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TITLE

Gross rearrangements of the *MECP2* gene are found in both classical and atypical Rett syndrome patients

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ABSTRACT

INTRODUCTION: *MECP2* mutations are identifiable in ~80% of classic Rett syndrome (RTT), but less frequently in atypical RTT. METHODS: We recruited 110 patients who fulfilled the diagnostic criteria for Rett syndrome and were referred to Cardiff for molecular analysis but in whom an *MECP2* mutation was not identifiable. Dosage analysis of *MECP2* was carried out using MLPA or QF-PCR. RESULTS: Large deletions were identified in 37.8% (14/37) of classic and 7.5% (4/53) of atypical RTT patients. Most large deletions contained a breakpoint in the deletion prone region of exon 4. The clinical phenotype was ascertained in all 18 of the deleted cases and in four further cases with large deletions identified in Goettingen. Five patients with large deletions had additional congenital anomalies, which was significantly more than in RTT patients with other *MECP2* mutations (2/193 $p<0.0001$). CONCLUSIONS: Quantitative analysis should be included in molecular diagnostic strategies in both classic and atypical RTT.

INTRODUCTION

Rett Syndrome (RTT; MIM#312750) is an X-linked dominant neurological disorder of development, first described by Andreas Rett in 1966. It affects 1 in 10,000 females and possibly 1 in 100,000 males. [1-3] Classic RTT describes a characteristic presentation where all of the following clinical features are present: subtle abnormalities in development from birth, a period of stagnation, followed by regression with loss of hand and social skills and development of hand stereotypies, deceleration in head growth, severe learning difficulties and gait dyspraxia. [4-6] By contrast, atypical RTT defines those patients who meet most, but not all, of the criteria of the classic picture yet still conform to the expected profile of the disorder. [6]

Pathological mutations in the *MECP2* gene (MIM# 300005), which encodes methyl-CpG-binding protein-2, were first reported in RTT in 1999. [7] There are eight common mutations which arise at CpG hotspots. [7] *MECP2* mutations are identifiable in ~80% of classic RTT patients [8-10] but less frequently in atypical RTT. [9;11-16] The *MECP2* gene contains four exons and encodes two major functional domains; the methyl binding domain (encoded by exons 3 and 4) [17] and the transcription repression domain (encoded by exon 4) which contains a nuclear localisation signal. [18] The *MECP2* gene is flanked at the 3' end by the *IRAK1* gene and at the 5' end by the *OPN1LW* gene. The *IRAK1* gene has been postulated to play a role in atherosclerosis [19] whereas *OPN1LW* gene variants have been associated with colour vision variation. [20] The *MECP2* translational initiation site was originally identified in exon 2 but a second translation initiation site has recently been described in exon 1, which yields a new and predominant MeCP2 isoform. [21] Mutations in exon 1 have been reported [22], although they are rare. [23]

Gross rearrangements of the *MECP2* gene, which are not detectable by sequencing or DHPLC, have been successfully identified by Southern blot analysis [16;24-25] and more recently by using dosage assays including quantitative fluorescent PCR [26], real time PCR [26], and multiplex ligation-dependent probe amplification (MLPA). [27] So far, no gross rearrangements of the *MECP2* gene causing RTT have been identifiable by FISH. [28] In previous studies, quantitative methods have shown deletions in ~29% of females with classical RTT who had no detectable *MECP2* mutation by sequence analysis or DHPLC. [16;24-27;29-32]

We investigated our cohort of RTT patients, in whom no *MECP2* mutation had previously been detected by sequence analysis, for large deletions using QF-PCR or MLPA.

METHODS

Patient recruitment

Patients were recruited, with informed consent, from those referred to the Cardiff diagnostic laboratory for *MECP2* analysis since 1999. Of 337 female patients recruited, 227 had a pathological mutation identified by sequencing exons 2, 3 and 4 of the *MECP2* gene. Clinical data for these patients were ascertained through a clinical questionnaire. The remaining 110 patients, whose clinical phenotype was consistent with RTT, were recruited for quantitative analysis of the *MECP2* gene. In addition to the Cardiff cohort, four patients with large deletions identified in Goettingen were recruited for X-inactivation studies and clinical assessment.

