a TDT design which provides a robust test for association and which may be more powerful at detecting loci in complex diseases than linkage analysis,[21 22] at least when attention can be restricted to a small region. Association based studies can also provide a more precise localisation than linkage studies since they are based on the presence of linkage disequilibrium, which in outbred human populations only exists between markers within a few hundred kb of DNA sequence (probably <300 kb).[23–25] *CTLA-4* and *CD28* are both plausible candidate genes and are separated by only 25-150 kb. However, a polymorphism within these genes that predisposes to CD has not yet been identified. Disease predisposition may lie in undiscovered polymorphisms of *CTLA-4* or *CD28*, or alternatively within another gene in very close proximity. A database search of the region using Genemap 99 (http://www.ncbi.nlm.nih.gov/genemap99/) and Ensembl (http://www.ensembl.org/) did not show any genes with known immunological or gut related functions. Future work will therefore involve detailed mapping of the region with identification of further SNPs within *CTLA-4/CD28* and in the surrounding region in order to identify the aetiological polymorphism that confers susceptibility to coeliac disease.

Key points

- Genetic susceptibility to coeliac disease is not entirely explained by known HLA associations.
- Association of coeliac disease with the *CTLA-4/CD28* gene region on chromosome 2q33 has been reported in some European populations but not in others.
- This study of UK families did not show significant association with any of the three known polymorphisms of the *CTLA-4* gene.
- The study did, however, show significant association with D2S2214 (p=0.007), a microsatellite marker 0.3 cM centromeric of the *CTLA-4* gene.

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A common ancestral haplotype in carrier chromosomes from different ethnic backgrounds in vacuolating megalencephalic leucoencephalopathy with subcortical cysts

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Vacuolating megalencephalic leucoencephalopathy with subcortical cysts (VL) is a newly described, inherited leucodystrophy (MIM 604004). Clinically, the disease is characterised by accelerated head growth beginning in the first year of life and resulting in extreme macrocephaly and mild delays in gross motor milestones. In most cases, these early manifestations are followed by evolution of pyramidal symptoms and signs, cerebellar ataxia, epilepsy, and in older patients dystonia and athetosis.[1] Cognitive function is relatively spared in most patients. Brain imaging with computed tomography (CT) or magnetic resonance imaging (MRI) shows diffuse cerebral white matter swelling with progressive cystic-like changes, prominent in the frontotemporal regions, with preservation of grey matter structures.[1 2] Pathological specimens from VL patients showed splitting of the myelin sheaths between the lamellae consistent with an oedematous process, with sparing of the exons.[3] Although progressive in nature, VL is characterised by a relatively mild clinical course compared to the severity of the neuroradiological findings.[4 5] About 70 cases of the disease have been described in different ethnic groups. The molecular basis of this disorder remains unknown. The inheritance is autosomal recessive and the disease gene was recently mapped to a 3 cM interval between D22S1161 and the telomere of chromosome 22q.[6] Linkage was established in a group of 13 Turkish families all originating from rural areas of central and south eastern Anatolia. No shared alleles or shared haplotypes were detected between the Turkish families.

METHODS

Six of the seven families included in this study (fig 1) have been described in detail by Ben Zeev *et al*.[7] Families 1, 2, 4, and 6 are of Libyan Jewish origin and family 3 is of Turkish Jewish origin. Family 7 is of non-Jewish Indian origin and family 5 is of mixed ancestry. The father is of Libyan Jewish origin and the mother is Ashkenazi. The parents in families 2, 3, and 4 are first cousins, while the parents in the other families are unrelated. The study was approved by the Helsinki Committee at the Sheba Medical Centre and participants gave informed consent. Computed screening of the full chromosome 22 sequence, telomeric to D22S922, showed two new CA repeats located in clones WI14811 and STS28616. These polymorphic repeats were amplified with the primers 5′-GGAGAAT CACTTAAACTCAG-3′ and 5′-TTCAGCAGTTTTTCTGTCCC-3′, 5′-TGGAAGAAAGAAATCTCAAA-3′ and 5′-TGAACTCAAGGT TTGCTAAG-3′, respectively. The markers N66C4 and *ARSA*[6] were amplified with primers 5′-TGTACATCCTTACTGCTCG-3′ and 5′-ACGGCAGTGGGGAAACACAA-3′, 5′-CCGGCCAAA AATGACTTTTA-3′ and 5′-CTGGAAAGAGCAAGACCCTG-3′, respectively. Amplification was carried out as described elsewhere.[8] Lod scores were calculated with the LINKAGE (version 5.1) package of programs, assuming recessive inheritance and a disease allele frequency of 0.004. Haplotypes were

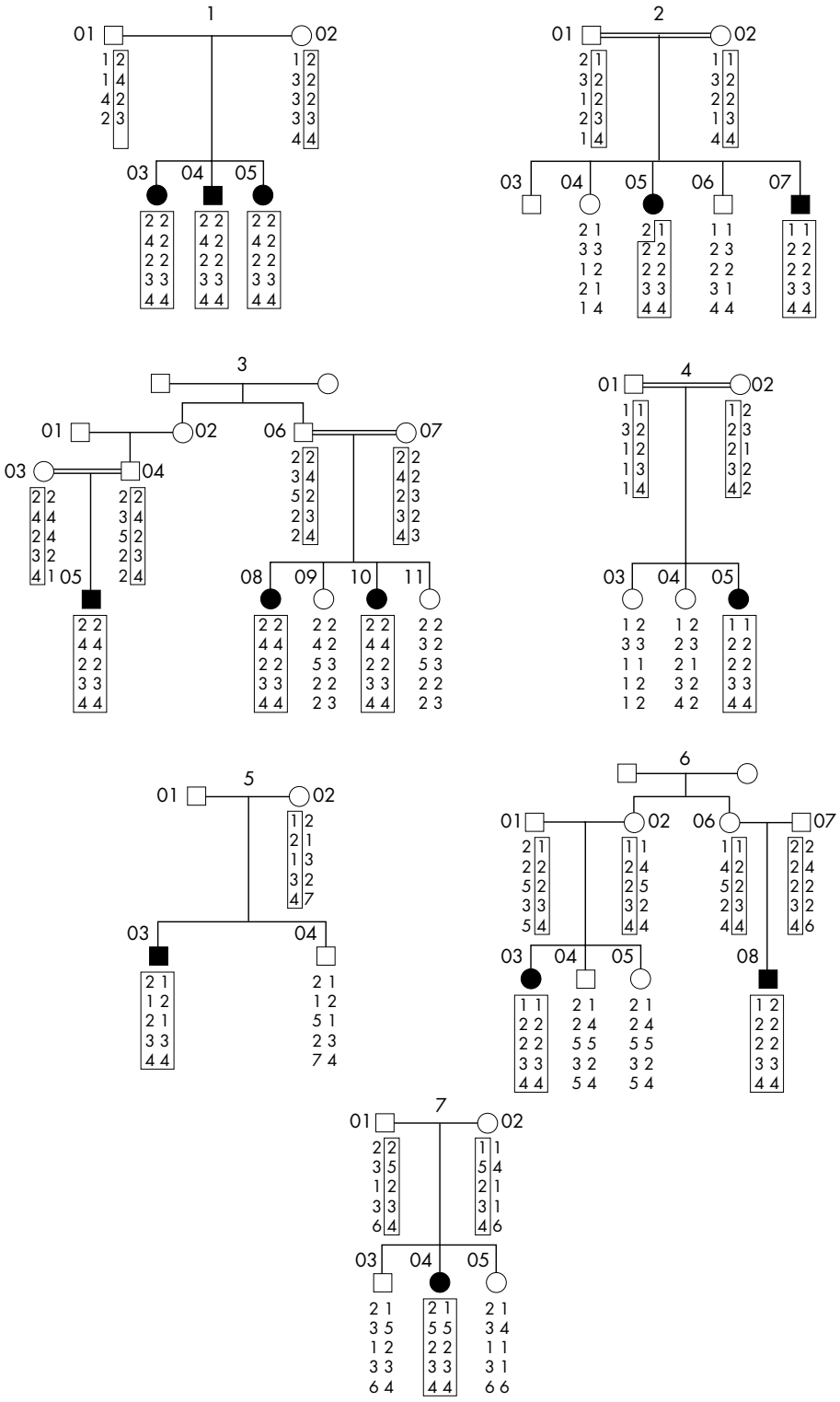

Figure 1 Typing for five markers on chromosome 22q (from top to bottom): D22S922, WI14811, STS28616, *ARSA*, and N66C4. Carrier chromosomes in the parents and affected subjects are shown in boxes.

constructed so as to minimise recombinants. In the consanguineous families, a single carrier chromosome was counted, while two such chromosomes were counted in the non-consanguineous families. DNA samples from 25 normal Libyan Jews were also typed for these markers and, together with the non-carrier chromosomes from the VL families, were used as controls (overall 58 control chromosomes).

RESULTS

A schematic physical map of the region stretching between D22S922 and the telomere is presented in fig 2. In order to place the markers accurately on the map, genomic clones containing these markers were compared to the full sequence of chromosome 22q. Two point lod scores between the disease and five chromosome 22q markers are presented in table 1. A maximal two point lod score of 5.93 was obtained with STS28616 at $\theta=0.00$. Four of the five markers yielded peak lod scores >3.00 at $\theta=0.00$. Haplotype analysis presented in fig 1 is consistent with linkage to 22q in all of the families. A recombination event in subject 2-05 for D22S922 defines this marker as the centromeric boundary. A haplotype constructed with the four distal markers used in this study,

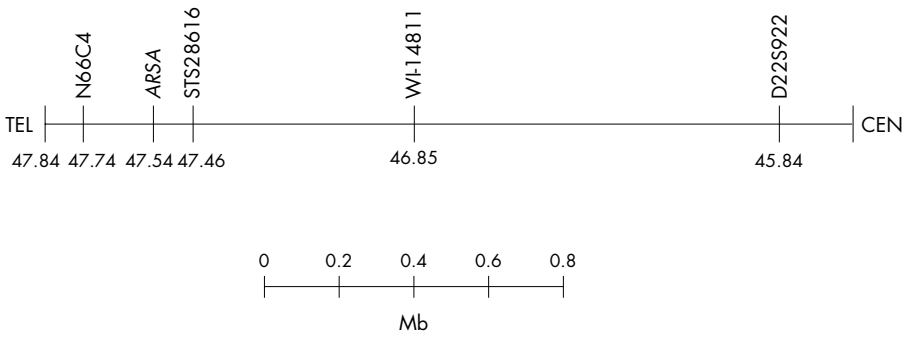

Figure 2 Schematic map of the interval containing the VL gene on chromosome 22q. Numbers under the horizontal line represent the distance in MB from the top of the chromosome.

Table 1 Two point lod scores between VL and chromosome 22q markers

	0.00	0.05	0.10	0.15	0.20	0.25	θmax	Zmax
D22S922	−∞	1.18	1.18	1.05	0.87	0.67	0.07	1.21
WI14811	5.88	5.21	4.47	3.73	2.98	2.27	0.00	5.88
STS28616	5.93	5.17	4.40	3.64	2.92	2.23	0.00	5.93
ARSA	4.60	4.04	3.45	2.87	2.29	1.74	0.00	4.60
N66C4	5.53	4.83	4.13	3.45	2.78	2.15	0.00	5.53

WI14811, STS28616, *ARSA*, and N66C4 (alleles 2, 2, 3, and 4, respectively), was present in seven of eight Libyan Jewish carrier chromosomes but in none of the 61 control Libyan Jewish chromosomes ($p<0.0001$). Even more interesting is the fact that 10 of the 11 carrier chromosomes in this study share the same alleles of the markers STS28616, *ARSA*, and N66C4 (alleles 2, 3, and 4, respectively), while none of the 68 control chromosomes share these alleles ($p<0.0001$). The only carrier chromosome that does not share these three alleles is that of the Ashkenazi mother in family 5 (5-02). This chromosome, however, does share with all the other carrier chromosomes the alleles of the two most telomeric markers, which appear only in two of 68 control chromosomes ($p<0.0001$).

DISCUSSION

Topcu *et al*[6] mapped the gene causing VL to a 3 cM interval between D22S1161 and the telomere, a region spanning 2 Mb. In this report we show that seven VL families from four different ethnic origins all map to the same region on chromosome 22q, thus providing evidence for genetic homogeneity. The gene causing the disease is located very close to the telomere of 22q, a region which is not well covered by the polymorphic markers provided in genome screening kits. Indeed, only by using markers developed by Topcu *et al*[6] and by ourselves were we able to detect linkage in our families. A haplotype constructed with the four telomeric markers was found in most of the Libyan Jewish carrier chromosomes but in none of the control Libyan Jewish chromosomes, suggesting a common founder. When only the three most telomeric markers were included, the haplotype extended to include all but one of the carrier chromosomes tested in this study, but none of the control chromosomes. This may reflect an ancient common mutation segregating in these families. If this is the case, the deviation from the historical haplotype at marker WI14811 in affected members from families 1, 3, and 7 may have resulted from recombinants that have occurred in past generations in these families. When attempting to narrow an interval that contains a candidate gene, analysis of recombinants is usually the first stage. A fairly large interval may be left after all recombinant events are used, especially when dealing with a small panel of families. In contrast, analysis of historical recombinants is a very powerful tool, owing to the fact that it takes advantage of recombination events that have occurred over multiple generations and not only the last one. The size of an interval retaining remnants of an ancestral chromosome is inversely proportional to the number of generations that have elapsed since a given mutation was introduced into a population and to the recombination rate. If the assumption that all the carrier chromosomes sharing alleles for N66C4, *ARSA*, and STS2861 have a common founder is correct, the deviation from the ancestral chromosome observed at WI14811 results from a historical recombination that has occurred between WI14811 and STS28616. This confines the disease gene to a 1 000 000 bp region between WI14811 and the telomere. When the haplotype is constructed only from the two most telomeric markers, N66C4 and *ARSA*, the haplotype formed is common to all carrier chromosomes. This theoretically could reflect an ancestral recombination between STS28616 and *ARSA*, thus reducing the gene interval to <400 000 bp. However, since this haplotype comprises only two markers, the data should be interpreted cautiously. A search for additional polymorphisms from this region, especially from the interval between *ARSA* and N66C4, may substantiate the reduction of the interval to less than 0.4 Mb.

