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Recessive mutations in *MCM4/PRKDC* cause a novel syndrome involving a primary immunodeficiency and a disorder of DNA repair

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Abstract

Background We present a study of 10 children with a novel syndrome born to consanguineous parents from the Irish Traveller population. The syndrome is characterised by a natural killer (NK) cell deficiency, evidence of an atypical Fanconi's type DNA breakage disorder and features of familial glucocorticoid deficiency (FGD). The NK cell deficiency likely accounts for the patient's recurrent viral illnesses. Molecular tests support a diagnosis of mosaic Fanconi's anaemia (FA) but the patients do not present with any of the expected clinical features of the disorder. The symptomatic presentation of FGD was delayed in onset and may be a secondary phenotype. As all three phenotypes segregate together, we postulated that the NK cell deficiency, DNA repair disorder and FGD were caused by a homozygous mutation in a single recessive disease gene.

Methods SNP homozygosity mapping and targeted next-generation sequencing of 10 patients and 16 unaffected relatives.

Results We identified a locus for the syndrome at 8p11.21-q11.22. Targeted resequencing of the candidate region revealed a homozygous mutation in *MCM4/PRKDC* in all 10 affected individuals. Consistent with the observed DNA breakage disorder, *MCM4* and *PRKDC* are both involved in the ATM/ATR DNA repair pathway which is defective in patients with FA. Deficiency of *PRKDC* in mice has been shown to result in an abnormal NK cell physiology similar to that observed in the patients.

Conclusion Mutations in *MCM4/PRKDC* represent a novel cause of DNA breakage and NK cell deficiency. Our findings suggest that clinicians should consider this disorder in patients with failure to thrive who develop pigmentation or who have recurrent infections.

Keywords: failure to thrive, DNA repair defect, Irish Traveller, natural killer cell deficiency, familial glucocorticoid deficiency, *MCM4*

Introduction

Members of three clans from the Irish Traveller population have presented to a variety of clinical specialists including paediatric endocrinologists, haematologists and geneticists with intra-uterine growth retardation (IUGR) and failure to thrive (FTT). All affected individuals from the first presenting family (pedigree 1) had clinodactyly, some had episodes of hypoglycaemia and those tested had delayed bone age. Some patients had relative macrocephaly with head circumference measurement on the 3rd centile and height and weight $<3^{rd}$ centile. In others, all three parameters were below the 3rd centile.

Members of a second clan (pedigree 2) from the Irish Traveller population presented to endocrinologists with IUGR, FTT, hypoglycaemia and clinodactyly. When investigated, the affected children were found to develop hyperpigmentation (after 2 years of age) and elevated adrenocorticotropin hormone (ACTH) levels over time (mean age of 5 years) with low-normal cortisols, confirming a diagnosis of familial glucocorticoid deficiency (FGD). However, symptomatic presentation of FGD was later than normal for the condition. At initial diagnosis the patients had a tanned appearance typical of FGD. Most patients who were regularly taking their hydrocortisone had normal skin colouring, while those who were not compliant with medication continued to have a tanned appearance. Due to a history of recurrent infections, some of the children from pedigree 2 were investigated for disordered immunological function which revealed low levels of NK cells and evidence of a DNA repair disorder. Clinical features of the DNA repair phenotype in members of this clan were described by Eidenschenk et *al.* [1]. Members of a third clan (pedigree 3) were referred to clinical geneticists with suspected Russell-Silver syndrome but a diagnosis of FGD was made based on the development of increased pigmentation and subsequent biochemical investigations.

As some members of the original clan (pedigree 1) were later found to have high ACTH levels, we postulated that the NK cell deficiency, DNA repair disorder and FGD were due to the result of a single recessive genetic event. We proceeded to test members of all the clans for the three different phenotypes and found that those with a diagnosis of FGD also had low NK cells and some showed defective DNA repair. The DNA repair disorder was classified as mosaic Fanconi's anaemia (FA) but the patients do not have the typical mosaic FA test result or the expected clinical features of the disorder. In mosaic FA, patients have two subpopulations of cells, one of which is hypersensitive to cross-linking agents (DEB: diepoxy-butane) while the other behaves normally in response to these agents. Upon testing, patients with mosaic FA have some cells with high levels of DNA damage and others that are completely normal. However, often the patients in the current study have a relatively low level of DNA damage in a minority of cells. The observed chromosome breakage is greater than that expected from a healthy individual but less than that of mosaic FA.

