European Journal of Human Genetics (2013) 21, 460–464

www.nature.com/ejhg

SHORT REPORT

Fine mapping of whole *RB1* gene deletions in retinoblastoma patients confirms *PCDH8* as a candidate gene for psychomotor delay

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Retinoblastoma (Rb) results from inactivation of both alleles of the *RB1* gene located in 13q14.2. Whole-germline monoallelic deletions of the *RB1* gene (6% of *RB1* mutational spectrum) sometimes cause a variable degree of psychomotor delay and several dysmorphic abnormalities. Breakpoints in 12 Rb patients with or without psychomotor delay were mapped to specifically define the role of chromosomal regions adjacent to *RB1* in psychomotor delay. A high-resolution CGH array focusing on *RB1* and its flanking region was designed to precisely map the deletion. Comparative analysis detected a 4-Mb critical interval, including a candidate gene protocadherin 8 (*PCDH8*). *PCDH8* is thought to function in signalling pathways and cell adhesion in a central nervous system-specific manner, making loss of *PCDH8* one of the probable causes of psychomotor delay in *RB1*-deleted patients. Consequently, we propose to systematically use high-resolution CGH in cases of partial or complete *RB1* deletion encompassing the telomeric flanking region to characterize the putative loss of *PCDH8* and to better define genotype/phenotype correlations, eventually leading to optimized genetic counselling and psychomotor follow-up.
European Journal of Human Genetics (2013) **21,** 461–465; doi:10.1038/ejhg.2012.186; published online 22 August 2012

Keywords: retinoblastoma; *RB1*; *PCDH8*; CGH array; psychomotor delay; large-scale rearrangement

INTRODUCTION

Retinoblastoma (Rb) is a rare embryonic neoplasm of retinal origin resulting from inactivation of both alleles of the *RB1* gene (MIM no.180200) located in chromosome band 13q14.2.[1] Predisposition to Rb must be suspected independently of family history and regardless of the clinical presentation, because 100 and 10% of bilateral and unilateral cases, respectively, carry an inherited or *de novo* germline mutation. Screening for the predisposing *RB1* mutation should therefore be proposed to all Rb patients.[2,3] The pattern of mutations found in molecular studies revealed the existence of 6% of complete deletions of the *RB1* gene that are associated with variable phenotypes.[4,5]

Interstitial 13q deletions involving *RB1* and its flanking regions, initially revealed by karyotype analyses in Rb patients, were found to be associated with dysmorphic, cranial and hand/foot abnormalities, psychomotor delay and hypotonia.[6,7] Correlations between the size of the deletion and the phenotype were therefore investigated. Although a correlation between the size of the deletion and a specific pattern of malformations and dysmorphism was not established, psychomotor delay was suspected to be restricted to patients harbouring a deletion that encompasses more than the 13q14 band.[7] The size and location of the deletion may therefore define the risk of psychomotor delay in a context of contiguous gene syndrome as previously demonstrated, for example, in neurofibromatosis type 1.[8] The correlation between the size of the deletion and psychomotor delay in Rb has not yet been determined because of the limited resolution of karyotype analysis. High-resolution analysis of deletions, for example, by CGH, allows this issue to be properly addressed. This work is a nice follow-up of previous studies,[9,10] as it specifically tackles for the first time the issue of psychomotor delay in *RB1*-deleted patients. We used a dedicated *RB1*-customized CGH-array designed to define a critical interval and consequently identify candidate genes. This study also provides clues concerning the role of CGH array in Rb molecular diagnosis and parent/patient information regarding genetic counselling.

PATIENTS AND METHODS

Patients

Diagnosis of Rb was established on the basis of examinations by an ophthalmologist and a paediatrician, and by histopathological criteria when the tumour was available. Rb patients were offered genetic counselling, and individual written consent was obtained from all sampled individuals or their legal guardians. In this series of 1160 consecutively ascertained cases, 320 mutations were found, and a total of 17 patients were diagnosed with a complete deletion of the *RB1* gene by QMPSF or karyotype analyses. A sufficient amount of DNA was available for CGH analysis in 12 of these patients. Psychomotor delay was reported either when a paediatrician, geneticist and/or psychometrician observed a delayed motor development or speech acquisition delay, or when the patient was taken into care by a specialized educational structure (reported in Table 1 as a binary variable 'yes' or 'no').