The region between WI14811 and the telomere contains 12 known genes and five ESTs. All of the 12 genes and one of the ESTs are expressed in the CNS, as well as in many other tissues. Sequencing of these genes and other expressed sequences from the region will eventually identify the gene causing this disease.

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A late onset variant of ataxia-telangiectasia with a compound heterozygous genotype, A8030G/7481insA

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Ataxia-telangiectasia (A-T) is a multisystem autosomal recessive disorder, with an estimated frequency of 1/40 000-1/100 000 live births.[1] The ataxia-telangiectasia mutated gene (*ATM*), located on chromosome 11q22-23,[2] encodes a nuclear 370 kDa phosphoprotein, homologous to a family of phosphatidylinositol kinase related proteins involved in DNA damage response and cell cycle regulation.[3–6] Classical A-T patients show progressive cerebellar degeneration with onset in childhood, oculocutaneous telangiectasia, variable immunodeficiency, recurrent sinopulmonary infections, and high levels of serum α-fetoprotein, chromosomal instability, and predisposition to lymphoid malignancies. The majority of patients are compound heterozygotes for two truncating *ATM* mutations with no detectable ATM protein. The A-T phenotype, therefore, is most commonly the result of null alleles, although missense mutations can also destabilise the protein, with similar consequences.[7–9]

Milder cases, designated A-T variants, are a heterogeneous group characterised by a combination of one or more of the following: later onset of clinical signs, slower progression, extended life span when compared to most classical A-T patients, and decreased levels of chromosomal instability and cellular radiosensitivity.[10–12] In these patients telangiectasia and/or immunodeficiency can be absent while secondary features of A-T, such as peripheral neuropathy, dysarthria, chorea and/or dystonia, are present. Cancer and recurrent sinopulmonary infections may also be absent or reduced. The genotype of A-T variants is mostly compound heterozygous for a severe mutation together with a mild or leaky mutation, which is expected to express some ATM protein with residual function.[13–15]

Table 1 Clinical and cellular phenotype in proband II.1 compared to A-T and NBS syndromes

	A-T	A-T var	NBS	II.1
Onset in childhood	+	+/–	+	–
Ataxia	+	+	–	+
Polyneuropathy	–	+/–	–	+
Choreoathetosis	+	+	–	+
Dystonia	+	+	–	+
Immunodeficiency	+	+/–	+	–
Telangiectasia	+	+/–	–	–
Stunted growth	+/–	ND	+	+
Microcephaly	–	+/–	+	–
High α-fetoprotein	+	+	–	+
Sinopulmonary inf	+	–	+	–
Mental impairment	–	ND	+	+
Cancer susceptibility	+	+/–	+	–
Chromosomal instability	+	+	+	+

+, present; +/–, sometimes present; –, absent; ND: not determined.

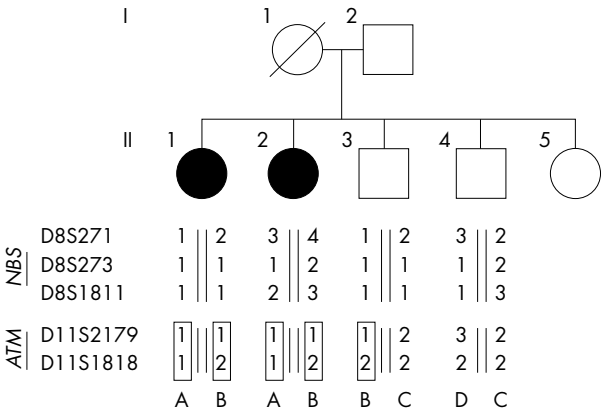

Figure 1 Pedigree of the family and linkage analysis at *NBS* and *ATM* loci. Haplotypes have been reconstructed from segregation analysis and show that affected sisters II.1 and II.2 share the same genotype A, B at the *ATM* locus.

A normal level of ATM protein in A-T variants can also be suggestive of mutations in other genes such as *hMRE11*, mutated in an A-T-like disorder with a classical A-T phenotype without telangiectasia.[16–17] Other syndromes, such as Nijmegen breakage syndrome (NBS), include some A-T signs combined with a typical facies, sinopulmonary infections, microcephaly, progressive deterioration of intellectual function, early retardation of height growth, normal serum α-fetoprotein levels, and lack of telangiectasia[18] (table 1). A-T and NBS are almost indistinguishable at the cellular level. The gene mutated in NBS is p95-*NBS1* which encodes a component of the complex (p95/hMre11/hRad50) involved in DNA double strand break repair.[19–20]

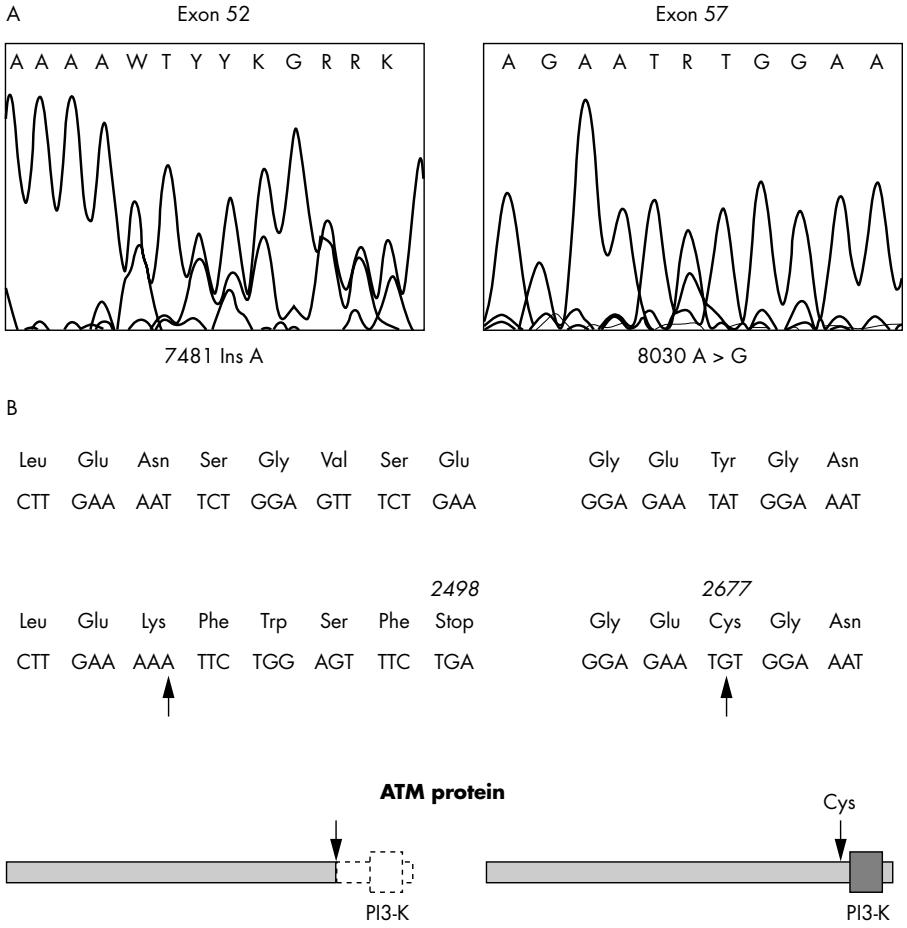

Figure 2 *ATM* gene mutation screening in II.1. (A) Genomic sequences of exons 52 (reverse primer) and 57. Mutation analysis of the *ATM* gene was initially performed by PTT and subsequently by cDNA sequencing. cDNA synthesis was performed under standard conditions.[42] The entire coding region of the *ATM* gene was divided into six overlapping fragments: 1 (1634 bp, exons 4-13), 2 (1707 bp, exons 12-24), 3 (1668 bp, exons 22-32), 4 (1736 bp, exon 31-44), 5 (1820 bp, exons 41-53), 6 (1825 bp, exons 52-65). Forward primers were designed to include a T7 promoter sequence for the initiation of transcription by T7 RNA polymerase, as well as a eukaryotic translation-initiation sequence (T7: 5′-TAATACGACTCACTATAGGAACAG-ACCACCATC- specific primer 3′; specific primer sequences are available upon request). Protein truncation test was performed using the TnT coupled reticulocyte lysate system (Promega).[43] Each fragment was gel purified (Qiaquick gel extraction kit, QIAGEN) and sequenced using internal primers and the Dye-terminator chemistry on a 377 ABI-Prism automatic sequence analyser (Applera). DNA from peripheral blood was extracted from all available sibs (II.1, II.2, II.3, II.4) using the Qiamp blood kit (QIAGEN). The 7481insA mutation was confirmed by genomic sequencing of exon 52; this was amplified using primers ex52f (5′-tcatgtgtgattttgtagttctgtta) and ex52r (5′-aagcacagggtagaatattggg) in standard conditions and the following cycling parameters: 94°C for three minutes, 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds, 72° for 40 seconds, 72°C for 10 minutes. The 8030 A to G mutation was confirmed by allele specific oligonucleotide hybridisation (ASO) (primers 8030-A: 5′-aggagaatAtggaaatc and 8030-G: 5′-aggagaatGtggaaatc) after ex57 amplification in standard conditions (primers ex57f: 5′-aagtgcaaatagtgtatctgacc and ex57r: 5′-ttcatcactaaaactctaaggc)[42] and the following cycling parameters: 94°C for three minutes, 35 cycles at 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 40 seconds, and 72°C for 10 minutes. (B) Changes produced at nucleotide and amino acid level (codon numbering is consecutive from ATG). On the bottom a schematic representation of the two ATM mutated proteins is shown. Ficoll extracted peripheral blood lymphocytes from patient II.1 were transformed with Epstein-Barr virus to establish a lymphoblastoid cell line.

Here we describe two sisters affected by a variant form of A-T with onset of ataxia at 27 years, showing polyneuropathy, choreoathetosis, and absence of telangiectasia, immunodeficiency, sinopulmonary infections, or cancer. Mutation screening of the *ATM* gene showed a compound heterozygous genotype with one missense mutation (8030 A→G in exon 57 which causes Y2677C) and a frameshift mutation (7481 ins A in exon 52). Neither mutation had previously been reported in A-T patients. Western blot analysis showed a low level of ATM protein which was able to phosphorylate p53ser15 significantly. We cannot exclude that neurological signs were present in II.1 before the age of 27 years but certainly these must have been very mild to have escaped medical observation. Therefore, patients II.1 and II.2 are among the mildest A-T variant phenotypes so far described. We suggest that the late onset of the disease and the milder phenotype can be explained by residual activity of the mutated protein.

CASE REPORTS

The proband (II.1, fig 1) was born to non-consanguineous parents originating from the same town in southern Italy. She was well until the age of 27 years, when she presented with unsteady gait and dyskinetic movements of the trunk, head, and limbs. At 42 years she was admitted for neurological assessment, with marked paretic gait and "stéppàge", hypotrophy and hyposthenia of the four limbs, moderate dysarthria, chorea, and dystonia, and mild impairment of mental function (15% decrease with the Wechsler-Bellevue scale). Ankle and radial reflexes were absent, deep and shallow sensitivity was intact, and the cutaneous plantar reflex was normal. No alterations of the segmental cerebellum were evident and MRI of the brain showed moderate enlargement of subarachnoid spaces and normal cerebellum. EMG showed moderate signs of neurogenic impairment of the lower limbs. The patient did not show telangiectasia or abnormal eye

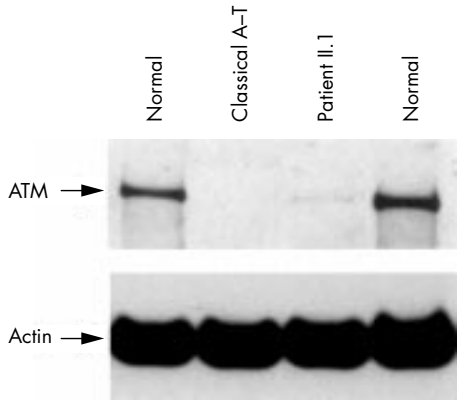

Figure 3 Western blot analysis of the ATM protein in patient II.1. Ficoll extracted peripheral blood lymphocytes from patient II.1 were transformed with Epstein-Barr virus to establish a lymphoblastoid cell line, and protein preparation and western blot were performed as described previously.[15] ATM antibody recognises the N-terminal portion of the ATM protein (antibody FP8[22]). Actin was used as a protein loading control. ATM is considerably reduced compared to controls (normal), but retains a minimal amount of full length product (<10%) as compared to a classical A-T patient.

movements, but an exudative maculopathy (right eye) with strabismus since childhood was noted. Haematological parameters were normal (IgM 1.41, IgA 3.35, and IgG 11.9 g/l), apart from serum α-fetoprotein, which was greatly increased (328.2 ng/ml, normal <10.9). Acanthocytes were absent and endocrinological assessment was in the normal range. The patient was given a generic diagnosis of "encephaloneuropathy of genetic origin".

The 44 year old sister (II.2) with a similar phenotype except for strabismus refused to undergo further diagnostic investigation. Both sisters have short stature (137 cm, <5th centile) compared with their sib. Sibs II.3, II.4, and II.5 and the father are in good health while the mother died from a stroke at 63 years. A female first cousin of the proband was affected by dyskinetic movements at 40 years and her brother was mentally retarded; their parents are the brother and the sister of the proband's father and mother, respectively.

Cytogenetic and molecular studies

Cytogenetic analysis was performed on peripheral blood lymphocytes of patient II.1. Cells were separated using Emagel (Hoechst, Germany), enriched with red cells, and incubated with phytohaemagglutinin (HA15, Murk, France) for 72 hours. Colcemid was added for the last two hours. Metaphase spreads were G-Wright banded and 27.4% of mitoses (23/84) showed translocations involving chromosomes 7 and 14. This finding of chromosomal instability is indicative both of A-T and NBS syndromes. The phenotype of our proband (II.1), while consistent with an A-T variant, also partially overlapped NBS for short stature and mental impairment. Moreover, a similar clinical phenotype with typical features of A-T and in addition microcephaly and mental retardation has also been described and named A-T-Fresno.[21]

Linkage of the patient's phenotype with the *NBS* locus (8q21) (markers D8S1811, D8S271, and D8S273, kindly provided by Professor C Sperling) and the *ATM* locus (11q23) (markers D11S2179 and D11S1818) was performed using fluorescently labelled primers with the ABI PRIM 377 sequencer and Genescan 2.1 software (Applera). This analysis excluded the *NBS* but not the *ATM* locus (fig 1).