The current study involves 10 individuals from three consanguineous Irish Traveller families whom were diagnosed with a combination of a NK cell deficiency, mosaic FA and FGD (Figure 1 A-C). Many of these children would not have been investigated and diagnosed were it not for their family history. Clinical and laboratory details of the 10 patients are discussed (Table 1 and Supplementary Material). Details of the presentation and endocrine findings of some of these patients were previously described by O' Riordan and colleagues [2]. We performed SNP homozygosity mapping and targeted next-generation sequencing to identify the underlying risk gene in these families.

Subjects and Methods

Patients and DNA samples

DNA was available from 10 affected and 16 unaffected members of the three Irish Traveller families. The 10 patients were diagnosed with a NK cell deficiency, mosaic FA, and FGD. Clinical information for each patient is provided in the supplementary material. Ethical approval was obtained from Our Lady's Children's Hospital Ethics Board (Dublin, Ireland) and written informed consent was obtained from all patient guardians.

SNP Homozygosity mapping

DNA samples from the 10 affected and 16 unaffected individuals were genotyped for 1 million SNPs on the Illumina 1M array (Illumina, California). SNP homozygosity mapping was performed using the HomozygosityMapper programme [3].

Target enrichment, exome sequencing and data analysis

DNA from 6 of the patients (Pedigree 1: III:11, IV:4, IV:9, IV:12, Pedigree 2: IV:1 and Pedigree 3 II:1) and 4 unaffected siblings (Pedigree 1: III:12, IV:5, IV:11 and Pedigree 2: IV:2) was selected for targeted resequencing of the exons within the 8p11.21-q11.22 candidate region. Target enrichment was performed using a SureSelect XT Custom MP2 kit (Agilent, Santa Clara) and libraries were sequenced on an Illumina HiSeq at GATC. The reads were mapped against UCSC hg18 using BWA version 0.5.7 [4]. Duplicates were removed and the quality scores for the aligned reads were recalibrated using GATK [5]. Variants and indels were detected using SAMtools [6].

Analysis of MCM4 splice variant

The MCM4 c.71-2A>G variant was analysed with Human Splicing Finder (HSF) version 2.4.1 (http://www.umd.be/HSF/) [7]. The mutation designations are based on *MCM4* transcript ENST00000262105 from Ensembl. A in the ATG start codon is defined as c.1.

Results

Homozygosity mapping and targeted next-generation sequencing

SNP homozygosity mapping identified a single homozygous segment at 8p11.21-q11.22 that was shared by the 10 affected individuals (Figure 1D). The candidate locus is 10.5 Mb in size and contains 34 candidate genes. Targeted resequencing of the 34 candidate genes was performed in 6 patients and 4 unaffected siblings (Supplementary Table S1). To identify potential disease mutations, we prioritised variants that (i) are homozygous, (ii) segregate with the phenotype and (iii) are novel or have a frequency <1% in dbSNP. The prioritisation strategy narrowed the search to two novel variants; one is located in an intron of *GOLGA7* (NM_001002296.1:c.367-82C>T) and the second variant is located within both the 3' acceptor splice site of *MCM4* intron 1 (NM_005914.2:c.71-2A>G) and the 5' untranslated region (UTR) of *PRKDC* (NM_006904.6:c.-57-u1331T>C) (Supplementary Figure S1).

The intronic variant in *GOLGA7* is located 82 base-pairs 3' of the acceptor splice site sequence and is of unknown significance. Analysis of the candidate *MCM4* splice variant using HSF shows that the mutated splice site has a consensus value (CV) <70 (52.59) and a ΔCV >10% (40.46%) which is predicted to result in a broken and inactive splice site (Supplementary Table S2). In addition, mutations with a CV <70 combined with a ΔCV reduction >10%, such as the *MCM4* variant identified in this study, are expected to completely block the production of wild-type transcript. The *MCM4* variant is also located within the 5'-UTR of two *PRKDC* transcripts (NM_001081640 and NM_006904). The 5'-UTR harbours numerous binding sites for proteins that either repress or promote translation and impairment of any of these features can alter translational regulation leading to altered gene expression and, in some cases, susceptibility to disease [8].

Discussion

The 10 affected individuals are from three separate Irish Traveller clans and were initially assessed for query Russell-Silver syndrome due to IUGR, FTT and hypoglycaemia. The natural history in our patients varied with affected patients from one clan being prone to recurrent infection and some of them developing bronchiectasis. It is possible that environmental factors, such as poor housing, are contributing to the recurrent infections in this family. Further investigations revealed that three different phenotypes (NK cell deficiency, mosaic FA and FGD) were segregating together in these families suggesting that they may be caused by a single recessive disease gene.