Patients were designated either classic or atypical according to Hagberg criteria for RTT. [6;33] Since development in babies with RTT is abnormal from birth, the criteria were modified to take this into account. [34] Common atypical features included preserved head growth (no departure from the birth centile), absence of regression, late regression (after 30 months) or the early onset of

seizures (in the first 12 months of life or before regression). Where clinical data were insufficient to permit classification or data were unavailable, patients were assigned to the "not-classified" group.

Molecular Methods

Exon 1 of the *MECP2* gene was sequenced as previously described. [23]

Quantitative analysis was carried out using one of three dosage-based assays to identify gross deletions of the *MECP2* gene: quantitative fluorescent PCR (amplicons from exons 2, 3 and 4), real time PCR (amplicons from exons 2, 3 and 4) or multiple ligation-dependent probe amplification (amplicons from exons 1, 2, 3 and 4 of the *MECP2* gene and amplicons from the flanking genes *IRAK1*, *L1CAM* and *SYBL1*) (MRC Holland, Amsterdam) as previously described (Figure 1). [23;26-27]

Analysis of X-chromosome inactivation was performed in patients from both Cardiff and Goettingen with exonic deletions as described. [35] In summary, lymphocyte genomic DNA was restricted with *Hpa*II or McrBC enzymes prior to PCR amplification of the polymorphic CAG repeat of the androgen receptor (*HUMARA*) gene. Restriction fragments were run on an ABI 3100 DNA sequencer and peak areas compared.

Clinical Assessment

All 18 Cardiff patients and 4 Goettingen patients in whom a deletion was identified were invited to attend for clinical assessment by one of the authors (HA), and this was accomplished for 11 patients. For the remaining patients, clinical information was collected by a questionnaire completed by the patient's physician or family. A severity score, devised by A Kerr, was assigned to each patient, scoring 0, 1 or 2 for each of the following: muscle tone, locomotor ability, feeding ability, seizures and scoliosis; therefore the most severe score would be 10. [36] The score generally increases until the age of 15 years and then plateaus. [37]

RESULTS

Large deletions of the *MECP2* gene accounted for 37.8% (14/37) of classic RTT patients and 7.5% (4/53) of atypical RTT patients where no mutation had been identified by sequencing of all 4 exons of the gene (Table 1). Intra-exonic, single exon and multi-exon deletions were found (Figure 1). More than 80% of the deletions involved exons 3 and/or 4 (Table 2). Among 14 deletions involving the 3' amplicon of exon 4, four extended to involve the *IRAK1* gene. Large deletions were among the most frequent *MECP2* mutations in the whole Cardiff cohort accounting for 7.3% of all mutations found (Table 3).

X-inactivation studies, in lymphocytes, were carried out on all Cardiff and Goettingen patients (total 22 patients) with large deletions. Analysis was informative for 16/22 patients with large *MECP2* gene deletions and uninformative in one. DNA was insufficient for X-inactivation analysis in three patients. X-inactivation was skewed more than 70% in six patients. The direction of skewing was not ascertained. Although numbers were insufficient for statistical analysis, there did appear to be a trend for those with skewing to be either more mildly or more severely affected (Table 2).

Of all 22 patients (18 Cardiff, 4 Goettingen) with large *MECP2* gene deletions, 18 had classic RTT and 4 atypical RTT (Table 2). Five patients had additional congenital anomalies, which in patients 1, 19 and 20 were considered to be severe (Table 2). Both patients with a cleft palate (patients 18 and 20) had normal G-banded karyotype and FISH studies for a 22q11 microdeletion.