Mutation screening of the *ATM* gene by PTT did not show any alteration. Each fragment was therefore sequenced and showed two variants (fig 2). One was a frameshift in exon 52 (7481insA) which produces a stop codon at position 7493 (codon 2498) causing protein instability and loss (fig 2A). Retrospectively, we noted that our PTT conditions had failed to detect this alteration in fragment 5, since the 66.7 kDa normal and the 61.4 kDa mutated protein were unresolved, and in fragment 6, since the 4.8 kDa protein had run out of the gel. The mutation was confirmed by direct sequencing of exon 52 from genomic DNA.

The second mutation was an A to G transition at nucleotide 8030 in exon 57, which changes a Tyr to a Cys at codon 2677 corresponding to a residue near the PI-3 kinase domain (fig 2B). Y2677C, confirmed at the genomic level, does not change ATM charge and hydrophobicity, and was excluded as a polymorphism because it was the only substitution observed in the complete coding sequence and it was not present in 100 control chromosomes screened by allele specific oligonucleotide hybridisation. The importance of Y2677 is also supported by its orthologous conservation. Neither mutation has been reported previously. Both mutations were confirmed in II.2 sharing the same genotype with II.1 and the 7481insA was also found in the heterozygous brother II.3.

DISCUSSION

To date, five missense mutations have been described in A-T variants: 7271T>G (V2424G in exon 51),[14–22] 8480T>G (F2827C in exon 60),[14] 7967T>C (L2656P in exon 56),[23] 6047A>G (D2016G in exon 43), and 1709T>C (F570S in exon 13)[24]; like Y2677C reported here, all reside outside the PI-3 kinase domain (aa 2857-2915). This could suggest that missense mutations outside the catalytic domain are associated with a residual ATM activity and result in the attenuated neurological signs observed in A-T variants.

Western blot analysis on patient II.1 showed a low level of full length protein (<10%, fig 3) probably produced by the allele carrying the Y2677C missense mutation. These findings suggest that the milder phenotype observed in A-T variants and II.1 could be the result of some residual function of the ATM protein. Many of the functions of ATM occur via modulation of p53, which is phosphorylated in ser15 after ionising radiation exposure mainly by ATM and after a longer interval by its related kinase ATR.[25–27] As a means of investigating the residual kinase activity of this mutant ATM protein, we assayed its ability to phosphorylate p53ser15 in the lymphoblastoid cells of II.1. The results showed a significant ability to phosphorylate p53ser15 at short times after irradiation, characteristic of phosphorylation by ATM, but less than is seen in normal cells (fig 4).

A major role of ATM is to regulate p53 responses which result in G1 arrest after exposure of the cell to some forms of DNA damage, particularly DNA double strand breaks caused by ionising radiation (IR). ATM also has role in the S phase and G2/M phase checkpoints following exposure of cells to IR.[28–29] Activated ATM may exert its effect on p53 activity and stability by mediating simultaneous phosphorylation of both partners of the p53-MDM2 autoregulatory feedback loop and also Chk2.[30] In A-T cells, where functional ATM is lost, the G1/S checkpoint is altered and cells accumulate genomic alterations. This instability could lead to a survival of damaged neuronal cells and to a selective neurodegeneration, as seen in cerebellar Purkinje and granular layer cells.[31] The ability of the Y2677C protein to phosphorylate p53ser15 is an indication of retained kinase activity which may also allow some phosphorylation of other targets of ATM. The partial retention of p53ser15 phosphorylation in our patient might directly explain the mild symptoms and slow progression of the disease. However, while the stabilisation of p53 following ionising radiation is delayed in A-T cells, the kinetics and magnitude of their response in NBS and A-TLD cells are essentially unaffected,[16–20] suggesting that ATM/p53 is not the sole pathway involved in cerebellar degeneration.

Since p53 loss is also critical to tumour development, retention of partial p53 phosphorylation could also explain the

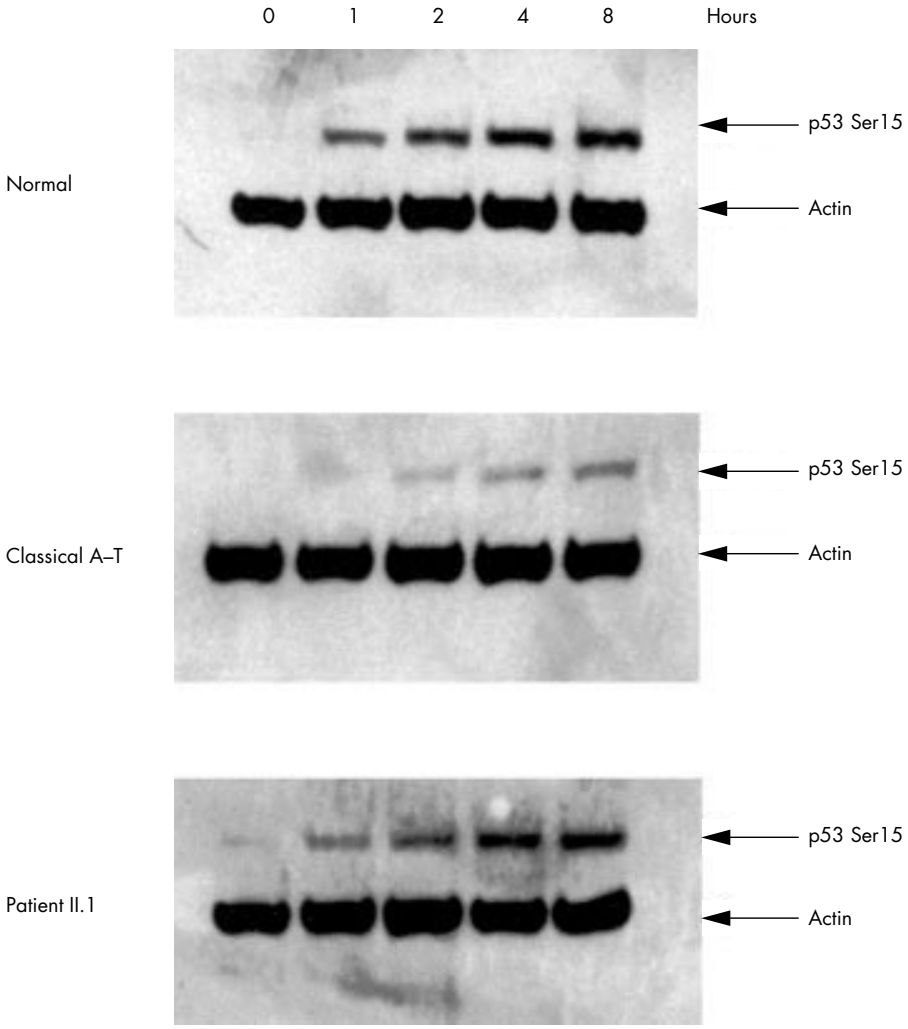

Figure 4 p53ser15 phosphorylation in an irradiated cell line of II.1. A residual p53ser15 phosphorylation is evident in cells derived from patient II.1 compared with a classical A-T patient and a normal subject, following exposure to ionising radiation. Cells were irradiated with 5 Gy of ionising radiation and harvested at one, two, four, and eight hours after irradiation. Whole cell lysate was separated on a SDS-PAGE gel and western blots were hybridised with p53 phosphoserine-15 (NEB) and actin.[15]

absence of lymphoid tumours in this variant. A functional link between ATM and p95/Nbs1 has been reported; after ionising radiation p95/Nbs1 is phosphorylated on ser343 in an ATM dependent manner in vitro and in vivo.[32] It is noteworthy that p95 is part of a multiprotein complex hRad50/hMre11/Nbs1 involved in the DNA damage response to double strand breaks[33] and *hMRE11* is the gene mutated in A-TLD.[16] The alteration of this pathway results in loss of the ionising radiation induced S phase checkpoint that is a common phenotype of both A-T and NBS cells.[32–34] ATM is, however, a key protein involved in several networks as proven by the many reported interacting partners (BRCA1, c-Abl, p34, CtIP, β-adaptin, RPA).[35–40] Therefore, other roles of ATM, such as alteration in cell membranes or cell signalling that depend on ATM, could be important and partially maintained in our patient. For instance, since both A-TLD and NBS patients do not show telangiectasia, the altered hRad50/hMre11/Nbs1 complex might not be responsible for this phenotype. The absence of this clinical feature in our patient suggests that the ATM dependent pathway leading to telangiectasia is not compromised.

A-T patients have very high levels of chromosome translocations in their peripheral lymphocytes involving breakage in the T cell receptor genes. This shows involvement of ATM in V(D)J recombination.[41] The high frequency of t(7;14) translocations in this patient is interesting when compared with other milder cases of A-T, where the frequency of translocations tends to be lower than in classical A-T patients. The absence of a lymphoid tumour in these sibs may suggest some protection against such a tumour, especially T-PLL, but this is not certain and the presence of mutant protein may be an indication of risk of another tumour type.

Further molecular analyses of this A-T variant are needed to identify the pathways that regulate the severity of clinical symptoms. Their characterisation, also through expression profiles from microarray analysis, could help our understanding of the several functions of the ATM protein.

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In vitro analysis of aminoglycoside therapy for the Arg120stop nonsense mutation in RP2 patients

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X linked retinitis pigmentosa (XLRP) is a heterogeneous disease causing a severe form of retinal degeneration. Patients typically present with night blindness and constricted visual fields within the first two decades of life owing to peripheral photoreceptor degeneration. As the disease progresses, impairment of central vision occurs resulting in loss of visual acuity and complete functional blindness often by the age of 40-50 years.[1] The gene that causes one form of this disease, *RP2*, has recently been positionally cloned and has been shown to account for between 15-20% of XLRP.[2] The *RP2* gene consists of five exons encoding a polypeptide of 350 amino acids and is ubiquitously expressed. There are currently few functional data available about this protein. One functional clue is a similarity to cofactor C with the predicted RP2 amino acid sequence having 30.4% identity over 151 amino acids.[2] Cofactor C was initially thought to play a role in the folding of β-tubulin,[3 4] suggesting that RP2 could also be involved in tubulin biogenesis. A recent study has shown, however, that RP2 undergoes N-terminal acyl modification and thus has a predominantly plasma membrane localisation in cultured cells.[5] For this reason it seems unlikely that RP2 functions exclusively in tubulin folding and its precise function and any specific role in the retina are at present unknown. Mutation screening in XLRP patients has identified over 20 different pathogenic mutations in the *RP2* gene, including missense, frameshift, insertion, and deletion changes.[2 6–11] There are also three different identified nonsense mutations causing a premature stop within the first two exons of the gene.[2 6–8]

In this study, we have investigated a potential drug mediated therapy to restore RP2 function in patients with nonsense mutations, in particular the opal nonsense mutation converting CGA (arginine 120) to TGA (termination codon) at nucleotide position 358 in exon 2, which is present in a large pedigree from Moorfields Eye Hospital.[7] The aminoglycoside antibiotics, such as gentamicin, have been shown to suppress premature stop codons both in transcription/translation reactions, cultured cells, and whole animals.[12–20] This interesting and potentially clinically beneficial phenomenon is believed to be caused by aminoglycoside antibiotics interacting with ribosomes during translation, reducing the usual stringency of codon-anticodon pairing.[12 13] This sometimes results in the insertion of alternative amino acids at the site of the internal stop codon of the mutated gene, thus permitting the ribosomes to continue reading through to the end of the gene and make the full length polypeptide. It has been suggested, therefore, that aminoglycoside antibiotics could be used to treat almost any genetic disease caused by nonsense mutations. As there are several large pedigrees of subjects with this particular type of mutation in *RP2*, we have investigated the therapeutic potential of aminoglycosides for the *RP2* mutation Arg120stop as the first therapy for X linked retinitis pigmentosa. This type of treatment would have several potential advantages over a traditional gene replacement approach. There is already a large amount of pharmacological data available on the use of aminoglycoside antibiotics and the approach requires little information on the function or expression profile of RP2, so this potential therapy could be taken into the clinic relatively quickly.

In this study, we investigated whether treatment with gentamicin could restore full length protein production in an in vitro model. As RP2 appears to be ubiquitously expressed,[2 5] we used human lymphoblastoid cells from affected males, with the Arg120stop mutation, as a model to assess the effect of gentamicin on RP2 protein expression. These cells harbour the actual patient mutation in context, not engineered mutations constructed for the experiments which may not have had the same significance for the treatment of our patient group. Demonstration of effective stop codon read through in this clinically relevant mutation would have promised a new potential therapy for the clinic; however, our results suggest that aminoglycoside therapy is not a practical treatment at this stage for the Arg120stop nonsense mutation in RP2.

METHODS

Cell culture

Human lymphoblastoid cell lines corresponding to a large Moorfields pedigree with the Arg120stop nonsense mutation in RP2[7] and randomly selected control males were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). Cells were maintained in suspension culture in RPMI 1640 Glutamax-I (Life Technologies, Paisley, UK) supplemented with 10% fetal bovine serum (Sigma, Poole, UK) with media changes every two to three days. For aminoglycoside antibiotic treatment, cells were cultured for 12 hours, three days, and 10 days in the presence of 0-500 μg/ml gentamicin (Sigma), with media changes, where appropriate, every three days.

Transfections and dual luciferase reporter assay

Chinese hamster ovary cells were maintained in Dulbecco's modified Eagle's medium/F12 supplemented with 10% fetal bovine serum. Transfections were performed using lipofectamine reagents (Life Technologies). The reporter plasmid p2luc constructs were a gift from Professor John Atkins (University of Utah). Briefly, 1×10^5 Chinese hamster ovary cells were plated on to 12 well plates and grown for 48 hours. Cells were transfected with 300 ng of plasmid DNA for 15 hours in serum free media. Fresh media containing serum and varying levels of gentamicin were added for 24 hours before being assayed. Cells were lysed and luciferase activity was determined using the dual luciferase reporter assay (Promega, Southampton, UK) according to the manufacturer's protocols. Stop codon readthrough was calculated by comparing the ratio of firefly to renilla luciferase activity in cells transfected with p2luc stop codon constructs, relative to the ratio of luciferase activity in cells transfected with p2luc control constructs.[19 21]

Preparation of cell lysates

Lymphoblastoid cells were Dounce homogenised on ice in 20 mmol/l Tris-HCL, pH 7.5, 500 mmol/l NaCl, 12.5 mmol/l KCl, 1 mmol/l EDTA, 1 mmol/l dithiothreitol containing a protease inhibitor cocktail (Sigma). The concentration of protein in the homogenates was determined using the Bio-Rad (Hemel Hempstead, UK) DC assay, following manufacturer's protocols.