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Received 3 April 2012; revised 20 June 2012; accepted 17 July 2012; published online 22 August 2012

Table 1 Patient characteristics and cytogenetic results

Patient ID	Rb phenotype	Origin of deletion	Age at diagnosis (month)	Psychomotor delay	Age at last examination (years)[a]	Other	Cytogenetic analysis	CGH array analysis: oligonucleotide		Sequencing analysis	
								Last present proximal	First present distal	Breakpoint nomenclature (Hg 18)	Size of deletion (bp)
1	Bilateral	*De novo*	n.r.	No	22	n.r.	46, XX,	47530000	49383000	n.d.	1 853 000[b]
2	Bilateral	*De novo*	3	No	12	Macrocrania	n.p	47644400	49592400	g.47645203_49591383del	1 946 179
3	Bilateral	Inherited	1	No	28	Bifid uvula	46, XY	47358800	50134000	g.47359006_50133362del	2 774 357
4	Bilateral	Inherited	2, 5	No	17	n.r.	n.p	45962800	48755600	g.45962864_48748560del	2 785 697
5	Bilateral	*De novo*	4	Yes	18	Documented fetal suffering	46, XX	45471400	52239000	g.45471625_52237785del	6 766 161
6	Unilateral	Inherited	10	No	14	n.r.	46, XY	42449700	50716400	g.42449743_50715540del	8 265 798
7	Bilateral	*De novo*	<12	Yes	15	Epilepsy	46, XX, del(13)(q14.1q14.3) t(14;19)(p13;q12)	43212100	54502000	g.43213373_54501464del	11 288 092
8	Bilateral	*De novo*	60	No	31	n.r.	46, XY (13 mitosis) 46, XY, del(13)(q14) (7 mitosis)	44701200	61288700	n.d.	16 587 500[b]
9	Unilateral multi-focal	*De novo*	11	Yes	8	n.r.	46, XX, del(13)(q13q21.3)	39587600	66345200	n.d.	26 757 600[b]
10	Bilateral	*De novo*	30	Yes	14	Facial dysmorphism	46, XY, del(13)(q13q21.1)	38819600	67215300	g.38819991_67214419del	28 394 429
11	Bilateral	*De novo*	12	Yes	2	Epilepsy	46, XY, del(13)(q13q21) (3 mitosis) 46, XY (9 mitosis)	38447600	>74000000	n.d.	>35 552 400[b]
12	Unilateral	*De novo*	12	Yes	6	Multiple dysmorphism	46, XY, del(13)(q13q32)	36383200	>74000000	n.d.	>37616800[b]

Abbreviations: n.r., not reported; n.p., not performed; n.d., not determined.
[a]Age at last follow-up exam by a geneticist and/or a paediatrician.
[b]Size of deletion estimated by CGH analysis.

Cytogenetic analysis

Karyotype analyses with RHG banding and FISH with an *RB1* probe (Vysis, Downers Grove, IL, USA) were performed according to standard cytogenetic procedures. A customized CGH array centred on the *RB1* locus was designed on a 1×385-K oligonucleotide CGH microarray (Roche NimbleGen, Madison, WI, USA). The covered region corresponded to the genomic position Chr13:34000000–74000000 (Hg18), for example, a 100-bp resolution. Data were analysed using VAMP software.[11]

Characterization and sequencing of breakpoints

First, MP/LC[12,13] was used to refine CGH analysis. MP/LC is a technique for the detection of chromosomal rearrangements, which combines the advantages of semiquantitative multiplex PCR and quality of separation of DHPLC. Long-range PCRs were then performed using TripleMaster PCR System (Eppendorf, Hamburg, Germany). Amplicons were sequenced using the BigDye Terminator V1.1 Cycle Sequencing Ready Reaction kit (Life Technologies, Carlsbad, CA, USA), followed by electrophoresis in an ABI 3130xl (Life Technologies).