SNP homozygosity mapping and targeted next-generation sequencing of the 8p11.21-q11.22 candidate locus identified two novel homozygous variants that segregated with the phenotype; 1) an intronic variant in *GOLGA7* of unknown significance and 2) a variant located within the acceptor splice site of *MCM4* intron 1 that also overlaps the 5'-UTR of *PRKDC*. *MCM4* encodes a highly conserved mini chromosome maintenance protein that is essential for DNA replication [9] and important in maintaining genome stability [10]. *MCM4* has evolved to integrate several protein kinase regulatory signals to control progression through the S phase of mitosis. Interfering with the rate of DNA synthesis in the S phase can produce a FA-like phenotype in normal cells. The *MCM4* c.71-2A>G variant is predicted to have a significant effect on splicing with no production of wild-type transcript. This variant is also positioned within the 5'UTR of *PRKDC* and, while the functional consequences of UTR variants are currently difficult to predict, they can affect protein translation and gene expression. The presence of a single mutation affecting two genes may account for the diverse clinical features observed in the patients.

MCM4 and *PRKDC* are both involved in the ATM/ATR DNA repair pathway which is dysfunctional in patients with FA. Similar to other Fanconi genes, *MCM4* and *PRKDC*

have been shown to function in the mitochondria [11, 12]. Furthermore, mice homozygous for the spontaneous Severe Combined Immunodeficiency (SCID) mutation in the *Prkdc* gene (B6.CB17-Prkdc/ScJ) show defects in DNA repair and have an abnormal NK cell physiology characterised by markedly elevated NK cell activity in the first 10-14 weeks of life, and reduced NK cell numbers at 10-12 months. The similarity of the *Prkdc* mutant mouse to the phenotype of the patients in this study adds support to the involvement of *PRKDC* as a contributing risk factor for FA and NK cell deficiency.

Despite a diagnosis of mosaic FA, none of the patients have the typical FA phenotype. They have not developed bone marrow failure and they do not have typical Fanconi malformations. While many of them had anaemia, this was microcytic and related to poor diet. Upon breakage analysis in patients with typical mosaic FA, some cells are found to have a full mutation phenotype with high numbers of breaks and exchanges per cell, while other cells are normal with little or no chromosome damage. In the current study, the patients' cells do not show a mixture of highly damaged and normal populations. Instead, in most cases, a minority of cells demonstrate a relatively low level of breaks and exchanges, not far above that of healthy controls. One of the 12 affected family members has shown a full DEB response typical of classic FA. However, similar to the 11 patients with low levels of chromosome breakage, she also shows no clinical features of FA. This generally low level and variable expressivity for a FA type breakage syndrome has not been previously described.

The FGD phenotype in the patients is atypical in that the onset is delayed and the individuals remain small. The pigmentation improves upon treatment with hydrocortisone. However, in some families the compliance with medication has been poor. Despite lack of compliance, withdrawal of hydrocortisone has not resulted in an adrenal crisis, unlike other patients with classic FGD. Sequence analysis of the known FGD disease genes (*MC2R*,

MRAP and *STAR*) was undertaken in the current study but no causative mutations were identified. Other causes of adrenal insufficiency were also excluded. The late onset of FGD symptoms in these patients together with the absence of adrenal crisis suggests that the elevated ACTH levels may be a secondary phenotype in these patients, stemming from the NK cell deficiency and DNA repair disorder.

The biochemical and immunological results from these individuals were not necessarily present on initial testing. Many of these children were only diagnosed because the clinicians were aware of the family history. We recommend considering this diagnosis in children from consanguineous pedigrees who present with failure to thrive, recurrent viral illnesses and features of FGD. Assessment using ACTH +/- NK cell and DEB testing should be adequate to identify most future cases. We identified variation in the *MCM4* and *PRKDC* genes as the most likely cause of mosaic FA, NK cell deficiency and secondary FGD in these families. Further investigation into the molecular mechanisms of *MCM4/PRKDC* mutations and how they result in this complex phenotype is warranted.