Immunoblotting

The cell lysates were prepared for electrophoresis by the addition of sample buffer (100 mmol/l Tris-HCl, pH 6.8, 2% glycerol, 0.4% SDS, 1% 2-β mercaptoethanol, 0.01% bromophenol blue, final concentration) and heating to 96°C for five minutes. A total of 50 µg or 100 µg of total protein were loaded on a 12% SDS-polyacrylamide gel and after electrophoresis were electroblotted onto nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). Non-specific binding sites on the nitrocellulose were blocked by incubation with 5% marvel, 1 × PBS, 0.05% Tween 20 overnight at 4°C. The blots were hybridised with sheep polyclonal antisera S974, raised against full length recombinant His tagged RP2, and affinity purified[5] at a titre of 1:1000 for one hour at room temperature. Anti-sheep polyclonal secondary antibody conjugated to peroxidase (Sigma) was used at a titre of 1:2000. Immunoreactive bands were visualised using enhanced chemiluminescence (Amersham Pharmacia, Little Chalfont, UK). Standards containing known amounts of the recombinant His tagged RP2 were included to ensure sufficient minimum detection levels of protein. The immunoreactive bands were quantified and compared using imaging software (Kodak Digital Science™ 1D image analysis software, Eastman Kodak Company, Rochester, NY).

RT-PCR

RNA was extracted from patient and control lymphoblastoid cells using the RNeasy kit from Qiagen (Crawley, UK) following the manufacturer's protocols. RT-PCR was performed using the reverse transcription system from Promega (Southampton, UK) to obtain the first strand cDNA using oligo(dT) primers as per the manufacturer's protocol. PCR was performed using exonic primers RP2-5 (GCGGATCCGCCATGGGCTGCTTCTTCTCCAAGAG) and RP2-1-199 (GGATCCTCTAGATCAAACATAGTCCTGAACCACAGC). The following conditions were used: 94°C for five minutes, then 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for one minute, then a final extension of 72°C for five minutes.

Confocal microscopy of cells

Lymphoblastoid cells from RP2 patients and male controls were washed with PBS and trypsinised to reduce clumping of the cells. After further PBS washes, the cells were fixed in 3.7% formaldehyde followed by detergent permeabilisation in 0.05% Triton X-100. Non-specific binding sites were blocked by incubation with 3% bovine serum albumin, 10% normal rabbit serum, and PBS for one hour at room temperature. Affinity purified S974 was used at a titre of 1:250 for one hour at 20°C. Cy™3 conjugated anti-sheep secondary antibody (Jackson ImmunoResearch, West Grove, USA) was used at a titre of 1:150 for one hour at 20°C. The stained lymphoblastoid cells were fixed to glass slides by cytospin centrifugation and analysed using a Zeiss laser scanning confocal microscope.

RESULTS

RP2 expression in control and patient lymphoblastoid cells

The expression of RP2 was investigated in lymphoblastoid cells from an unaffected control male and compared with RP2 expression in human SH-SY5Y neuroblastoma cells and human retina by western blotting using antisera S974, as previously characterised[5] (fig 1). The predicted molecular weight of the 350 amino acid RP2 is 39.6 kDa. A band of approximately this size was observed in all of the samples. The expression levels of RP2 in the lymphoblastoid cells were much higher than in human retina but lower than in the SH-SY5Y cells; these differences agree with previously estimated RP2 expression levels in human retina and SH-SY5Y cells.[5] Interestingly, sera S974 appeared to cross react with another protein of 68 kDa that was present only in the human lymphoblastoid cells and not in retina, SH-SY5Y cells, or any other tissues examined. The reactivity of S974 with this 68 kDa protein and RP2 could be removed by blocking S974 with 100 µg of recombinant RP2 protein during the antibody incubations (data not shown), suggesting that this lymphoblastoid protein may share epitopes with RP2.

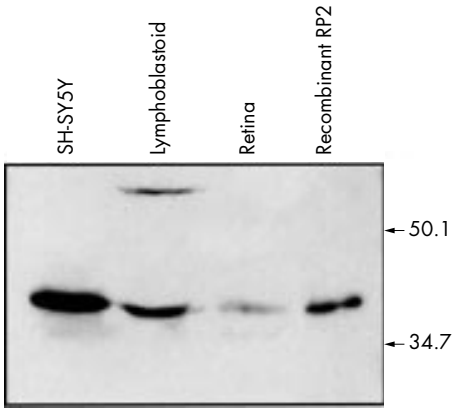

Figure 1 Detection of RP2 in control male lymphoblastoid cells (50 µg) compared with SH-SY5Y cells (50 µg) and human retina (50 µg). Ten ng of 9 × histidine tagged recombinant RP2 was loaded as a standard. Western blotting was performed with affinity purified polyclonal antisera S974.[5] The positions of molecular weight markers are indicated by arrows on the right.

RP2 mRNA expression in the lymphoblastoid cells from males affected with the Arg120stop mutation and cells from control males was also investigated. A full length *RP2* transcript was detected in both the male patient and the control male lymphoblastoid cells (data not shown). The expression levels of *RP2* mRNA, however, were greatly reduced in the male patient cells compared to the male control cells, suggesting that *RP2* containing the Arg120stop mutation may undergo nonsense mediated decay.[22]

RP2 protein expression in males affected with the Arg120stop mutation and control males was investigated in lymphoblastoid cells (fig 2A). The expression levels of RP2 protein were compared between the cells from five male RP2 patients and five control males. A band of the predicted size for RP2 was observed consistently in all of the control cells, but no band of the expected size for full length RP2 or the predicted 119 amino acid truncated protein product was detectable in any of the patient cells. We have recently shown that sera S974 is immunoreactive with the first 15 amino acids of RP2 (data not shown), indicating that any truncated protein produced is rapidly degraded. The immunoreactive band corresponding to the 68 kDa protein was observed in both the patient and the control lymphoblastoid cells, suggesting that, although the protein may share epitopes with RP2, it does not correspond to a post-translational modification of RP2 and is not a product of the *RP2* gene (fig 2B). Although the basis for the cross reactivity between S974 and this 68 kDa protein remains to be resolved, it did provide a useful internal loading and transfer control for the western blot assays.

Determining the minimum detection levels of RP2

It was necessary to determine the minimum detection level of the RP2 protein before analysing the effects of aminoglycoside therapy on RP2 expression. From comparison to recombinant RP2 standards, we estimate that RP2 protein represents approximately 0.085% of the total protein in lymphoblastoid cells. This figure is higher than in other human tissues where RP2 protein has been estimated to represent approximately 0.01% of the total protein.[5] A standard curve of recombinant

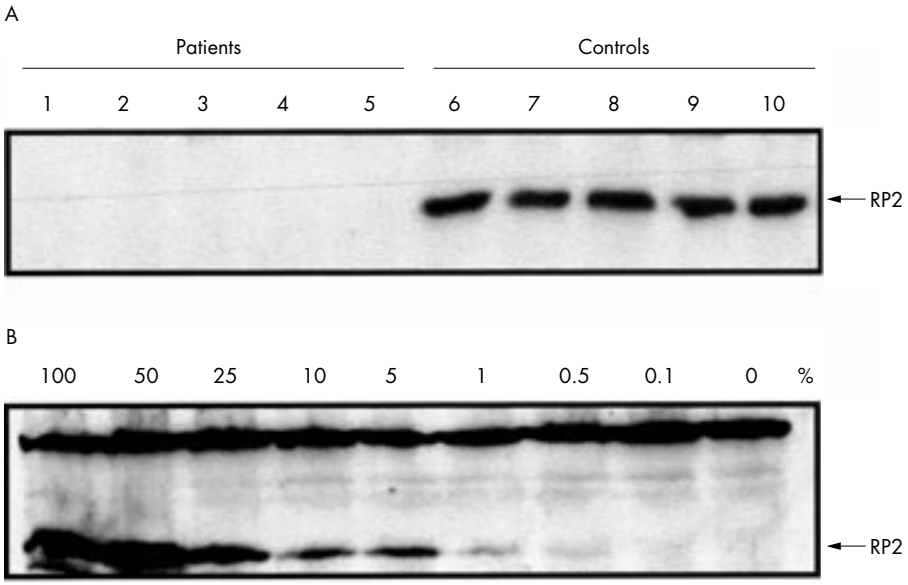

Figure 2 (A) Expression of RP2 in lymphoblastoid cells from RP2 patients with the Arg120stop mutation (50 µg) and control males (50 µg). (B) Minimum detection levels of RP2. Control lymphoblastoid cell lysates were mixed with RP2 patient lymphoblastoid cell lysates in a variety of ratios. The percentages of control cell lysate loaded into each lane were 100, 50, 25, 10, 5, 1, 0.5, 0.1, and 0 %; 100 µg of total protein was loaded into each lane. Western blotting was performed with affinity purified polyclonal antisera S974.[5]

His tagged RP2 protein of known concentration was used for immunoblotting (data not shown). Using our methodology, we could easily detect below 0.5 ng of recombinant RP2, or approximately 0.4% of the total RP2 expressed in control lymphoblastoid cells. This detection limit was confirmed by mixing RP2 patient lymphoblastoid cell lysates and control lymphoblastoid cell lysates in different fixed ratios and loading the same amount of total protein into each well. This confirmed that the western assay could detect at least 0.5% of control RP2 levels (fig 2B).

Subcellular localisation of RP2

The subcellular localisation of RP2 in lymphoblastoid cells from control males and RP2 Arg120stop patients was investigated by immunofluorescent staining and confocal microscopy. RP2 protein localisation in the lymphoblastoid cells from control males was predominantly on the plasma membrane of the cells (fig 3A). The intensity of plasma membrane staining was variable within each individual cell and also between cells. This observation is in agreement with recently published data on the subcellular localisation of RP2 in other cultured cell types.[5] In addition to the plasma membrane staining, a diffuse stain was also observed throughout the cells but at a much lower intensity. The plasma membrane staining was not seen in lymphoblastoid cells from patients (fig 3B). The staining pattern in the cells from the patients was much more diffuse and did not appear to have a specific subcellular localisation. A similar staining pattern was seen in lymphoblastoid cells from the control males and RP2 patients probed with the Cy™3 conjugated anti-sheep antibody alone, without a previous S974 primary antibody incubation (fig 3C, D). Cross reactivity with human tissues appears to be a problem in many commercially available anti-sheep antibodies. Therefore, the diffuse intracellular staining pattern may in some part correspond to cross reactivity between the human lymphoblastoid cells and the secondary antibody and the reactivity between S974 and the 68 kDa protein.

Gentamicin treatment of cells

To determine whether gentamicin could lead to suppression of the premature stop codon in RP2 patients, lymphoblastoid cells from male patients with the Arg120stop mutation were cultured in the presence of various gentamicin doses ranging from 0-500 µg/ml for 12 hours, three days, and 10 days. At doses above 500 µg/ml, significant cytotoxicity was observed within the 12 hour period. Even with sensitive, calibrated western blots with detection levels down to below 0.4% of control RP2 levels, we could not detect any increase in full length or truncated RP2 protein expression in these cells after treatment with gentamicin at all of the tested doses and time points (fig 4).

The efficacy of the gentamicin used in this study was confirmed using a dual luciferase reporter system, which uses firefly and renilla luciferase coding sequences separated by stop codons or control codons in different sequence contexts.[19 21] We examined the effect of gentamicin on two constructs encoding the opal stop codon UGA. We compared the intervening sequence that contained the sequence UGAG, the stop codon context in the *RP2* Arg120stop mutation, with the intervening sequence UGAC, the opal stop codon context found to achieve the greatest levels of aminoglycoside induced readthrough.[19] Using the same methodology used in these earlier studies, we achieved gentamicin induced stop codon readthrough of between 1.5% and 5% for the sequence UGAC, similar to readthrough levels achieved in previous studies, using gentamicin at concentrations of up to 500 µg/ml. However, the base immediately after the stop codon had a profound effect on the efficiency of gentamicin induced translation. By substituting the cytosine base for a guanine base, as found in the Arg120stop mutation, readthrough levels were reduced by over 75%.

DISCUSSION

Aminoglycoside antibiotics have been shown to suppress nonsense mutations both in vitro and in vivo.[12–20] The primary aim of this study was to investigate whether this phenomenon could be used as a potential treatment for RP2 patients with the Arg120stop nonsense mutation.[7] We have shown that, at present, aminoglycoside antibiotic therapy does not appear to be a viable therapy for RP2 patients with this mutation. No RP2 protein could be detected in cells from affected males treated with a wide range of doses and treatment time points of the aminoglycoside antibiotic, gentamicin.