RESULTS

The 13q deletion in all 12 patients was characterized by our *RB1*-customized CGH array (Table 1 and Figure 1). The largest deletion that was not detected by karyotype analysis measured 8.2 Mb and the smallest deletion detected by karyotype analysis measured 11 Mb. The karyotype resolution was therefore about 10 Mb, which is consistent with routine diagnostic practice. The sequencing experiment (Figure 2) demonstrated good accuracy of the CGH array, as the mean difference of location between the sequencing and CGH mapping results was equal to 1.08 kb ($\pm$ 1.7 kb, SD). Unfortunately, long-range PCRs and breakpoint sequencing failed in five cases due to low complexity and/or repeated regions. Nevertheless, CGH resolution was sufficient to allow breakpoint location, for example, for *PCDH9* (see below).

Patients 1 to 6 presented a molecular microdeletion (not detected by karyotype analysis), and patients 7 to 12 presented a cytogenetic deletion centred around the 13q14.2 band. One of the 6 patients with a molecular microdeletion and 5 of the 6 patients with a cytogenetic deletion presented psychomotor delay (Table 1). Cytogenetic deletions in a context of Rb were therefore associated with psychomotor delay ($P = 0.03$; Fisher's exact test; two-sided). To define the minimal deletion associated with psychomotor delay, patient 5 (with psychomotor delay) and patient 8 (without psychomotor delay) were excluded from the following analysis because they presented documented fetal suffering or mosaicism documented by karyotype, respectively (Table 1; see Discussion).

The largest deletion found without psychomotor delay measured 8.2 Mb (Table 1, patient 6). Sequencing analysis (Figure 2) showed that the breakpoint were located inside the *EPSTI1* and *FAM124* genes. This interval (chr13:42449743_50715540) of deletion was inherited from the patient's father. In patients 2 and 4, who did not present psychomotor delay, sequencing analysis localized breakpoints inside the *DLEU2* and *CDACC1* genes. The deletion identified in patient 7 (chr13:13213373_54501464) was the smallest deletion (11.2 Mb) associated with psychomotor delay. The breakpoint was found inside the *ENOX1* gene (Figure 2). Combining these results with those from patient 6, the chr13:50715540_54501464 interval defined an *RB1*-flanking telomeric region where candidate genes for psychomotor delay may be found (Figure 1). Twenty-five Refseq genes have been reported inside this region, including *PCDH8* (Supplementary Table 1).

DISCUSSION

High-resolution CGH reliably defined a deleted interval not associated with psychomotor delay (chr13:42449743_50715540; Table 1