The frequency of non-familial congenital anomalies was statistically significant when the Cardiff group with large deletions was compared with patients with other *MECP2* mutations (Fishers exact test, $p<0.0001$).

All deletion patients had severe intellectual disability and most were unable to walk, to use their hands, or speak. Only a single patient spoke one or two words with meaning. Over half of the deletion patients were microcephalic, which in three had been apparent from birth. All had marked hand stereotypies and only three were able to finger feed. Only seven patients could walk independently. All had abnormal muscle tone and dyspraxia. Scoliosis was reported in 19 cases. All had poor peripheral circulation, and breathing irregularity was present in all but one case. In the patients classified as atypical RTT, one had onset of seizures before regression, one had preserved head growth, one had complex congenital anomalies, and one had abnormal neuroimaging (Table 2). The large deletion group were indistinguishable from other mutation positive Rett patients in terms of clinical severity.

		Classic RTT n=188	Atypical RTT n=123	Not classified n=26
MECP2 mutation identified by sequencing		143	58	26
Dosage assay results	deletion	14	4	
	normal	23	49	
	fail / insufficient DNA	8	12	

Table 1: Results of *MECP2* analysis in 337 female patients with RTT from the Cardiff study group.

Patient	Quantitative Result	Quantitative Methods	*IRAK1* gene also deleted	X-inactivation ratio	Age (Yrs)	Rett Type	Atypical Features	Congenital Anomalies	Severity	Walk	Hand use	Speech	Scoliosis	OFC
1	Deletion 1	MLPA	Yes	54:46	14	Atypical	Abnormal MRI	Hypoplastic cerebellum and cerebellar vermis	9	No	Grasp briefly	No	Mild	<3rd
2	Deletion 1 – 2	MLPA	No	65:35	4.2	Classic			3	Yes	Finger feeds	No	No	2nd– 9th
3	Deletion 1 – 2	QF-PCR	Nk	57:43	45	Classic			6	With support	Finger feeds	No	Moderate	50th
4	Deletion 1 – 2	MLPA	No	80:20	16.0	Classic			9	No	None	No	Severe	<3rd
5	Deletion 3 – 4.1	QF-PCR	Nk	67:33	4.3	Classic			5	No	None	No	No	2nd
6	Deletion 3 – 4.1	QF-PCR	Nk	58:42	12.9	Atypical	Epileptic seizure onset before regression		10	No	Grasp briefly	No	Severe	<0.4th
7	Deletion 3 – 4.1	QF-PCR	Nk	75:25	5.2	Classic		Developmental dysplasia of the hip (DDH)	3	Yes	None	No	Mild	<0.4th
8	Deletion 3 – 4	RT-PCR	No	90:10	20.3	Classic			5	With support	None	No	Moderate	0.4th- 2nd
9	Deletion 3 – 4	RT-PCR	No	90:10	14.6	Classic			3	Yes	None	No	Mild	9th
10	Deletion 3 – 4	RT-PCR	No	60:40	21.4	Classic			4	No	None	No	Mild	0.4th- 2nd
11	Deletion 3 – 4.3	QF-PCR MLPA	Yes	57:43	10	Classic			6	No	None	No	Mild	0.4th- 2nd
12	Deletion 3 – 4.3	QF-PCR MLPA	Yes	61:39	7	Classic			6	No	Grasp briefly	No	Moderate	<0.4th
13	Deletion 3 – 4.3	MLPA	No	NP	19.2	Classic			5	With support	None	No	Mild	<3rd
14	Deletion 3 - 4.3	MLPA	No	56:44	15.0	Classic			7	No	None	No	Mild	0.4th– 2nd
15	Deletion 3 - 4.3	MLPA	Yes	NP	6.5	Classic			3	Yes	None	Possibly	Mild	2nd- 9th
16	Deletion 4.1 – 4.3	MLPA	No	67:33	12.3	Atypical	Preserved head growth		6	No	None	No	Moderate	50th
17	Deletion 4	RT-PCR	Yes	70:30	4.6	Classic			3	Yes	Grasp briefly	No	Moderate	50th
18	Deletion 4.2	MLPA	No	58:42	4.6	Classic		Cleft palate	9	No	Grasp briefly	No	Mild	<0.4
19	Deletion 4.2 – 4.3	QF-PCR MLPA	No	NI	38.0	Classic		Atretic right ear and absent auditory meatus	4	Yes	Finger feeds	No	Moderate	<0.4th
20	Deletion 4.2 – 4.4	QF-PCR MLPA	No	85:15	4.4	Atypical	Multiple congenital anomalies Very abnormal early development	Transposition great arteries, right choanal stenosis, laryngeal web, cleft palate	8	No	None	No	Moderate	<0.4th
21	Deletion 4.3	MLPA	No	58:42	14.3	Classic			*	Yes	Nk	No	Nk	<3rd
22	Deletion 4.3	QF-PCR MLPA	No	NP	12.4	Classic			8	No	Grasp briefly	No	Severe	<0.4th