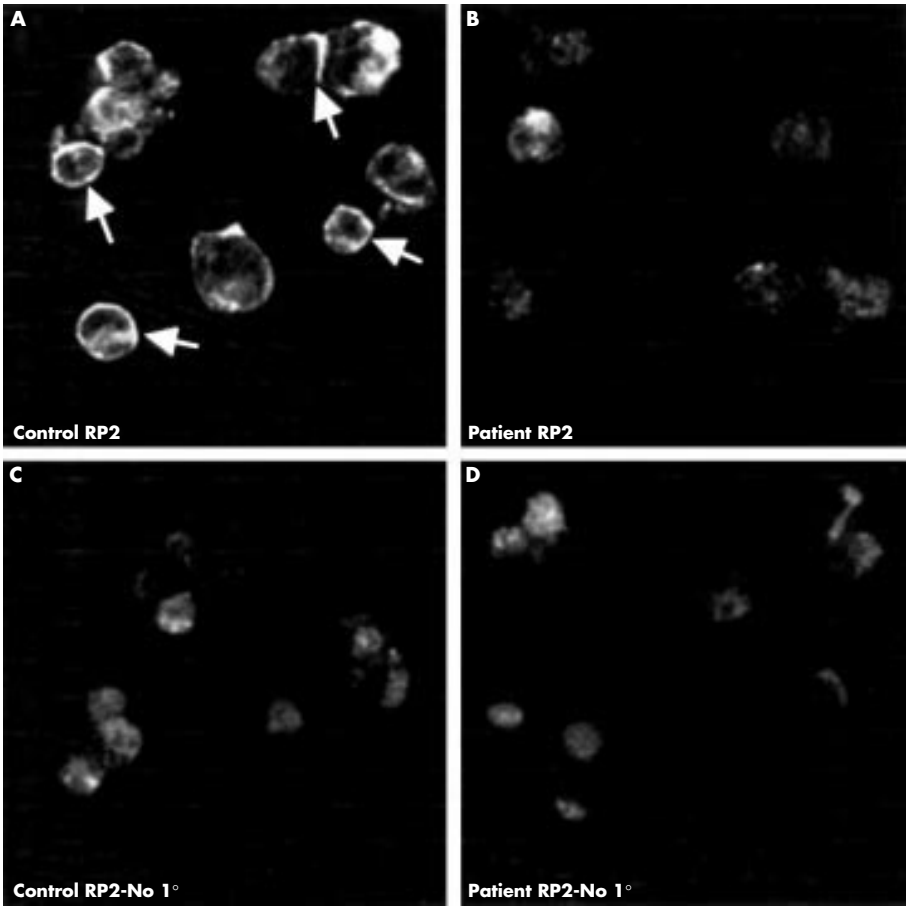

Figure 3 Subcellular localisation of RP2 in lymphoblastoid cells. Confocal immunofluorescence showing localisation of RP2 in control male lymphoblastoid cells (A) and in RP2 patient lymphoblastoid cells (B). Control staining with the Cy™3 labelled anti-sheep antibody alone in the control male and patient lymphoblastoid cells are shown in (C) and (D) respectively. Arrows highlight plasma membrane staining. All images are 72 μm × 72 μm.

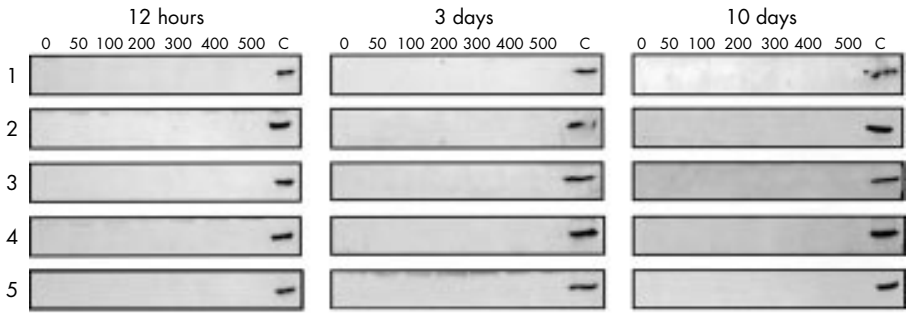

Figure 4 Gentamicin treatment of cells. Representative western blot showing RP2 expression in lymphoblastoid cells of five RP2 patients with the Arg120stop mutation. The patient cells were treated with gentamicin at the three different time points shown. The concentrations of gentamicin used to treat the cells were 0, 50, 100, 200, 300, 400, and 500 μg/ml. Control RP2 expression in lymphoblastoid cells (C) from a control male is also shown; 100 μg of total protein was loaded into each lane. No RP2 expression could be detected in the patient cells at any exposure; recombinant standards (not shown) were loaded to ensure sensitivity of RP2 detection of less than 0.4% of RP2 in control cells.

Studies on the cystic fibrosis transmembrane conductance regulator (*CFTR*) suggested that full length CFTR protein production in transfected HeLa cells and bronchial epithelial cells expressing an opal (UGA) nonsense mutation could be restored to levels of 20-35% of that in wild type cells after treatment with aminoglycoside antibiotics at doses of 100-200 μg/ml.[14 15] Furthermore, the CFTR produced appeared to be functional by investigating cyclic AMP-activated chloride channel activity in the cells. Similar observations were made in an in vivo model for Duchenne muscular dystrophy, the *mdx* mouse,[16] where, before aminoglycoside antibiotic treatment, muscle expressed no full length dystrophin protein. After treatment with gentamicin, however, dystrophin levels were detectable at up to 10-20% of that in muscle of control mice expressing the wild type dystrophin protein, and the mice displayed a significant degree of protection against contractile induced damage, suggesting some functional recovery. Recent studies, however, have brought into question the accuracy of

these high levels of protein restoration and suggest that the true level of readthrough may be much lower, possibly less than 5%, while highlighting the importance of the context of the stop mutation.[18 19] This lower level of protein restoration may still have clinical applications. A study of premature stop codons in the Hurler syndrome gene, *IDUA*, has suggested that restoration of activity to 3%, mediated by readthrough, may be sufficient to mediate some cellular recovery.[20] In our study, however, we observed no restoration of full length RP2 protein production to less than 0.5% by western blotting in our opal mutation cell lines on gentamicin treatment at the doses and times that have been shown to be effective in these paradigms. As the function of RP2 is unknown at present, it was not possible to investigate the restoration of any function in the cells or to know what levels of RP2 expression would be necessary for some function to be restored. Once the function of RP2 has been elucidated, a more sensitive assay may be used to test the effectiveness of aminoglycoside antibiotic treatment for the Arg120stop nonsense mutation.

The data in this study have shown a clear difference in RP2 subcellular localisation between the RP2 patient and control lymphoblastoid cells and this could have been used to determine if any full length protein produced was correctly targeted. However, owing to the background cross reactivity of the secondary antibody and the potential problems caused by the cross reacting 68 kDa protein, immunoblotting represented a more sensitive detection method for RP2. By immunoblotting we were able to detect full length protein down to levels of less than 0.4% of wild type protein.

Although the potential use of aminoglycoside therapy for nonsense mutations is very attractive, our observations that full length protein production was not restored in cells with the Arg120stop nonsense mutation show that there may be severe limitations to their application. A major consideration is that mRNAs with premature stop codons may be subject to their own quality control via nonsense mediated decay (NMD).[22] We have observed that although the patient cell lines do express the *RP2* mRNA, it appears to be present at much lower levels in the patient cells than in controls. This level of RNA surveillance reduces the amount of mutant mRNA available for translation and readthrough assistance from the aminoglycoside. Furthermore, the type of nonsense mutation and composition of the RNA sequence flanking the stop codon may also have a major effect on the efficiency of aminoglycoside mediated readthrough.[12 13 18 19 23 24] Although the opal premature stop mutation, UGA, which was investigated in our study, appears to show greatest translational readthrough, the nucleotide in the position immediately downstream from the stop codon appears to be a major modifier (in the order C > U > A > G).[18 19] The presence of a G immediately after the stop codon, as in the Arg120stop patients, reduced readthrough levels by up to 75%. On the basis of the type of mutation and its immediate context, it could be predicted that readthrough of up to 3% could occur in our model.[19] However, the effects of other context characteristics in the mRNA on readthrough are unknown, such that the combination of mutation context and the lower levels of *RP2* mRNA caused by NMD result in levels of readthrough below the sensitivity of our western assay.

It should also be noted than in the in vivo model of *mdx*, not all animals with the same nonsense mutation responded to the gentamicin treatment.[16] This raises the possibility that even if the "context" of the mutation is conducive for the aminoglycoside treatment to be effective, other factors may modulate the beneficial effect of the drug. As variation within different people may be important, we investigated the effect of gentamicin on the Arg120stop mutation covering all of the previously tested doses and time points in lymphoblastoid cells from five male RP2 patients. It should be noted that doses of gentamicin higher than 500 μg/ml were not used in this study as there was evident toxicity to the lymphoblastoid cells at 500 μg/ml and higher doses. It is possible, however, that the lymphoblastoid cells have cell line specific differences in RP2 expression, NMD, and aminoglycoside mediated readthrough distinct from retina. Nevertheless, it must be taken into consideration that these patient derived cell lines possess the stop mutation within the actual context of the RP2 gene and within the context of the patients' genetic background. Therefore, they represent a much better in vitro model system in which to study these compounds' therapeutic potential than cell free transcription/translation or transfection of standard cell lines.

Even if aminoglycoside therapy had proven to be a potential treatment for *RP2* patients, it would still have been important to consider their possible toxicity. Barton-Davies *et al*[16] have suggested that administration of gentamicin below the maximum recommended human dosage (for antibiotic use) could prove effective in restoring protein function. There are, however, no data available on the consequences of the long term use of this drug, particularly at the high doses that may be required for effective readthrough therapy. Retinal toxicity of gentamicin should also be given careful consideration before any treatment of patients with ocular disease. The toxicity of gentamicin in the retina is well documented, the majority of cases being reported after its prophylactic use in vitrectomy or routine ocular surgery, at doses considered to be safe.[25] There is also evidence of gentamicin toxicity in primates after intravitreous injection, leading to damage within the inner retinal layers.[26] Aminoglycoside antibiotics cause full length polypeptides to be made as they interfere with the usually stringent codon-anticodon pairing during translation, causing alternative amino acids to be inserted in the place of the premature stop codon. This means that as well as inserting the correct amino acid to produce functional full length protein, it may also introduce erroneous insertions of other incorrect amino acids leading to the possible production of aberrant proteins with unknown toxicity. Such proteins may not fold correctly and form aggregates, or may exhibit gain of function effects.

Another possible treatment for diseases caused by premature stop mutations could be the use of suppressor tRNA gene therapy,[27] as opposed to conventional gene replacement therapy. This is enabled by engineering mutant tRNAs that can read premature stop codons as sense codons and hence restore full length polypeptide production.[28] This may represent a more focused approach to the treatment of diseases caused by nonsense mutations, but may be unsuitable for the treatment of RP2 at present. As the function and localisation of the protein are at present unknown, it would be difficult to direct this treatment towards the necessary specific target cell, and similar problems of erroneous protein production would need to be considered as a possible side effect. However, as more information becomes available about the function of RP2, this may be a viable therapy, as there are a large number of patients with nonsense mutations in RP2.

An extremely useful finding of this study is that it should be possible to use immunoblotting or immunocytochemistry as a diagnostic test for mutations in *RP2*. As the majority of *RP2* mutations are protein truncating, most patient mutations could be detected by using immunoblotting for the presence of full length protein. Alternatively, immunocytochemistry could be used to detect mutations that also affect correct protein targeting.[5] Immunoblotting and/or immunocytochemistry with a suitable antibody, such as the sheep sera S974, as a primary screen would be relatively inexpensive and less time consuming than sequencing the whole of the *RP2* and/or the phenotypically indistinguishable *RP3* gene for every potential XLRP patient. The RP2 protein appears to be ubiquitously expressed[2 5] and we were able to show that protein truncations can be detected in lymphoblastoid cells. Therefore, a diagnostic test could be carried out using lymphocytes from blood samples that would usually be taken for DNA analysis. Choroideremia, another X linked retinal dystrophy, already

Key points

- The clinical potential of using aminoglycoside antibiotics to suppress premature stop mutations has been heightened by recent results showing their effectiveness both in vitro and in vivo. The purpose of this study was to determine whether aminoglycoside therapy could be a viable treatment for X linked retinitis pigmentosa patients with the Arg120stop nonsense mutation in *RP2*.
- The expression of *RP2* mRNA and protein in Arg120stop patient lymphoblastoid cells were compared to control cells from unaffected males. Lymphoblastoid cells from male patients with this mutation were treated with a range of gentamicin doses in an in vitro system and expression levels of RP2 protein were compared to those in control lymphoblastoid cells from males unaffected by *RP2* mutations using immunoblotting. Differences in expression levels of RP2 were determined by densitometry. *RP2* mRNA was detectable in both patients and controls, although expression levels were reduced in the patient cells. The RP2 protein was only detectable in control lymphoblastoid cells and not in patient cells by both western blotting and immunocytochemistry. When cells containing the premature stop mutation were treated with gentamicin, under a wide range of conditions, no induced expression of full length RP2 protein could be detected down to 0.4% of control levels.
- Aminoglycoside antibiotic therapy does not appear to be a viable treatment for RP2 patients with the Arg120stop nonsense mutation at the present time. The use of immunoblotting or immunocytochemistry of peripheral blood cells could, however, be a useful tool for the rapid diagnosis of new patients with protein truncating or targeting defect mutations in RP2.

has a protein based diagnostic test[29] and, as more genes are cloned, many other diseases may be diagnosed using this type of method.

This study has shown that, unfortunately, aminoglycoside therapy does not appear to be a viable treatment for Arg120stop RP2 patients at this time. We propose, however, that immunoblotting or immunocytochemistry for RP2 may be a potentially useful diagnostic tool. As more becomes known about the function of RP2, and more specifically its function in the retina, more potential therapies may be investigated.

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Okihiro syndrome and acro-renal-ocular syndrome: clinical overlap, expansion of the phenotype, and absence of *PAX2* mutations in two new families

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The Okihiro syndrome consists of Duane anomaly, radial ray defects, and deafness. There are similarities with the acro-renal-ocular syndrome in which there are radial ray and renal abnormalities and colobomas which mostly involve the optic nerve. Both malformation syndromes are dominantly inherited. We report two families with an overlapping phenotype, suggesting a common aetiology. The combination of optic nerve coloboma and renal disease is also seen in families with mutations of the *PAX2* gene. We did not find any evidence of *PAX2* involvement in our families.

The main features of the autosomal dominant Okihiro syndrome are radial ray defects of variable severity and Duane anomaly.[1 2] Sensorineural hearing loss and spinal and other skeletal abnormalities also occur, and polydactyly, hemifacial microsomia with skin tags, cardiac defects, and Hirschsprung disease have also been reported.[1 3–5] The radial ray defects may be associated with vascular abnormalities such as hypoplasia of the radial artery[6] and abnormal nerve conduction studies with reduced or absent motor response from the median nerve.[1 6] The acro-renal-ocular syndrome, which is also autosomal dominant, consists of radial ray defects, renal anomalies, and ophthalmological abnormalities, mainly colobomas, but also microphthalmia, ptosis, and Duane anomaly.[7–12] There is considerable overlap between the two syndromes, and it has been suggested previously that they are one clinical entity.[8] In addition, mutations in the *PAX2* gene have been documented in patients with optic nerve colobomas and renal malformation.[13–15] There are also similarities to Wildervanck syndrome, Goldenhar syndrome, and thalidomide embryopathy. This can lead to considerable difficulties with classification in individual families. We describe four affected subjects in two new families with features of both Okihiro syndrome and the acro-renal-ocular syndrome and investigate the possibility of *PAX2* involvement.