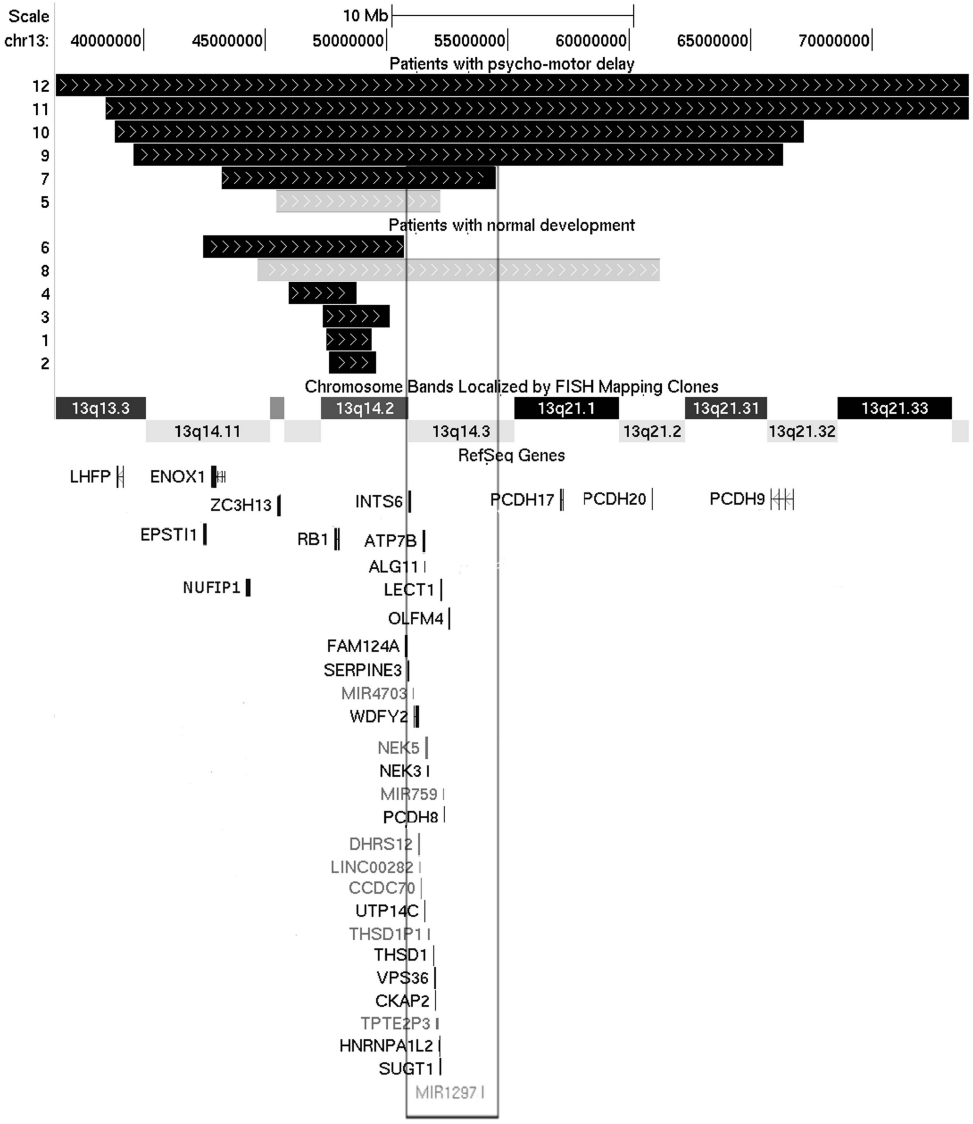

Figure 1 CGH array results from 12 Rb patients harbouring a complete RB1 deletion. Adapted view from UCSC. From top to bottom, schematic view of chromosome, deletion mapped by CGH array labelled with the patient number, representation of cytogenetic bands and Refseq genes. Deletions represented by a grey bar were excluded from the definition of 'the zone of interest' (flanked by the black frame) because of mosaicism or confounding diagnosis. *PCDH8* loss was always associated with psychomotor delay, whereas a deletion encompassing *NUFIP1* cosegregated in patient 6 and his affected relatives, without any detectable impact on psychomotor development (see text for details). The Refseq genes shown are only those cited in the text, as well as all those included in the zone of interest.

and Figure 1). This deletion was inherited (Table 1), conferring a high degree of confidence to our results, by excluding the mosaicism bias. On the other hand, mosaicism led to exclusion of patient 8, as a mosaic status can be associated with an attenuated phenotype and should not be considered in terms of the genotype/phenotype relationship.[14] As Rb is a disease with a high rate of *de novo* mutations, an attenuated phenotype (ie, an absence of psychomotor delay in a patient harbouring a cytogenetic deletion) in first-generation mutation carriers is not unexpected.[15,16] Patient 5 was also excluded from the analysis because fetal suffering can be responsible for psychomotor delay, thereby introducing another analysis bias.