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Figures

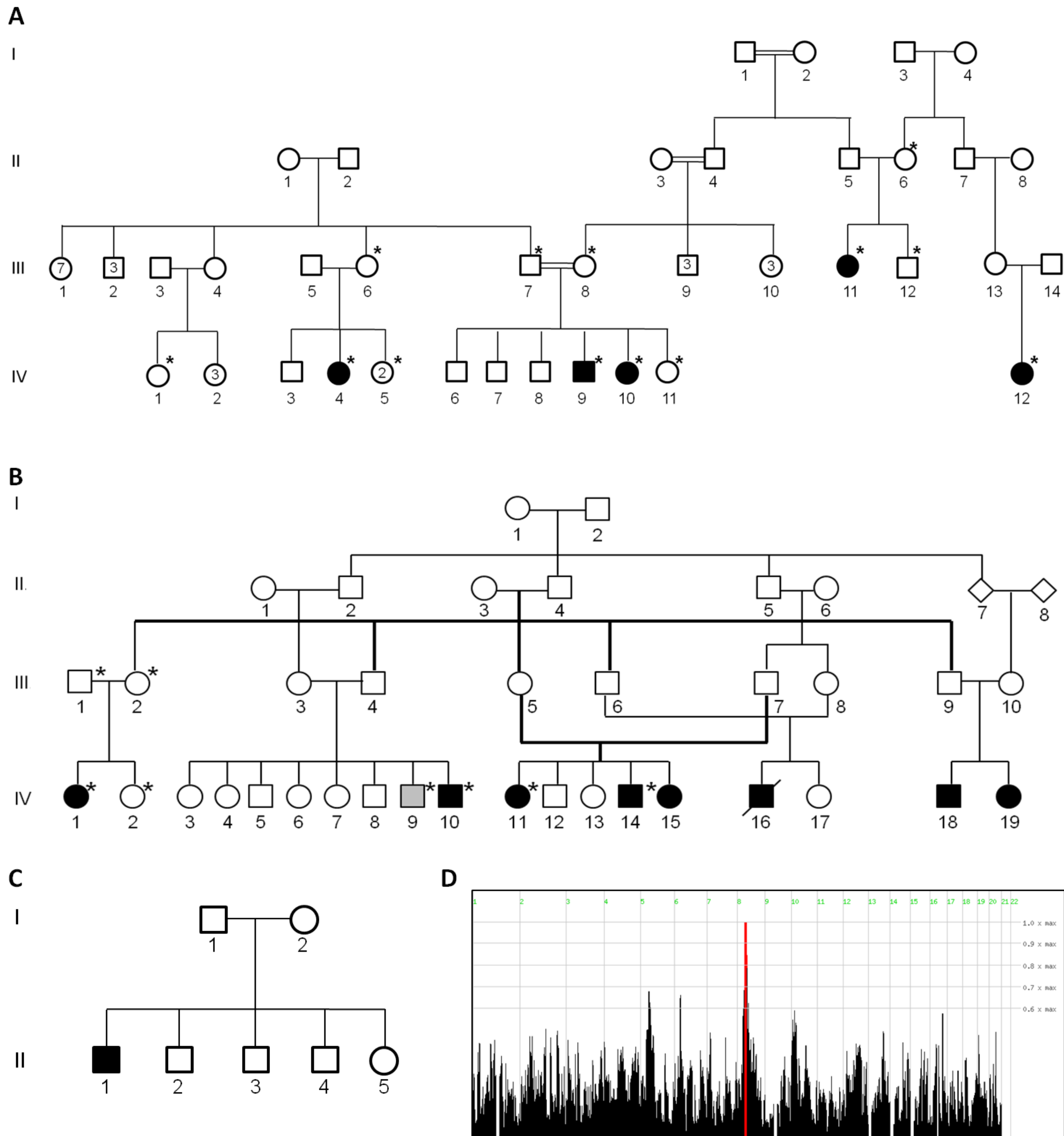


Figure 1 Pedigrees of Irish Traveller families presenting with a novel syndrome involving a primary immunodeficiency and a disorder of DNA repair. Individuals from whom DNA was available are marked with a *. (**A**) Pedigree 1 includes 5 affected individuals initially assessed for query Russell-Silver syndrome. DNA from 5 affected and 8 unaffected individuals was available for homozygosity mapping. Patients III:11 and IV:4 were also

diagnosed with a mild mosaic form of FA. Patient IV:9 has a NK cell deficiency but the results of the FA test are of unclear significance. Patient IV:10 was diagnosed with a NK cell deficiency. (**B**) Pedigree 2 includes 8 affected family members. One affected individual (IV:16) died aged 11 years from bronchiectasis. DNA was available for homozygosity mapping from 4 affected and 3 unaffected individuals. All of the affected individuals were diagnosed with mosaic FA (IV:19 has classic FA), a NK cell deficiency and FGD. (**C**) Pedigree 3 includes one affected proband initially suspected to have Russell-Silver syndrome. Further investigation led to a diagnosis of a NK cell deficiency and FGD. (**D**) SNP homozygosity mapping using the HomozygosityMapper programme identified a single candidate locus at 8p11.21-q11.22 (chromosome 8:40,802,495-51,349,159) which includes 34 positional candidate genes.