Table 2: Clinical and molecular results for 22 RTT patients with large deletions of *MECP2*. Quantitative results: exon 1, 2, 3 and 4 refer to amplicons derived from these *MECP2* exons (Figure 1). MLPA – multiplex ligation dependent probe amplification; QF-PCR – quantitative fluorescent PCR; RT-PCR – real time PCR. X inactivation (NI – no informative, NP not performed due to insufficient DNA). Severity score – composite severity score

for muscle tone, locomotor ability, scoliosis, feeding and seizures 0 = not present, 1 = mild / moderately affected, 2 = severely affected so that most severe score = 10. OFC- current head circumference percentile. * - data unavailable, nk- not known, na – not applicable. Patients 9, 12, 13 and 20 have been previously reported [26]

Mutation	Frequency	Proportion of all mutations
R168X	37	15.1%
C-terminal deletions	28	11.4%
T158M	25	10.2%
R255X	24	9.8%
R270X	19	7.8%
R133C	18	7.3%
Large exonic & multi-exonic deletions	18	7.3%
R306C/R306H	13	5.3%
R294X	12	4.9%
R106W/R106G	12	4.9%
Other	39	15.9%
	245	100%

Table 3: Frequency of *MECP2* gene mutations detected in the Cardiff study group.

DISCUSSION

Within this cohort of patients with Rett but without a mutation in *MECP2* identified by sequencing, 37.8% of classic RTT and 7.5% of atypical RTT patients had deletions of the *MECP2* gene, predominantly involving exons 3 and 4. To our knowledge this is the first study to determine the frequency of large deletions in patients with atypical RTT, and to highlight their importance in RTT patients with additional congenital anomalies.

There are nine published studies which have looked for large rearrangements of the *MECP2* gene in patients with suspected RTT. [16; 24-27; 29-32] The frequency of large deletions in clinically defined classic RTT alone was 28.8% (30/104 patients). [24-27;29-31] The overall deletion detection rate, combining results from published studies, in suspected RTT (classic, atypical and unclassified) was 11.9% (26/218 patients). [16;25-27] The majority (140) of these patients were reported in one large study and were selected on the basis of having strongly suspected RTT rather than meeting diagnostic criteria for RTT; the need for careful clinical selection in future studies was highlighted by the authors. [26] Another study found no deletions among a group of 17 patients selected only on the basis of referral for *MECP2* analysis. [31] The lower deletion frequencies found in these less well selected groups provide support for our policy of strict clinical selection on the basis of diagnostic criteria. We consider that the higher deletion frequency as reported here is a more accurate reflection of the true deletion rate in clinically defined RTT.