Figure 2 Arms of patient 2.

FAMILY 1

Patient 1

The female proband was the second child of unrelated parents. She was born with severe upper limb defects, had a preaxial extra digit surgically removed from the right hand, and was diagnosed as having bilateral Duane anomaly in early childhood. She was referred to the Genetics Centre at the age of 24 years. On examination, she had a longer arm on the right with an elbow, a shortened forearm, and a hand with four digits. The left upper arm was severely shortened and three digits were directly attached to the shortened forearm. The thumbs were absent bilaterally. Both humeri were shortened and the radii were absent. Ophthalmological examination showed bilateral latent fine nystagmus and slightly dysplastic optic discs, and orthoptic assessment showed bilateral Duane anomaly with severe limitation of eye abduction (fig 1A, B, C). ECG and renal ultrasound did not show any abnormalities and an audiogram showed mild bilateral conductive hearing loss only. Her karyotype was 46,XX.

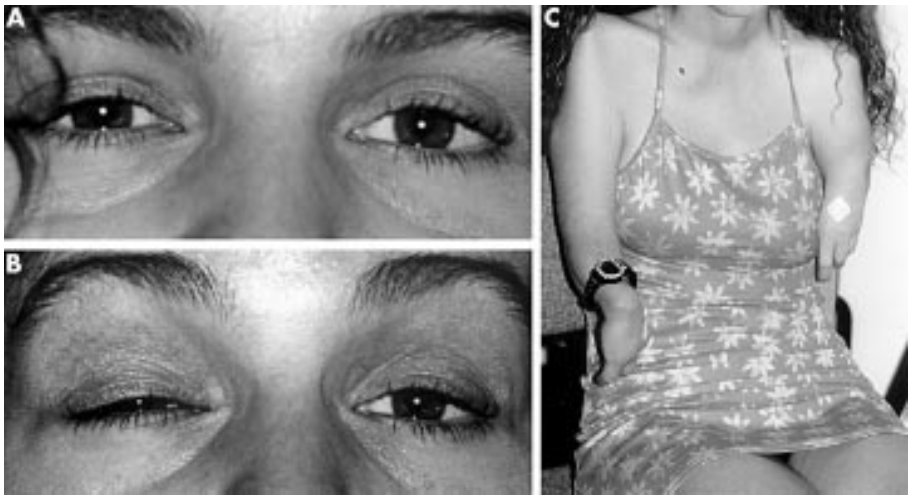

Figure 1 (A) Eyes of patient 1 in neutral position. (B) Duane anomaly on left lateral gaze. (C) Arms of patient 1.

Patient 2

The mother of case 1 was noted to have absent thumbs and shortened forearms at birth (fig 2). She was otherwise well and ophthalmological examination was normal. A renal ultrasound showed a normal right kidney and a smaller left kidney in pelvic position, situated in the left iliac fossa.

An audiogram showed bilateral, moderate, low frequency conductive loss and significant bilateral, high frequency sensorineural hearing loss, of which the patient had previously been unaware. An ECG was normal and chromosomes were 46,XX. Her parents had died and no other family members were known to have any limb abnormalities.

FAMILY 2

Patient 3

The female proband was the second child of unrelated parents. She presented as a neonate with absent left thumb and hypoplastic right thumb (fig 3A, B). Visual inattention and abnormal eye movements were noted by the parents in the first few weeks of life. Ophthalmological examination showed a right "morning glory" optic disc (a major dysplastic disc with a deep central pit) and a left dysplastic optic disc with a large inferior retinal coloboma. In the Genetics Clinic, unilateral, left Duane anomaly was noted, which was subsequently confirmed by detailed ophthalmological and orthoptic assessment.

She has since then developed bilateral nystagmus secondary to the visual impairment. Her development has otherwise been age appropriate to date.

An EEG, ERG/VER, cranial ultrasound, and MRI scan of the brain were all normal. A renal ultrasound showed mild left pelvicalceal dilatation and an MCUG showed grade 1 vesicoureteric reflux. Audiometry showed bilateral moderate hearing impairment which appeared to be conductive in origin, as there was middle ear effusion on the left and right total meatal occlusion with wax; a sensorineural component, however, cannot be excluded at present. The karyotype was 46,XX.

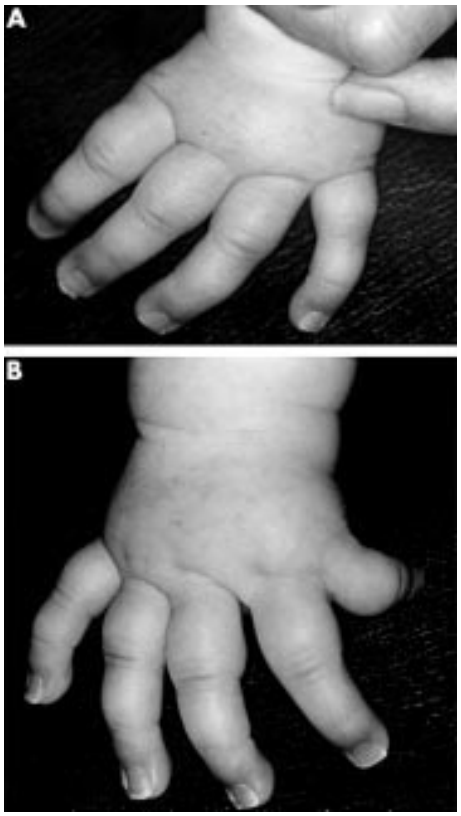

Figure 3 (A) Left hand of patient 3. (B) Right hand of patient 3.

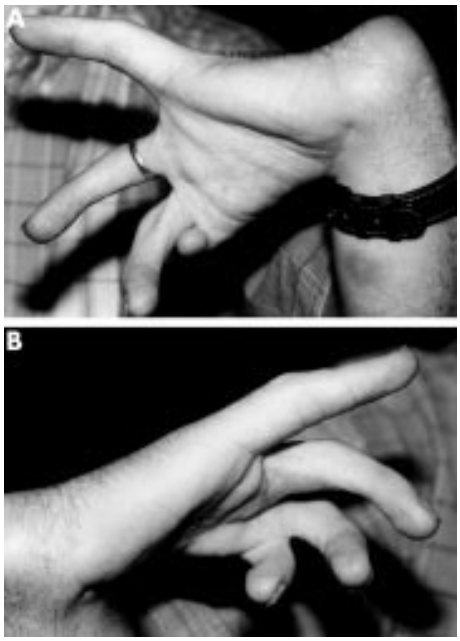

Figure 4 (A) Left hand of patient 4. (B) Right hand of patient 4.

Case 4

The father of case 3 presented with severe upper limb defects at birth which were thought to be the result of thalidomide exposure in utero (fig 4A, B). His mother had taken one single thalidomide tablet during the pregnancy. He was noted to have unilateral, right Duane anomaly in the Genetics Clinic, which was later confirmed by the orthoptist. Ophthalmological examination showed bilateral segmental optic disc hypoplasia. A renal ultrasound was normal. The patient declined a hearing test. His mother had a hearing impairment from an early age, and a female cousin had a left hypoplastic optic disc, but did not have the Duane anomaly.

MUTATIONAL ANALYSIS OF THE *PAX2* GENE

Exons 2, 3, and 4 (containing the paired box domain) and exon 5 (containing the octapeptide sequence) of the *PAX2* gene were investigated for mutations with a combination of single strand conformation polymorphism analysis and direct sequencing as previously described.[15] No mutations in the two functional domains were found in patients 2 and 4.

DISCUSSION

Six families with Okihiro syndrome and six families with acro-renal-ocular syndrome have been reported. Radial ray defects, vertebral anomalies, pre- and postaxial polydactyly, Duane anomaly, and deafness occur in families with either syndrome. Urinary tract anomalies have been described in two families with some features of Okihiro syndrome. One family presented with upper limb defects, Duane anomaly, renal agenesis, and malrotation, but the report preceded delineation of the Okihiro syndrome and the acro-renal-ocular syndrome.[7] In the second family, the affected family members were found to have a supernumerary bisatellited marker chromosome derived from chromosome 22. In addition to renal agenesis, the proband in this family showed other features such as absence of the cervix and uterus with blind ending fallopian tubes, which have not been reported in other Okihiro syndrome families.[16] The lack of reports of renal abnormalities in Okihiro syndrome families may be a reflection of the lack of renal investigations in this patient cohort. Urinary tract abnormalities are a defining feature of the acro-renal-ocular syndrome. Colobomas of the iris, choroid, and optic nerve and other ophthalmological findings

such as optic nerve hypoplasia, microphthalmia, microcornea, cataract, ptosis, and nystagmus are only mentioned in connection with the acro-renal-ocular syndrome, but retinal abnormalities may not have been specifically looked for in the Okihiro patients. The "morning glory" optic disc anomaly has not previously been reported in either syndrome; the association of the Duane anomaly and the "morning glory" anomaly is rare, but has been reported previously.[17] The "morning glory" optic disc anomaly also occurs in renal-coloboma syndrome, which is caused by mutations in the *PAX2* gene, in combination with renal disease. A locus for isolated autosomal dominant Duane anomaly has been mapped to chromosome 2q31.[18 19] It also occurs as part of a contiguous gene syndrome in patients with interstitial deletions of chromosome 8q.[20 21]

Both our families show a combination of radial ray defects, Duane anomaly, renal abnormalities (malrotation, vesicoureteric reflux), and abnormal retinal findings (optic nerve hypoplasia or dysplasia, retinal coloboma, and "morning glory" anomaly of the optic nerve). One of four affected subjects had a significant sensorineural hearing loss and two had preaxial polydactyly. Radiological examinations of the spine were not carried out in our families. No cardiac lesions were identified.

Our two families are the first to show an overlapping phenotype clinically, which suggests that the two syndromes are one clinical entity. To date, no mapping studies have been published and molecular confirmation of this clinical hypothesis is awaited. It has been pointed out by Naito *et al*[9] and Aalfs *et al*[11] that the pattern of malformations is consistent with a disturbance of embryonic development around the fourth to eighth week. The phenotypic resemblance to the pattern of malformations induced by thalidomide is interesting. The mode of action of this well known teratogen is still poorly understood, but as in chondrodysplasia punctata and warfarin embryopathy[22] teratogens and genes can act through common pathways.

Diagnostic difficulties often arise when not all features are present in an affected subject or within a family. In Okihiro syndrome, subjects with radial ray defects only[3 4] or Duane anomaly only[1 2 4] have been described. Clinically normal obligate gene carriers also occur.[4] In the acro-renal-ocular syndrome, renal abnormalities are frequent, but radial ray defects and/or ophthalmological findings may be absent.[8 9] The main features distinguishing Goldenhar syndrome from Okihiro syndrome and the acro-renal-ocular syndrome are the hemifacial microsomia and epibulbar dermoid; in Wildervanck syndrome, it is the absence of limb abnormalities. Epibulbar dermoid and preauricular tags have been described in acro-renal-ocular syndrome[8] and facial asymmetry and microtia have been reported in a subject with Okihiro syndrome.[4] Goldenhar-like features including Duane anomaly can occur in subjects with Townes-Brocks syndrome and mutations in the *SALL1* gene.[23] Renal anomalies, sensorineural hearing loss, and thumb abnormalities are also part of this syndrome, but our families did not show any anal anomalies, and the limb involvement was more extensive. Until molecular studies are carried out to identify the gene(s) involved in the pathogenesis of these syndromes, careful clinical examination and further investigation of affected subjects and family members are the only means of attempting classification. We have excluded a coding sequence mutation in exons 2-5 of the *PAX2* gene in our families by single strand conformation polymorphism analysis and direct sequencing. Most of the mutations reported to date in *PAX2* are within exons 2-5 for as yet unknown reasons.[24 25]

Renal and retinal anomalies need to be specifically looked for in subjects presenting with Duane anomaly and/or radial ray defects.

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Branchio-oculo-facial syndrome and branchio-otic/ branchio-oto-renal syndromes are distinct entities

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Branchio-oculo-facial syndrome (BOF, MIM 113620[1]) is a rare autosomal dominant disorder. The symptoms of this disorder include bilateral postauricular cervical branchial sinus defects with haemangiomatous, scarred skin, cleft lip with or without cleft palate, pseudocleft of the upper lip, nasolacrimal duct obstruction, low set ears with posterior rotation, a malformed, asymmetrical nose with a broad bridge and flattened tip, and, occasionally, prematurely grey hair. Father to son transmission of this disorder has been observed,[2] which indicate autosomal dominant inheritance. Another disorder with hearing loss resulting from bilateral branchial cleft fistulas is branchio-oto-renal syndrome (BOR, MIM 113650). Common features of both syndrome are summarised in table 1. Some characteristics of both BOR and BOF syndromes have been reported in a father (BOF) and his son (BOR), but the constant features of BOF syndrome were not observed in either of them. This observation led to the conclusion that BOF and BOR might be allelic variants of the same gene.[3] It was suggested that, in both syndromes, penetrance and expression could be variable, and it was concluded that BOF and BOR syndromes are the variable results of mutations in the same autosomal gene.[3] However, it was pointed out later that both subjects in fact should be considered as BOF syndrome rather than BOF and BOR syndrome, and that these syndromes are distinct entities and may not be allelic.[4] Another related disorder is branchio-otic syndrome (BO, MIM 602588), which comprises branchial fistulas, preauricular pits, and hearing impairment, but lacks renal anomalies (table 1).