Table 1 Summary of the clinical features in 10 patients with a Russell-Silver like phenotype

Patient	Birth weight in kg (centile)	OFC	Failure to thrive	Hyper-pigmentation	Short stature	Low growth hormone	Hypo-glycaemic	Mosaic FA	NK cell deficiency
P1 III:11	2.38 (0.4^{th}-2nd)	NA	+	+	+	-	UK	+	NT
P1 IV:4	3.09 (25th)	<3rd centile	+	+	+	+	+	+	NT
P1 IV:9	2.64 (2nd)	3rd centile	+	+	+	UK	UK	Unclear	+ (3%)
P1 IV:10	2.58 (2nd)	NA	+	+	+	UK	UK	Unclear	+ (2%)
P1 IV:12	1.57 (9th)	9th centile	UK	+	+	UK	UK	NT	NT
P2 IV:1	2.38 (0.4^{th}-2nd)	NA	+	+	+	UK	UK	UK	NT
P2 IV:10	1.73 (<0.4^{th})	NA	+	UK	+	UK	UK	+	+ (1%)
P2 IV:11	1.64 (0.4^{th}-2nd)	<3rd centile	+	UK	+	UK	UK	+	+ (2%)
P2 IV:14	1.84 (<0.4^{th})	<3rd centile	+	+	+	+	+	+	+ (<1%)
P3 II:1	1.56 (<0.4^{th})	<3rd centile	+	+	+	-	-	-	+ (5%)

Clinical characteristics of 10 patients from three unrelated Irish Traveller families with a novel syndrome. Affected individuals were diagnosed with mosaic Fanconi's anaemia (FA), natural killer (NK) cell deficiency (normal NK cell levels = 9-16%) and familial glucocorticoid deficiency (FGD). Individuals from pedigrees 1, 2 and 3 are represented as P1, P2 and P3 respectively. The birth weight centiles are provided in (). Abbreviations are as follows: OFC; occipitofrontal circumference, NA; not available, UK; unknown, NT; not tested; + (present) and – (absent)

Supplementary Material

Clinical description of cases

Pedigree 1 III:11 [1995] At 9 years of age, elevated ACTH levels led to a diagnosis of familial glucocorticoid deficiency (FGD) in patient III:11. She had a history of recurrent infections, poor weight gain and pigmentation and failure to thrive (FTT). Her coeliac screen was negative and her growth hormone levels were normal. At age 14 years and 5 months, she remained pigmented and continued to show poor weight gain. Her bone age was delayed by 1 year and 2 months. Chromosome fragility studies identified evidence consistent with increased sensitivity to the mutagen diepoxybutane (DEB). The increased sensitivity is below that expected from a patient with classic Fanconi's anaemia (FA) but above that expected from an individual that does not have FA. Therefore she was diagnosed with mosaic FA. At age 15 years and 8 months, her weight was between the 0.4th and 2nd centile and her height was on the 9th centile.

Pedigree 1 IV:4 [1994] Patient IV:4 was a full term delivery and weighed 3.09 kg at birth (25th centile). At age 3 months, patient IV:4 was admitted to hospital with a minor episode of vomiting and diarrhoea. She was readmitted at age 7 months with bronchial pneumonia, gastroenteritis, bilateral cervical adenopathy and continuing FTT. Her weight was below the 10th centile. Endocrine examinations at age 8 months were clinically normal. In subsequent years she suffered from vomiting and diarrhoea for 24 hour periods and hypoglycaemia. She presented at clinic at age 8 years with an episode of profound hypoglycaemia during intercurrent gastroenteritis. Her complexion was sallow, similar to other members of the Irish Traveller population with FGD. The patient's height and weight were just below the 3rd centile and she had FTT. Her bone age has been consistently delayed at ~2 standard

deviations behind the mean for her age. She had a normal cardiology exam, ophthalmology exam and brain MRI. Ultrasound of the abdomen and pelvis showed no abnormalities. Her growth hormone was low but oestrogen, hydroxyprogesterone and testosterone were all normal. Two of the patient's first cousins were diagnosed with FGD. Analysis showed that her ACTH levels were 1,800 ng/ml (normal <60 ng/ml). At age 12 years, the patient still showed a poor increase in height, poor weight gain, FTT, hypoglycaemia and ACTH resistance.