Prior to this study, there was only one reported atypical RTT patient with a large deletion. [26] Another atypical case had a duplication. [29] One study looked for large deletions in patients with the preserved speech variant of RTT, but none were found, which suggests that they will be rare in mild atypical presentations of RTT. [25] The relative lack of gross rearrangements in atypical RTT could reflect patient selection bias in that classic RTT patients may constitute not only the most frequently studied group, but also the most intensively investigated group in terms of the *MECP2* gene analysis performed. Our study has shown that large deletions of *MECP2* are an important and frequent cause of atypical RTT.

Large deletions frequently involve either exon 4 or both exons 3 and 4 of the *MECP2* gene. They probably arise by unequal recombination caused by mis-pairing of homologous repetitive elements. [26] There is a highly repetitive region in exon 4 of the *MECP2* gene (deletion prone region), [26] located 3' to the TRD, where many intra-genic deletion breakpoints occur (C-terminal deletions). Recombination between this and a second highly repetitive region, located in intron 2, could be responsible for mediating the commonly identified large deletions encompassing both exons 3 and 4. [26] It is likely that there are also recombinogenic repetitive elements in the 3'UTR which give rise to the large intra-genic deletions noted in exon 4.

Late-truncating mutations of the *MECP2* gene arising 3' to the TRD (in the terminal exon 4) are often associated with a milder clinical phenotype. [10;38] Therefore, one might expect that the 6 patients with deletions involving only the 3' amplicons of exon 4 would have presented with a milder phenotype than actually observed. Detailed clinical information was however only available for four patients. One patient had additional significant congenital anomalies, but also skewed X-inactivation, and these factors probably added to the severity of her phenotype. The remaining patients had classic RTT, with two patients retaining the ability to walk, and one older patient still able to walk with support. A 3' terminal exon 4 deletion would be predicted to lead to (i) loss of the termination codon in all cases and (ii) loss of at least one of the polyadenylation sites in most cases. Although the consequences at the level of protein expression of large 3' terminal *MECP2* deletions are unknown, such mutations are likely to disrupt the secondary structure of mRNA with resultant mRNA instability. Whether large deletions invariably constitute a null mutation or whether some

residual functional MeCP2 protein may be present in certain cases, still remains to be determined. No clear relationship was apparent between clinical severity and exonic deletions arising distal to the TRD in this study.

In our study, five patients had additional congenital anomalies, accounting for 22.7% of those with large deletions: four had deletions involving the 3' end of exon 4. The increased frequency of additional congenital anomalies in Cardiff cases with gross *MECP2* deletions (5/18), when compared with RTT patients with any other *MECP2* mutations (2/193), was statistically significant. Some deletions are likely to involve complex deletion-insertion events [26], and the potential disruption of a second gene by the inserted fragment. Extension of the deletion into the adjacent *IRAK1* gene, and potentially other genes in the immediate vicinity, may also play a role in extending the observable phenotype. We suggest that the complex nature of the mutation underlying some large deletions is manifesting in the additional anomalies found in this subset of patients with RTT.

Clinical Relevance

Our results have indicated that large deletions of the *MECP2* gene are an important and frequent cause of *both* atypical and classic RTT. Such deletions may be found in patients with additional congenital anomalies and features of RTT. Quantitative analysis of the *MECP2* gene is an important part of the diagnostic assessment of all patients with clinically confirmed RTT in whom *MECP2* gene sequence analysis has not demonstrated a mutation.

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ETHICAL APPROVAL

Ethical approval was granted by MREC (Wales): reference 02/9/33.

LEGENDS FOR FIGURES

Figure 1: A representative diagram of single and multi-exonic deletions of the *MECP2* gene identified in this study (not to scale). A: The *MECP2* and adjacent genes are shown at the top of the diagram, with the exons of *MECP2* shown in more detail. The methyl-binding domain (MBD), transcription repression domain (TRD) and untranslated regions (UTR) are in their approximate location. B: The position of the amplicons used for quantitative analysis of *MECP2* for each of the three methods: MLPA, QF-PCR and RT-PCR. C: The extent of deletions identified is represented by a thick black bar. The broken line extending from the thick bars shows the unknown extent of each deletion. Note that the majority of the deletions include one potential breakpoint in the deletion prone region of exon 4.

