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SHORT COMMUNICATION

Further refinement of the critical minimal genetic region for the imprinting disorder 6q24 transient neonatal diabetes

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Abstract
Aims/hypothesis Transient neonatal diabetes (TND) is associated with overexpression of genes within a critical region on 6q24. This study aims to refine the boundaries of this region to reduce the number of potential candidate genes for 6q24 TND.
Methods Fifteen patients with transient neonatal diabetes and submicroscopic chromosome 6 duplications were investigated. The duplications were confirmed by microsatellite analysis and subsequently mapped using tiled chromosome 6 array Comparative Genomic Hybridisation (aCGH) and MLPA. Duplication boundaries were compared to identify the minimal shared region of duplication. These data were then used with available clinical data to identify associations between size of 6q24 duplication and severity of TND phenotype.
Results Alignment of the minimal region of duplication to the human genome reduced the minimal TND critical region, formerly estimated at 440 kb, to 160–173 kb, revealing *PLAGL1* (pleiomorphic adenoma gene-like 1) and *HYMAI* (imprinted in hydatidiform mole) to be the only genes wholly included therein. Additionally, the complete paternal duplication of a region containing the theoretical protein *FAM164B* was associated with the severe growth restriction observed in 6q24 duplication patients.
Conclusions/interpretation This study has significantly reduced the critical region associated with 6q24 TND. It has eliminated several previous TND candidate genes, leaving the overlapping imprinted genes *PLAGL1* and *HYMAI* as the only remaining complete candidate genes for 6q24 TND. Moreover, these data provide the first evidence that an additional region, encompassing the theoretical protein *FAM164B*, may have a critical role in the growth restriction phenotype observed in many 6q24 TND patients.

Keywords Duplication · *FAM164B* · *HYMAI* · Imprinting · *PLAGL1* · Transient neonatal diabetes

Abbreviations

aCGH	Array comparative genomic hybridisation
DMR	Differentially methylated region
IUGR	Intrauterine growth retardation
MLPA	Multiplex ligation-dependent probe amplification
msPCR	Methylation sensitive PCR
PLAG1	Pleiomorphic adenoma gene 1
SNP	Single nucleotide polymorphism
TND	Transient neonatal diabetes
UPD6	Uniparental disomy of chromosome 6

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Introduction

Transient neonatal diabetes (TND) has an incidence of approximately 1:400,000 live births and is defined as diabetes presenting within the first 6 weeks of life with recovery in the first 18 months. Neonates with TND are often growth restricted and usually require a short period of insulin treatment due to low or undetectable levels of insulin at birth.

While 26% of TND is caused by mutations of *KCNJ11* and *ABCC8* on chromosome 11p15, 71% of cases are caused by aberrations of the TND locus on chromosome 6q24 [1]. To date three mechanisms have been described in 6q24 TND: paternal UPD6 [2], paternal duplication of 6q24 [3], and maternal hypomethylation of the 6q24 TND differentially methylated region (DMR) [4]. Through the identification of these mechanisms, it was hypothesised that a paternally expressed imprinted gene(s) existed at 6q24, whose overexpression caused TND. As it is overexpression rather than gene deletion or mutation that causes 6q24 TND, candidate genes cannot be analysed by traditional mutation detection methods. In a previous study [3] the minimal region of duplication was refined to 440 kb, containing four protein-coding genes and one non-coding RNA. Among these, two have been confirmed to be imprinted, namely *PLAGL1* (also known as *ZAC* or *LOT1*) [5] and *HYMAI* [6].

Monoallelically expressed transcripts of *PLAGL1* and *HYMAI* originate from a promoter that coincides with the differentially methylated region associated with TND (TND DMR) [7]. Loss of maternal methylation at this DMR results in relaxation of the normal monoallelic expression of both genes [4]. *PLAGL1* has a second promoter approximately 55 kb upstream from the TND DMR, which is normally unmethylated and supports biallelic transcription [8].