The breakpoints of the deletion located in patients with normal development demonstrated that *DLEU2*, *CDACC1*, *EPST1* and *FAM124A* genes, disrupted by the deletion, therefore cannot be associated with psychomotor delay. A breakpoint in the *ENOX1* gene was also found in a patient with psychomotor delay. Inhibition of *ENOX1* has been reported to decrease angiogenesis in tumour growth.[17] *ENOX1* was found with a high but not exclusive expression level in fetal brain (see http://biogps.org), but no other data are available in the literature to incriminate this gene in the context of psychomotor delay. Comparative analysis of deleted intervals in delayed and non-delayed patients identified *PCDH8* as a candidate gene for psychomotor delay (Figure 1). *PCDH8* (MIM no. 603580), for protocadherin 8, is located in 13q14.3, and belongs to a subclass of cadherins.[18] *PCDH8* has a brain-specific expression making this gene a good candidate gene for psychomotor delay. Furthermore, previous linkage data suggested *PCDH8* as a candidate gene for schizophrenia.[19] Also of interest is that protocadherin *PCDH19* has been previously involved in the female-restricted

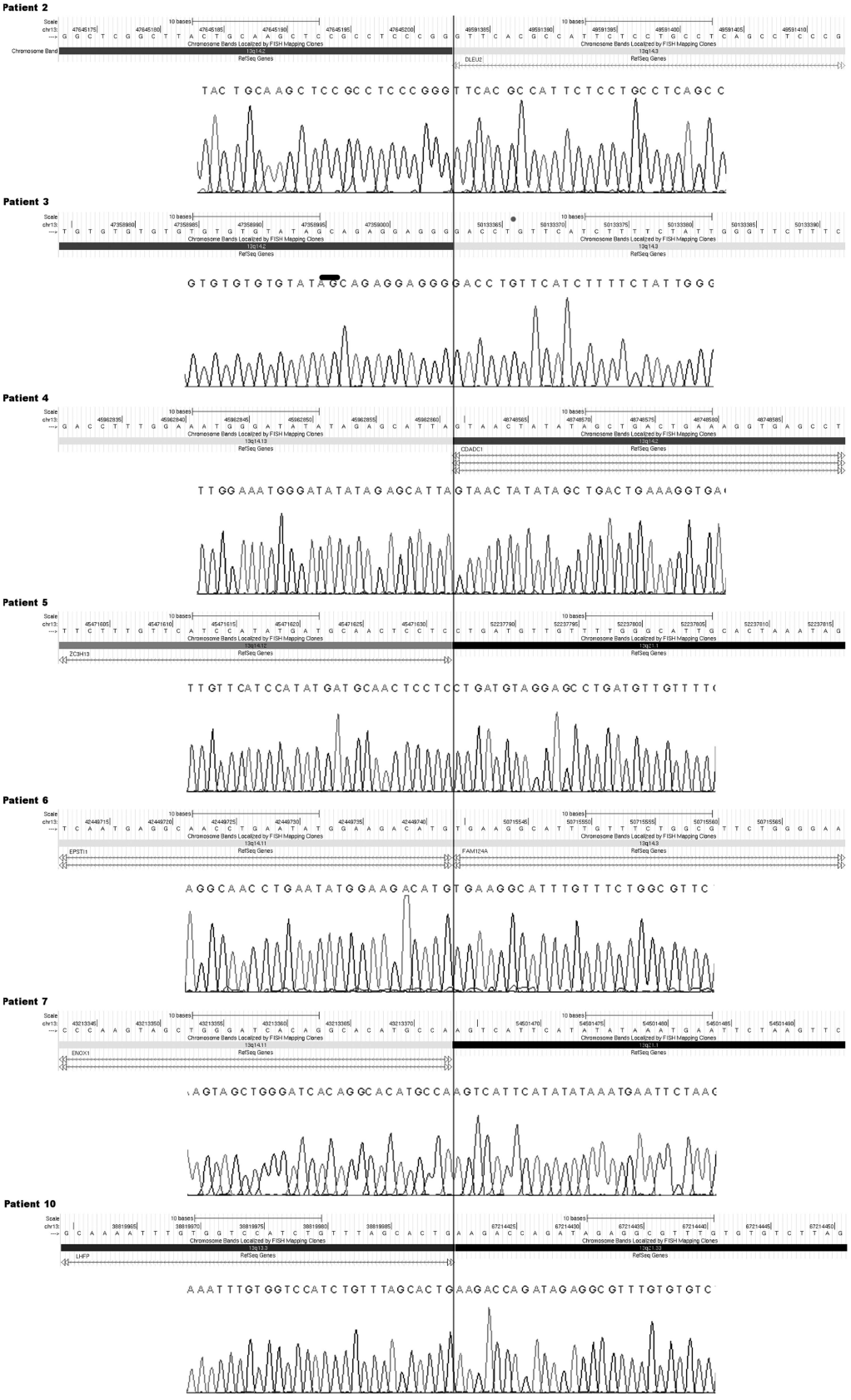

Figure 2 Breakpoint sequencing results. Sequencing electrophoregrams of the deletion breakpoints found in seven patients. The upper part represents an adapted UCSC view in which a schematic view of the chromosome and a Refseq gene representation are included, corresponding to the sequencing electrophoregram. The vertical black line represents the breakpoints.

epilepsy and mental retardation syndrome.[20] Interestingly, 2 patients (Figure 1; patient 7 and 11) with psychomotor impairment and loss of a *PCDH8* copy suffered from epilepsy. Also, our data clearly point to *PCDH8* as one of the putative genes responsible for psychomotor delay in the context of Rb, acting either directly or indirectly via regulatory mechanisms. An autosomic dominant model linked to *PCDH8* loss-of-function could be suspected, but a recessive model driven by epigenetic inactivation of the second allele of *PCDH8* cannot be excluded, as the *PCDH8* promoter has been found to be methylated.[21] Two previous studies also suggested the role of *PCDH8*, but did not formally map this gene, as confounding factors such as mosaicism and alternative causes of psychomotor delay were not evaluated.