Figure 2: Photographs of patients 19 and 20 with severe congenital anomalies. (a) Patient 19. (b) Atretic right ear of patient 19. (c) Patient 20.

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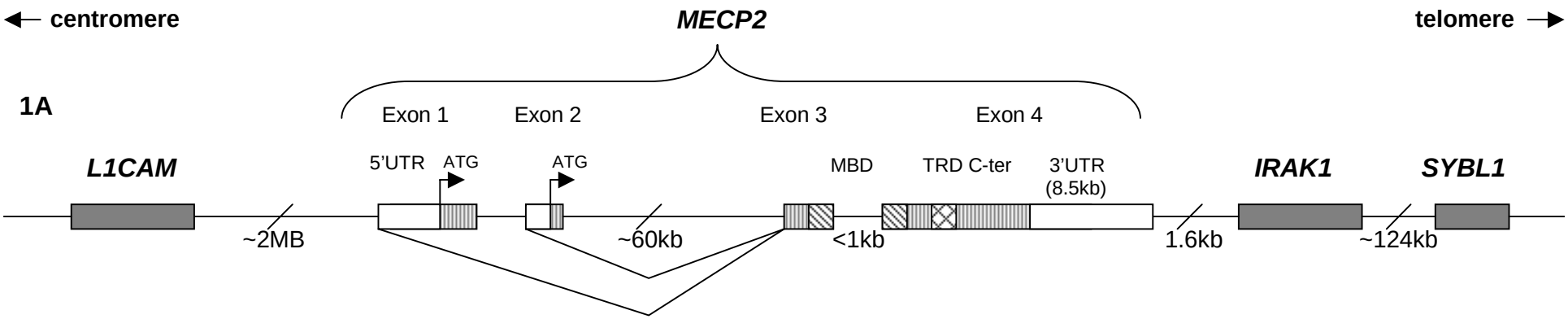
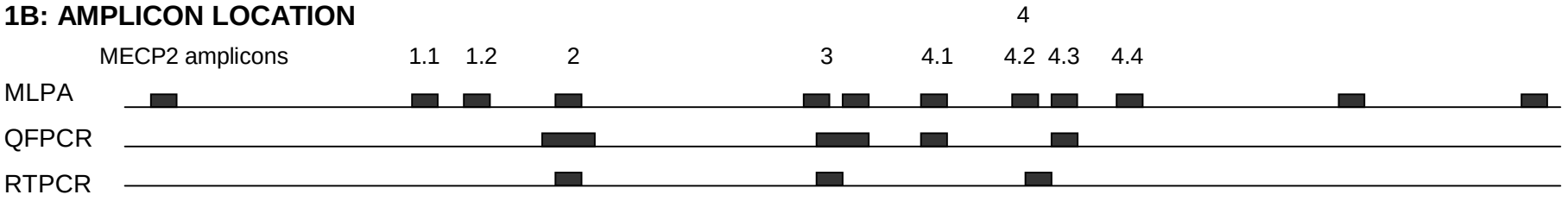
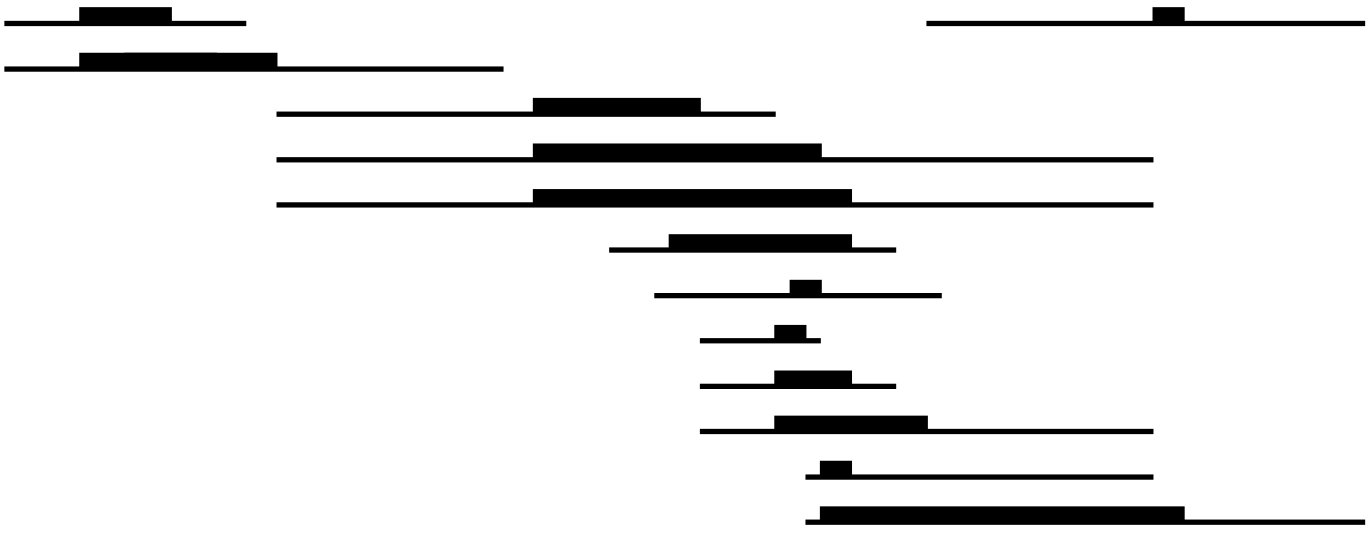