The first candidate gene for BOR has been mapped. This gene, *EYA1* ("eyes absent-like", a human homologue of the *Drosophila eyes absent* gene), was found by positional cloning[5] and maps to chromosome 8q13.3. Mutations in *EYA1* have been described,[6–8] which made it a candidate gene for BOR syndrome. The authors of the first report[8] concluded that BO and BOR syndromes are allelic. The hunt for a candidate gene in BOF syndrome was more difficult, because only a few familial cases exist[9] that could be studied. Since an allelic variant of BOF and BOR syndromes was not dispelled conclusively, several independent attempts have been undertaken to study the *EYA1* gene region as a candidate gene region for BOF syndrome. By sequence analysis, no mutations were found in the *EYA1* gene in five BOF syndrome patients.[7] This suggests once more that BOR syndrome might not be allelic to BOF syndrome. *EYA1* is a member of a gene family comprising at least four genes (*EYA1-EYA4*). *EYA1* is expressed during embryogenesis in the branchial arches and the somites and during limb development in connective tissue precursors.[10] At the tailbud stage of zebrafish, its expression is confined to cranial placodal precursors and, thereafter, to the anterior pituitary, olfactory, and otic placodes.[11] The expression of the other members of the *EYA* gene family, *EYA2-3*, is similar to the *EYA1* expression pattern.[10–12] The *EYA4* pattern, however, is confined to the dermamyotome and the limb, and expression was not found in the branchial arches.[13] The expression patterns in early embryogenesis together with the developmental defects in BOF syndrome prompted a segregation analysis for these four genes in a large pedigree with BOF syndrome, but no cosegregation of the disorder with genetic markers was found.[14] The latter study excluded the *EYA* genes as candidates for BOF syndrome.

Recently, a second gene locus (BOR2) for a BOR syndrome-like phenotype was mapped to human chromosome 1q31.3-q32.1.[15] Linkage between the BOR syndrome related disorder branchio-otic syndrome (BO) and marker D1S2757 was observed with a maximum lod score of 4.81 at a recombination fraction of zero.[16] The variability of the clinical phenotype of BOF syndrome and the overlapping symptoms with BOR or BO syndromes prompted us to perform a segregation analysis in this second candidate gene region of BO/BOR syndrome in order to verify whether the clinical differences reflect allelic variants of the same gene. In the present study, we show that BOF syndrome is not allelic to BOR/BO syndromes at this locus. This, taken together with previous reports, is a second proof based on genetic studies that BOR and BOF syndromes are distinctive entities. Our findings firmly support former hypotheses on the distinctiveness of these syndromes, which were based solely on the clinical phenotype.

MATERIAL AND METHODS

The analyses were performed on DNA of patients with BOF syndrome from one family, which represents the largest published BOF pedigree with five affected and two unaffected members (fig 1). The family studied here was described earlier in a review including photographs of the patients with the characteristic features of BOF syndrome[9] (ID 10-14). DNA was extracted from peripheral blood lymphocytes by standard techniques.[17] Screening for mutations in the candidate gene

Table 1 Branchiogenic disorders

Symptoms	BOF	BOR	BO
Inheritance	Autosomal dominant	Autosomal dominant	Autosomal dominant
Auricular pits/fistulas	+	+	+
Cleft lip±palate	+	–	
Pseudocleft of the upper lip	+	–	–
Upper lip pits	+		
Bilateral postauricular cervical branchial sinus defects	+	+	+
Branchial cleft/sinus/cysts	+	+	+
Cervical/supra-auricular defects with aplastic overlying skin	+	–	–
Haemangiomatous, scarred skin	+	–	–
Low set ears with posterior rotation	+		
Hearing loss (conductive, sensorineural, mixed)	+	+	+
Malformed, asymmetrical nose with broad bridge and flattened tip	+	–	–
Microphthalmia	+	+	
Nasolacrimal duct stenosis	+	+	
Prematurely grey hair	±	–	–
Renal agenesis	+	+	–
Renal aplasia, hypoplasia, dysplasia	+	+	–

region was impossible, because the gene itself is unknown.[16] Nevertheless, a segregation analysis with seven markers in an interval of 34 Mb is sensitive enough to exclude cosegregation of a putative candidate gene from this region with the disorder. For this segregation analysis, we used seven microsatellite markers from human chromosome 1q, the candidate region for BO syndrome. The markers span an interval of approximately 34 Mb and their map positions are (from proximal to distal) D1S2815 (204.84 Mb) - D1S461 (209.40 Mb) - D1S2757 (213.2 Mb) - D1S2640 (217.63-217.80 Mb) - D1S2622 (218.20 Mb) - D1S2668 (235.82-235.95 Mb) - D1S249 (238.72 Mb). All given map positions were extracted from NCBI's Entrez Genome Database (URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Genome). The markers include D1S2757 where the highest lod score for BO syndrome has been reported.[16] All markers contain dinucleotide repeats, and the maximum heterozygosity of the markers, as published in the database, was always greater than 0.65. Primer sequence information was picked from the GDB database (URL: http://www.gdb.org). The DNA of the family members was PCR amplified by a standard protocol with 2.5 mmol/l $MgCl_2$ for 30 cycles comprising three 30 second steps at 95°C, 51°C, and 72°C, respectively, in a

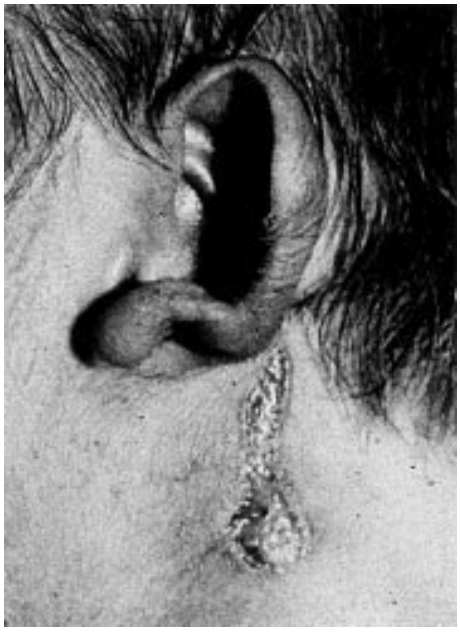

Figure 1 Patient II.3 of the pedigree in fig 2. The fistula of the cervical branchial sinus defect can be identified clearly in addition to the malformation of the low set ear of the newborn girl.

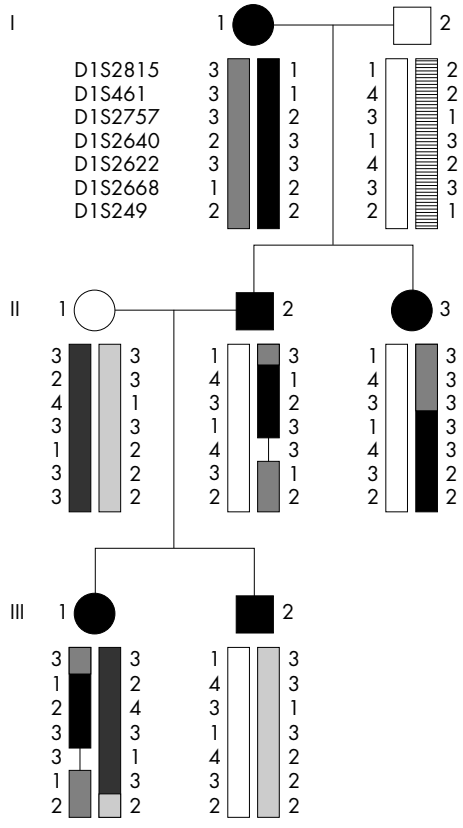

Figure 2 Pedigree of the family with BOF syndrome. The haplotypes around the BOR2 locus on chromosome 1q31.3-q32.1 are shown in a 34 Mb interval. The microsatellite markers are arranged (from top to bottom) from proximal to distal 1q. In the studied interval, the affected subjects II.2 and II.3 have inherited an identical haplotype from their healthy father (I.2) but only partially identical (D1S2640 and D1S2622) haplotypes from their affected mother (I.1), which might point to a candidate region. The children (III.1 and III.2) of II.2, however, only have alleles of marker D1S249 from their unaffected mother (II.1) in common, but they do not share any paternal (II.2) alleles. Therefore, the chromosome region around the BOR2 locus on chromosome 1q can be excluded as a candidate region for BOF syndrome, and BOF syndrome is not allelic with BOR syndrome.

"Robocycler Gradient 96" (Stratagene, Amsterdam, The Netherlands). The PCR products were separated on a denaturing 6% polyacrylamide gel in TBE buffer. The DNA was transferred from the gel to a nylon membrane and hybridised with a ^{32}P-labelled oligonucleotide $(CA)_{11}$. The results from the allele size calculation of the markers of the family were imported into Cyrillic 2.1.3™ pedigree analysis software (FamilyGenetix, Oxford, UK). The preliminary haplotypes were re-evaluated with SimWalk[18] to calculate the likelihood of crossovers. The resulting haplotypes were re-entered into Cyrillic for the delineation of the pedigree.

RESULTS

Our results clearly show that BOF syndrome does not cosegregate with markers from the studied region of chromosome 1q (fig 2). Subject III.1 inherited the chromosome with the assumed disease associated markers from her affected paternal grandmother (I.1), whereas III.2 inherited her paternal copy of chromosome 1 from the healthy paternal grandfather (I.2). This information from the haplotypes of grandchildren III.1 and III.2 definitely excludes this region as a BOF syndrome candidate region. The children, II.2 and II.3, inherited the same allele from their mother for marker D1S2815. From the other grandparental chromosome of their mother (these grandparents are not included in the figure), they share marker alleles D1S2640 and D1S2622, the latter with a 50% probability for II.2. Taken together, this clearly shows that BOF syndrome cannot be associated with genes from this region. As long as BOF syndrome is considered to be an autosomal dominantly inherited disorder, which is also evident from the pedigree, the alleles of the markers of the unaffected grandfather I.2 may be disregarded.

DISCUSSION

Although the phenotypes of the members of the family from our study show intrafamilial variability, their symptoms are all compatible with the diagnosis of BOF syndrome. The phenotypes of patients with BOF syndrome and BOR or BO syndromes have common symptoms, which not only justified the search for mutations in *EYA1*,[7] but also the present study. In addition to previous reports on mutations in the *EYA1-4* genes of BOR and BOF syndrome patients,[6 7 14] this is the second report, based on genetic analyses, which provides evidence that BOF syndrome and BOR or BO syndromes are phenotypically related but genetically completely distinct disorders which do not represent allelic variants. Based on the syndrome specific symptoms, it has previously been suggested that BOF and BOR/BO syndromes are distinct entities. Now, we provide additionally strong genetic evidence for this assumption with the exclusion of another candidate gene region from chromosome 1q. Unfortunately, the number of affected subjects and families is too small[9] to allow for a genome wide linkage analysis.

Obviously, BOF, BOR, and BO syndromes are caused by mutations in different genes. The pathogenetic mechanisms for overlapping and syndrome specific symptoms are unknown, but one might assume that the gene in interval BOR2 on chromosome 1q interacts with *EYA1*, leading to a cascade of gene activities that regulate proper differentiation of the branchial arches. *EYA1* is considered to represent a transcriptional activator, which might interact with downstream genes. Mutations in another transcription factor have been reported recently for the BOR-like Townes-Brocks syndrome (TB syndrome, MIM 107480[19 20]). This candidate gene *SALL1* on chromosome 16q12.1 is the human homologue of *Drosophila spalt* (*sal*) which is also a developmental regulator. Therefore, it may be assumed that BOF syndrome might also be caused by mutations in a transcription factor-like protein. A recent report shows expression of *SALL1* in the developing brain and the limbs, but also in the lens.[21] Expression of *EYA1* in the developing eye was excluded by RNA in situ hybridisations in mouse embryos.[5] Expression in the eye might be a hint to a candidate gene, because BOF patients occasionally also have anomalies of the eye like coloboma, strabismus, or microphthalmia.

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Infantile spinal muscular atrophy variant with congenital fractures in a female neonate: evidence for autosomal recessive inheritance

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We read with great interest the article published in this journal in 1991 by Borochowitz *et al*,[1] describing a new lethal syndrome consisting of infantile spinal muscular atrophy (SMA) and multiple congenital bone fractures in two sibs. Recently, another infant with a form of SMA and congenital fractures was reported by Kelly *et al*,[2] thus validating the suggestion of a distinct and rare form of SMA associated with congenital bone fractures. Autosomal recessive inheritance was suggested in the original report,[1] but no history of consanguinity was noted in the second.[2] X linked inheritance could, however, not be excluded since these three affected infants were male. Greenberg *et al*[3] reported four cases with infantile SMA, neonatal death, congenital joint contractures, and the presence of bone fractures in three of the four cases; these cases seem clinically to be similar to the originally reported cases,[1] but the pedigree in this report was consistent with X linked recessive inheritance and the gene in this family was mapped to Xp11.3-q11.2.[4]

Here, we report on a female neonate with a severe, acute, lethal form of SMA and congenital bone fractures, thus excluding X linked inheritance. Furthermore, since this girl was born to first cousin parents, this suggests autosomal recessive inheritance in this rare variant of SMA type 1 with congenital fractures.

CASE REPORT

The girl was born to a 35 year old, G5 P5 mother and a 41 year old father. The parents, of Moroccan origin, were consanguineous, as were the maternal grandparents. They had one healthy son and two healthy daughters, and another son who had died at the age of 3 months in Morocco. The pregnancy was not medically followed but reported by the mother as uneventful. Delivery, recorded as normal by both gynaecologist and mother, occurred spontaneously at 40 weeks of gestation with cephalic presentation. The Apgar scores were 3, 4, and 5 at one, five, and 10 minutes, respectively. Birth weight and length were normal (3700 g, 51 cm, respectively). She was severely hypotonic and was intubated and ventilated as soon as the paediatrician arrived (10 minutes after birth). A 4/6 pluriorificial heart murmur was noted. She was immediately transferred to the University Children's Hospital for ventilatory support and further evaluation. An abnormal position of the upper and lower limbs (in abduction) with swelling of the left upper arm and the left thigh and pain on mobilisation of the limbs were noted. She was very hypotonic and showed no spontaneous movements. Tendon reflexes were absent. She also had a prominent forehead, generalised oedema, an apparent accumulation of fat in the lower limbs, generalised hypertrichosis, a swollen abdomen, and camptodactyly of the fingers and toes (fig 1). Her sclerae were not blue. Head circumference could initially not be measured because of the presence of an important haematoma of the skull, but at the age of 13 days, it was 37.5 cm (>90th centile). Radiographs showed a mid-diaphyseal fracture of the left humerus and of the left femur. Ossification was normal. There were, however, abnormally straight femora with modelling defect, abnormally dense metaphyses of the lower limbs, dislocated hips, a

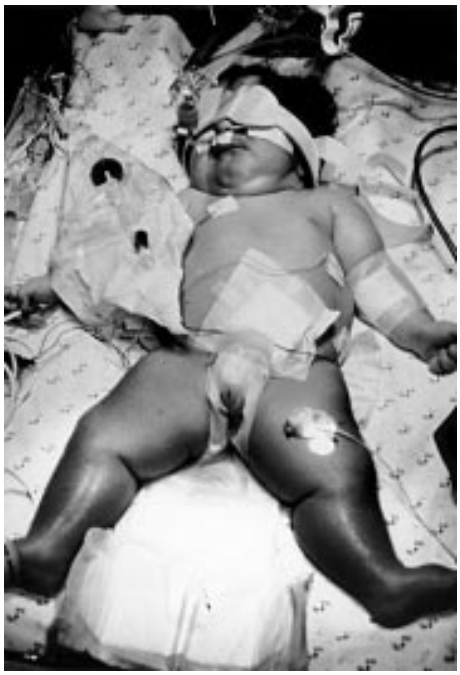

Figure 1 Clinical photograph of the proband.

thickening of subcutaneous tissue with loss of muscle density, and an impression of marked infiltration by fat (fig 2A, B). This fatty infiltration, confirmed by ultrasound, was also visible in the abdominal region extending into the mid-thoracic region (fig 2C). The ribs were thin and the skull was normal (no Wormian bones) except for the presence of a large parietal haematoma. Control *x* rays of the left femur and humerus at the age of 3 weeks showed the formation of a normal and large callus.