[9,10] Nevertheless, one study described a few deleted patients without *PCDH8* involvement, who did present psychomotor delay, leading authors to designate *NUFIP1* as another possible candidate gene.[10] Overall data show that loss of neither *PCDH8* nor *NUFIP1* can explain all delayed cases, but *PCDH8* loss is always associated with psychomotor delay, as opposed to *NUFIP1* (see patient 6, Figure 1).

Another interesting finding was that the deletion breakpoints in patient 9, who presented psychomotor delay, were located inside the *PCDH9* gene (Figure 1). On the basis of the role of protocadherins in neuronal development and neuronal plasticity,[22] a *PCDH* dose/effect in the expression of psychomotor delay, implying genetic heterogeneity, could be proposed in the context of complete *RB1* deletion.

In summary, we demonstrated that loss of *PCDH8* in the context of complete deletion of *RB1* should alert geneticists to the risk of psychomotor delay. Fine mapping of deletion breakpoints is therefore mandatory in Rb patients in case of the following: (i) complete or partial *RB1* deletion encompassing a flanking region and (ii) mental delay either isolated or associated with dysmorphic features. This could be performed by CGH array of the chromosome 13 or, in the near future, by a global approach such as massively parallel sequencing. This second line of investigation will precisely define the deleted genes flanking *RB1*, and thereby improve genetic counselling/information and define the most appropriate follow-up options.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by grants from the 'Programme Incitatif et Coopératif sur le Rétinoblastome' (Institut Curie) and RETINOSTOP. We thank Laurence Desjardins, Virginie Moncoutier, Carole Tirapo, Catherine Dubois d'Enghien, Anthony Laugé, Isabelle Eugène, Sandrine Miglierina, Catherine Rougeron and Catherine Gilbon for their helpful support during the study. We also thank clinicians from the GGC (Groupe Génétique et Cancer) for referring patients.

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Supplementary Information accompanies the paper on European Journal of Human Genetics website (http://www.nature.com/ejhg)