Pedigree 1 IV:9 [2005] Patient IV:9 was born full term and weighed 2.64 kg. At 3 weeks of age he was admitted to hospital with bronchiolitis, FTT and poor weight gain. Due to a family history of FGD, a metabolic screen was performed at age 5 months and indicated a probable ACTH resistance (ACTH 154 nmol/L; normal = 22-107 nmol/L). A repeat test at 10 months of age revealed significantly elevated ACTH levels (250 nmol/L) and the patient was diagnosed with FGD. The patient was admitted to hospital at age 4 years complaining of vomiting and diarrhoea (lasting 3 weeks), abdominal cramps, fever, lethargy, joint swelling and pain, weight loss and a rash. At 6 years of age, the patient underwent a lymphocyte population screen which showed reduced levels of NK cells (3%; normal = 8-15%) and a moderate reduction in B cells (14.1%; normal = 21-28%). He also had a chromosome fragility test to investigate the possibility of FA. Analysis of cultures exposed to a standard dose of DEB showed a level of damage within the normal range. However, analysis of cultures exposed to a double dose of DEB identified a higher proportion of metaphases with chromosome damage relative to a normal control sample. The results are of unclear significance as they are not consistent with those expected from a typical FA positive patient or those expected from a patient with a somatic mosaic form of FA. His weight and height at

6 years of age were on the 2nd and 0.4th centile respectively. His head circumerference was on the 3rd centile.

Pedigree 1 IV:10 [1994] Patient IV:10 was noted to be small at birth (2.58 kg). As a result, she underwent chromosome studies and a metabolic screen but both were normal. She had poor weight gain over the first two years of life. At 2 years of age, the patient was admitted with vomiting and diarrhoea. A jejunal biopsy was consistent with a diagnosis of coeliac disease. She commenced a gluten free diet for 2 years but subsequently reverted to a normal diet due to persistent diarrhoea. At age 7, the patient was investigated for FGD on account of her family history and had significantly elevated ACTH levels (259 nmol/L). On examination, she was noted to have mild facial dysmorphism with frontal bossing, FTT and pigmentation. At 9 years of age, the patient was admitted with recurrent chest infections, deep pigmentation, blurring of vision and headaches. Immunological investigations were undertaken when the patient was 15 years of age and identified a lack of NK cells (2%) and increased T helper cells. Analysis of chromosome damage in cultures exposed to DEB showed no evidence of the enhanced levels of chromosome fragility typically found in patients with classic FA. At age 15 years and 2 months, her weight was on the 0.4th centile and her height was between the 0.4th and 2nd centiles.

Pedigree 1 IV:12 [2009] Patient IV:12 weighed 1.57 kg at birth (34 weeks and 5 days) and initially presented with FTT. Biochemical tests confirmed high ACTH and low cortisol levels leading to a diagnosis of FGD. She did not have the typical tanned appearance observed in Irish Travellers with FGD but she was treated with hydrocortisone from an early age. She has not been tested or FA or NK cell deficiency. At age 4 years and 6 months, the patients'

weight was 4.01 kg (0.4th centile). Her occipitofrontal circumference (OFC) and height were on the 9th centile.

Pedigree 2 IV:1 [1990] Patient IV:1 was born by an emergency section following fetal distress and meconium liquor and weighed 2.38 kg (0.4th-2nd centile). She needed to be intubated at birth and a trachea lavage was performed. In the immediate neonatal period she developed feeding problems with persistent vomiting. A hiatus hernia and gastro-oesophageal reflux were diagnosed. Following her discharge from hospital after birth, the patient had 3 hospital admissions in quick succession. Her first admission was at 7 weeks of age for an upper respiratory tract infection and fever. Her white cell count and haemoglobin were low and she appeared hypochromic and microcytic. She was treated with IV antibiotics and packed red cells blood transfusion and discharged from hospital after 2 weeks. She was admitted two weeks later with vomiting, loose bowel motion and a generalised rash consistent with measles. Her stool examination was positive for cryptosporidium and a mid-stream urine test revealed significant growth of E. coli. She was treated with IV antibiotics and IV fluids. On examination at age 5 years, the patient was mildly hyperpigmented. She had a flat ACTH stimulation test with a reasonable base line cortisol level but no increase in response to an ACTH challenge. Biochemical test results were compatible with a diagnosis of FGD. In addition, she had a markedly delayed bone age (two and a half year delay), cardiomegaly, bronchiectasis and atopic dermatitis.

Pedigree 2 IV:10 [1997] Previously described by O' Riordan and colleagues (referred to as P1) [1].