Nerve conduction studies, performed on the 13th day of life, showed normal sensory and motor values. Electromyography of the biceps brachii, pectoralis major, and tibialis anterior muscles, done at 13 days, showed the presence of spontaneous fibrillations and positive sharp waves at rest and reduced interference pattern during contraction, changes compatible with a neurogenic disorder. The EMG of muscles innervated by cranial nerves (M orbicularis oris, M genioglossus) was normal.

A muscle biopsy from the quadriceps, performed at 16 days, showed almost exclusively atrophic fibres, with a small cluster of large fibres in only one area. The larger fibres were mostly of type 1; the atrophic fibres were of both histochemical types. The range of diameter fluctuations was 4-20 μm. There was no evidence of necrosis with myophagia. In order to ascertain the diagnosis, a second biopsy was performed at 32 days from the thenar muscle (fig 3), in a still moving hand, and confirmed the pronounced fibre size variation (diameters varied from 5 to 10 μm for type 2 and from 5 to 40 μm for type 1), but showed

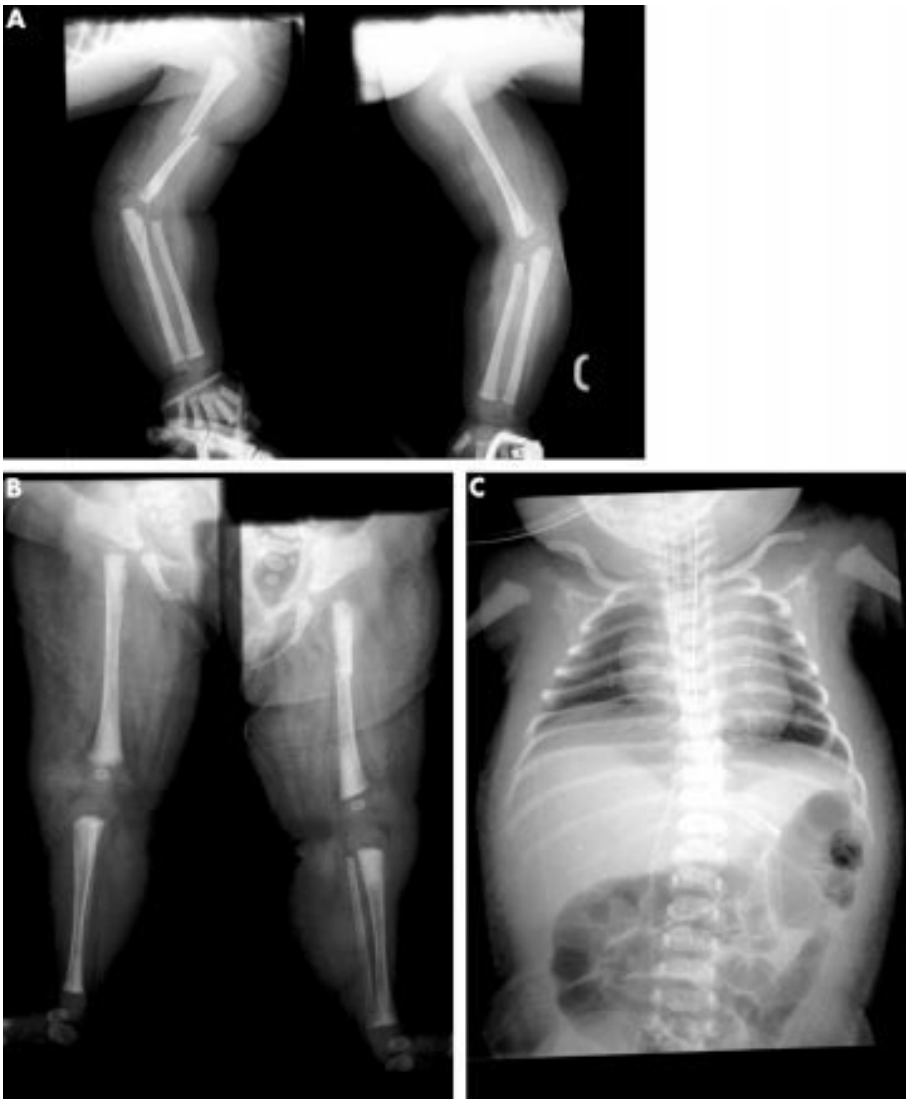

Figure 2 X rays of the proband showing (A) the upper limbs, with mid-diaphyseal fracture of the left humerus; (B) the lower limbs, with diaphyseal fracture of the left femur, abnormally straight femora with modelling defect, abnormally dense metaphyses, dislocated hips, a thickening of subcutaneous tissue with loss of muscle density, and an impression of marked infiltration by fat; (C) a marked fatty infiltration is also visible in the abdominal region extending into the mid-thoracic region; the ribs are thin.

less atrophy and more clumps of large fibres in comparison with the more proximal muscle.

Immunocytochemistry showed no deficit of dystrophin or merosin. There was no evidence of a mitochondrial disease. Electron microscopic findings showed a disorganised myofibrillar network in some fibres. The muscle biopsy findings were thus compatible with a severe and acute form of SMA.

Blood chemistry tests including calcium, phosphorus, alkaline phosphatase, electrolytes, viral serology, TORCH, and metabolic screening (including amino acids and very long chain fatty acids) were all within normal limits except for severe anaemia resulting from bleeding from the fractures and requiring red blood cell transfusions, severe neonatal asphyxia, and at birth a slightly raised CK level which normalised completely at the age of 25 days. Ultrasonography of the heart showed persistence of the arterial canal, moderate pulmonary hypertension, and a small ventricular septal defect. A haemodynamic exploration by cardiac catheterisation excluded a vascular malformation. Brain magnetic resonance imaging showed minor cortical atrophy and a slight delay of myelinisation. A CT scan confirmed the absence of intracerebral calcifications. Other examinations including eye fundus, ophthalmological examination, renal ultrasounds, and microscopic examination of the skin were normal. Her karyotype on high resolution banding was normal, 46,XX. DNA studies on a blood sample showed the absence of homozygosity for a deletion of exons 7 and 8 of the *SMNt* gene; congenital myotonic dystrophy or Steinert disease could also be excluded. Further molecular studies in the proband with 5q markers excluded homozygosity for the *SMN* region. Flanking markers used were D5S823 at less than 1 cM on the proximal side and D5S557 at less than 1 cM on the distal side. A hemizygous deletion of this region was also excluded by quantitative analysis of *SMN* by competitive PCR as described.[5] Point mutation analysis of the *SMN1* gene is continuing (HS).

During her stay in the neonatal unit, the proband continued to be dependent on a ventilator, furthermore requiring increasing ventilatory conditions. She had many bronchopulmonary infections. She showed no spontaneous movements, except some infrequent small movements of her fingers and face, even with treatment with strong analgesic drugs and even after healing of her fractures. There was, however, good visual contact. She developed decubitus wounds and had persistent generalised oedema. Parenteral feeding was needed throughout her life; enteral feeding by tube was tried at the

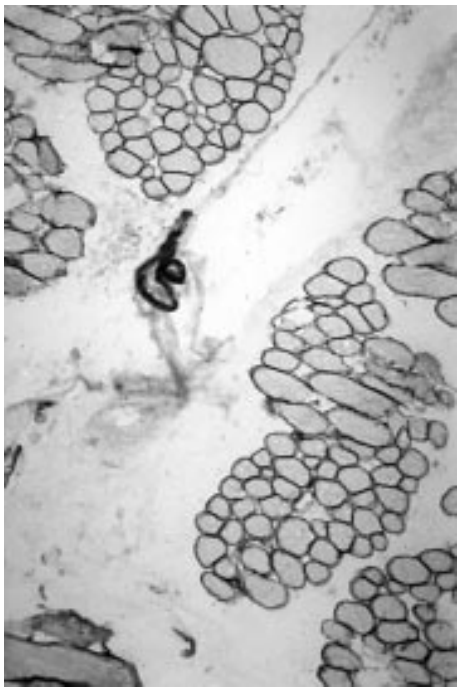

Figure 3 Muscle biopsy specimen of the thenar muscle of the proband at the age of 32 days, performed in order to ascertain the diagnosis, showed atrophic and hypertrophic fibres and confirmed the pronounced fibre size variation. It showed less atrophy and more clumps of large fibres, in comparison with the more proximal muscle (Spectrin, ×330).

age of 6 days but had to be stopped after three days because of ventilatory problems. She finally died at the age of 34 days from a cardiorespiratory arrest owing to massive bronchopneumonia. The family would not authorise a necropsy.

DISCUSSION

This female infant presented with a severe, acute form of SMA, confirmed by neurophysiological and muscle biopsy findings, with prenatal onset and early lethal outcome, together with congenital bone fractures, camptodactyly, bilateral hip dislocation, and a heart murmur resulting from persistence of the arterial canal, moderate pulmonary hypertension, and a small VSD. A distinct and very rare form of SMA associated with congenital bone fractures was proposed recently.[2] Three infants, similar to our case, have been reported so far.[1 2] Similarities between these three infants and our proband are a severe infantile form of SMA, profound hypotonia requiring artificial ventilation at birth, bone fractures (of the humerus/femur), joint contractures, normal sclerae, severe generalised oedema, and early death (all died before the age of 3½ months). Normal ossification and thin ribs, as present in the proband, have also been described in both originally described cases.[1] Additional findings in the proband are the presence of bilateral hip dislocation, abnormally straight femora with modelling defect, hypertrichosis, and a congenital heart defect. The association of SMA and hip dislocation, SMA and abnormally straight femora with modelling defect, and the association of SMA and hypertrichosis have not been described previously. To exclude the presence of abnormal collagen bundles, a skin biopsy was performed that did not show any alteration in collagen or elastin fibres. The hypertrichosis present in the proband could be familial. The association of SMA and congenital heart defect is usually seen as coincidental, but three sibs with SMA type 1 combined with atrial septal defect have been reported.[6] One of these sibs was a boy described as having a long bone fracture, arthrogryposis, distal oedema, and very early death from respiratory failure. The second and third sib, both girls, did not have long bone fractures, but presented with profound hypotonia at birth requiring artificial ventilation, early death owing to respiratory failure/bronchopneumonia, and other malformations, including valvular aortic stenosis with hypertrophic right ventricle (sib 2) and prenatal pleural effusion and brain malformation (sib 3). We think that the clinical features of these sibs, especially sib 1, resemble those of the proband and could belong to the same SMA variant we have described and reviewed here. A variable expression of this SMA variant seems probable. Additional cases with SMA and congenital fractures are needed for further delineation of the clinical phenotype. We suggest that the association of a congenital heart defect with SMA should prompt a search for additional anomalies.

In the differential diagnosis, a neonatal form of adrenoleucodystrophy[7 8] has been excluded.

Concerning the molecular studies, it is well known that homozygous deletions of exons 7 and 8 within the telomeric copy of the survival motor neurone (*SMN*) gene are detectable in about 95% of patients with early onset SMA.[9] It was not present in the proband or in the infant reported by Kelly *et al*.[2] Molecular studies were not reported in the two originally described cases.[1] We propose that all SMA patients, including variants, should be carefully investigated for *SMN* deletion because deletions of the *SMN* gene have also been shown in various "atypical" SMA patients with congenital joint contractures and in SMA patients with congenital heart disease.[10 11] Also, patients with severe SMA type 1, presenting at birth with asphyxia and severe weakness, characterising a new SMA type 0,[12] similar to our patient, have been reported with deletions of the telomeric survival motor neurone gene.[13] In our proband, we could exclude homozygosity for the *SMN* region (5q13) with DNA markers flanking the SMA locus, and we could also exclude a hemizygous deletion of this region. This supports non-linkage of SMA to chromosome 5 in this family, as was the case in a consanguineous family with two affected male sibs with SMA and arthrogryposis.[14] The probability of finding a *SMN* compound heterozygous genotype of two point mutations in this region is very low. However, further studies are continuing to rule out this possibility.

It seems probable that this variant with congenital fractures might be more common than the description of the three cases reported so far.[1 2] There are, indeed, the cases published by Möller *et al*,[6] discussed above, and the letter by Garcia-Alix *et al*,[15] briefly reporting six infants who were similar to the two originally described patients,[1] except that they had hypomineralised long bones. Osteopenia in SMA patients could occur secondary to the decreased load placed on the fetal skeleton owing to decreased fetal movement.

In conclusion, autosomal recessive inheritance should be considered in a rare and severe acute form of congenital SMA type 1, requiring early artificial ventilation and with early lethal outcome, and associated with congenital bone fractures, congenital joint contractures, generalised oedema, and sometimes also cardiopathy. Variable expression of this SMA variant seems probable. Since the primary defect is so far unknown, additional cases are needed to delineate the clinical spectrum further and to confirm the probability that this SMA variant is not linked to 5q.

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