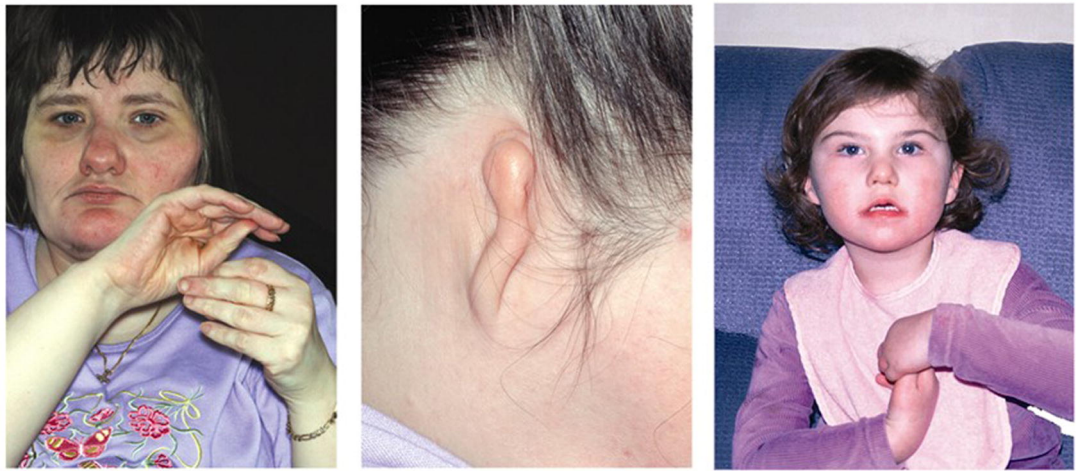




1C: EXONIC DELETIONS:

Patient	Deletion
1	Exon 1 and IRAK 1
2-4	Exon 1 & 2
5-7	Exon 3 & 4.1
8 -10	Exon 3 & 4
11-15	Exon 3 – 4.3
16	Exon 4.1 - 4.3
17	Exon 4
18	Exon 4.2
19	Exon 4.2 - 4.3
20	Exon 4.2 - 4.4
21	Exon 4.3
22	Exon 4.3 – *IRAK1*





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