Pedigree 2 IV:11 [1991] Patient IV:11 was born at 37 weeks gestation and weighed 1.64 kg (0.4^{th}-2nd centile). She was started on steroids at an early age because her sister died of an Addisonian crisis. Immunological investigations showed that patient IV:11 had no B cells and reduced NK cell activity. Subsequent immunological analyses at age 7 years showed a reduction in B cells (CD19+) and T cells (CD3+). She also has adrenal insufficiency which was categorised as an isolated corticosteroid deficiency. Similar to her brother, she has FGD, mosaic FA and short stature. Patient IV:11 was tested for a chromosome 22 deletion frequently observed in patients with DiGeorge syndrome, CHARGE association, familial or isolate congenital defects or velocardiofacial syndrome. No deletion on either of the chromosome 22 homologues was detected by FISH analysis with a probe (D22S75) mapping to 22q11.2. At age 12 years, the patient's height was considerably below the 3rd centile and she showed very poor growth over the previous year. Her bone age at 151 months (12.6 years) was 94 months (7.8 years).

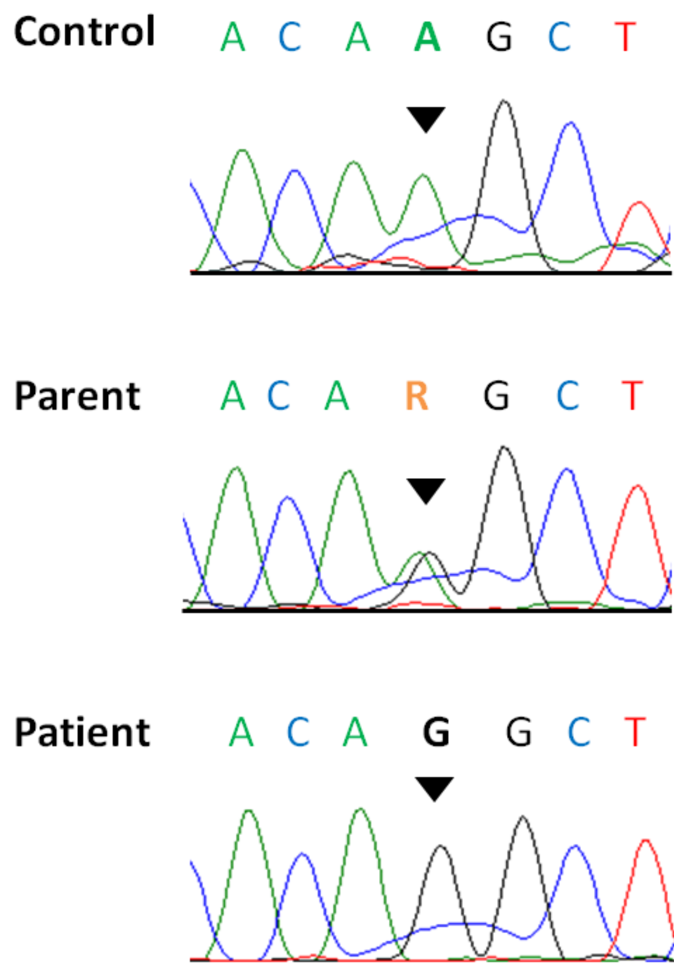
Pedigree 2 IV:14 [1996] Patient IV:14 was born by elected caesarean at 34 weeks gestation for intrauterine growth retardation (IUGR) and weighed 1.84 kg ($<0.4^{th}$ centile). He was investigated in hospital for FTT at 9 months of age, at which stage he showed no evidence of adrenal insufficiency and had normal 17-hydroxyprogesterone and testosterone levels. Patient IV:14 had one sister who died at two years of age from presumed adrenal crisis and overwhelming sepsis. She was also small with FTT but the test for FA was negative. However, it was found that she had an endoreplication of chromosome 22. Therefore, patient IV:14 was tested for a 22q11.2 deletion but no copy number variants were detected by FISH analysis. At age 2 years, the patient had haemophagocytic activity syndrome triggered by Epstein-Barr virus (EBV) or parvo virus. He was also admitted to hospital with severe bronchiolitis pneumonia where he was found to have pancytopenia and hypoglycaemia. A

year later, he had a small bowel resection because of an obstruction. This revealed an EBV-driven lympho-proliferative disorder. He was also regularly suffering from recurrent infections. At age 6 years the patient was referred to clinic because of his short stature, gross pigmentation and developmental delay. On examination it was noted that he had clinodactyly, a bone abnormality often observed in patients with FA. Biochemical and immunological investigations showed that he had low parathyroid and vitamin D levels, growth hormone deficiency and ACTH resistance. He was also diagnosed with mosaic FA and a NK cell deficiency. Consistent with a reduced level of NK cells, the cytotoxic activity of the patient's NK cells was also impaired. The NK cell deficiency is believed to be the predisposing factor to viral infections in this patient. At age 5 years and 6 months his weight was below the 3rd centile and his height was on the 3rd centile.

Pedigree 2 IV:16 [1997] Previously described by O' Riordan and colleagues (referred to as P4) [1].

Pedigree 3 II:1 [1992] Patient II:1 was born at 36 weeks gestation and weighed 1.56 kg (<0.4th centile). He showed signs of IUGR. He was admitted to hospital at 15 months of age with gastroenteritis and was noted to be below the 3rd centile for height, weight and head circumference. He also has a delayed bone age. At age 2 years and 6 months, the patient appeared pigmented and analysis of ACTH levels confirmed a diagnosis of FGD. Immunological investigations identified low NK cells (5%).

Supplementary Figure S1 Validation of MCM4 c.71-2A>G mutation by Sanger sequence analysis



The MCM4 c.71-2A>G mutation was validated by Sanger sequence analysis. The three rows represent Sanger traces corresponding to a control, parent and patient respectively. The control is an unaffected member of the Irish Traveller population. Sanger sequencing showed that all affected family members are homozygous for the MCM4 c.71-2A>G mutation. As expected, the unaffected parents are obligate carriers and unaffected siblings are heterozygous or homozygous for the normal allele.

Supplementary Table S1 Targeted resequencing of 34 positional candidate genes within the 8p11.21-q11.22 locus

Individual	Total Reads	Properly paired	Called bases overlapping with targeted exons	% coverage of targeted exons	Median coverage of targeted exons
P1 III:11	25,081,194	20,861,664	189,017,852	95.67	454
P1 IV:4	23,597,950	20,507,544	252,447,631	97.10	1,712
P1 IV:9	20,727,812	17,664,938	206,556,547	96.74	1,270
P1 IV:12	20,281,028	16,321,176	132,917,599	95.83	671
P2 IV:1	20,341,590	17,372,686	179,401,619	96.55	1,190
P3 II:1	26,584,196	22,737,288	254,229,824	96.96	1,778
P1 III:12	22,307,382	19,460,166	223,394,944	96.49	1,533
P1 IV:5	25,883,434	22,344,102	254,839,716	96.65	1,742
P1 IV:11	27,808,278	23,167,452	303,473,923	95.66	696
P2 IV:2	25,782,056	18,057,274	207,451,249	96.37	913
Average	23,839,492	19,849,429	220,373,090	96.40	1,196

DNA from six affected and seven unaffected relatives was selected for targeted resequencing of the 34 positional candidate genes. Target enrichment was performed using an Agilent SureSelect XT Custom MP2 kit and the libraries were analysed by paired-end sequencing on an Illumina HiSeq. The raw sequence reads were mapped to the reference genome (hg18) using BWA [2]. The percentage coverage of the targeted exons and the median fold coverage of the targeted exons were calculated. On average, the targeted regions were sequenced to a median fold coverage of 1,196x.

Supplementary Table S2 Software predicted effect of intronic *MCM4* variant at the cDNA level

Intronic sequence variant	**Transcripts**	**CV for WT sequence**	**CV for mutant sequence**	**ΔCV (%)**
c.71-2A>G	WTA	71.54	42.59	-40.46 (**SB**)
	A1/c.71-13	55.36	84.3	+52.29 (**NS**)
	A2/c.71-5	68.49	68.61	+0.17

The relative strength of the splice sites is given as the consensus value (CV), which ranges from 0 to 100. Splice sites with CVs higher than 80 are strong splice sites; less strong sites have CVs that range from 70 to 80. The effect of a splice site mutation does not solely depend on the CV value, but also on the relative change in ΔCV. Desmet et al. (2009) emphasised that ΔCV reductions of at least 10% for a mutation in any position, or of 7% for a mutation in position +4, are likely to affect splicing [3]. The MCM4 c.71-2A>G mutation was analysed using Human Splicing Finder to determine its effect on the given splice site based on the two parameters CV and ΔCV. Mutated splice sites with CVs >70 are likely to retain some activity while those with CVs <70 are considered inactive. ΔCV reductions <10% are likely to retain some wild-type splice site activity, whereas ΔCV reductions >10% are considered broken and inactive. The mutated splice site has a CV <70 (52.59) and a ΔCV >10% (40.46%) which is predicted to result in a broken and inactive splice site. WTA: wild type acceptor; A1, A2: alternative cryptic acceptor sites; CV: consensus value; ΔCV(%): CV variation between wild type and mutated sequence; **SB**: site broken; **NS**: new site

References

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