Pleiomorphic adenoma gene 1 (PLAGL1) functions in vitro as a regulator of cell cycle progression and apoptosis, and is regarded as the strongest candidate gene for 6q24 TND. However, this is in part due to the unknown function of *HYMAI* and absence of informative single nucleotide polymorphisms (SNPs) to determine the imprinting status of other genes within the 440 kb minimal region of duplication.

To refine the minimal duplication and reduce the number of candidate TND genes, we have used a combination of techniques for fine mapping of 6q24 duplications in 15 previously unreported patients.

Research design and methods

Patients Fifteen unrelated and previously unreported patients were eligible for this study, as defined by presentation with persistent hyperglycaemia within the first 6 weeks of life as a result of 6q24 duplication. These patients were recruited to the Wessex TND Cohort both directly and by onward referral from the Molecular Genetics Laboratory, Royal Devon and Exeter NHS Healthcare Trust, with informed consent for investigation of neonatal diabetes. Ethical approval for this study was obtained from the Wiltshire Research Ethics Committee (09/H0104/45).

Investigation Diagnosis of TND due to 6q24 duplication was made by methylation sensitive PCR (msPCR) [9] and confirmed by microsatellite analysis using standard methods and published microsatellite markers. Eligible patients were then anonymised with restricted clinical detail. Custom array CGH analysis used a custom 8 × 60 K Agilent array (Agilent Technologies, South Queensferry, UK) designed to densely cover the TND region, with broader coverage across the rest of chromosome 6. Probes were designed using Agilent Technologies' e-Array design tool (www.genomics.agilent.com/CollectionSubpage.aspx?PageType=Product&SubPageType=ProductDetail&PageID=1455) with the following filters applied: Tm filter, similarity filter and catalogue probe preference. Across the TND region (chr6:144126000-144728000) probes were tightly tiled to provide maximum resolution with an average spacing of 50nt. Between the tightly tiled TND region and the centromere (Chr6:136000000-144126000) and toward the telomere (chr6:160465000-170899992) average spacing of probes was extended to 5000nt (excepting a region at 6q27 including *IGF2R* [chr6:160250000-160465000] where average probe spacing was 50nt in relation to a different study). Chromosome 6p was represented by Agilent aCGH control probes, which provide a genome-wide backbone to enable efficient normalisation. aCGH was performed according to the manufacturer's instructions. Briefly, 500 ng of blood genomic DNA was labelled using the genomic DNA enzymatic labelling kit, hybridised to the array for 24 h and subsequently washed. Arrays were scanned using an Agilent Technologies' DNA microarray scanner G2539A and data extracted using Agilent Technologies' Feature Extraction software version 10.5.1. Duplications were mapped and endpoints determined by Agilent Technologies' DNA Analytics software version 4.0 with the following settings: data was analysed using the ADM-2 Algorithm with a threshold of 6.0 using the DLR error model, with fuzzy zero turned on and the continuous moving average applied.

The boundaries for each duplication were taken together to identify the minimal shared TND region of duplication, and 12 MLPA probes were designed across the region. The probes were designed to fit within the SALSA MLPA kit P200 human DNA reference-1 (MRC-Holland, Amsterdam, Netherlands) backbone to allow normalisation. For each

patient, 100–400 ng of DNA was hybridised to the probe mix (targeted probes at a final concentration of 4 fmol/l), with subsequent ligation and amplification all according to the manufacturer's instructions (MRC-Holland). MLPA amplification products were detected on an ABI 3130 capillary sequencer (Applied Biosystems, Warrington, UK). Using peak height values, data were analysed by competitive hypotheses testing (see MLPA spreadsheet analysis instructions at www.ngrl.org.uk/Manchester/sites/default/files/publications/MLPA/Gene%20Dosage/MLPA_analysis_spreadsheets_instructions_v10-06_0.pdf, accessed 1 November 2009) with duplicated probes defined as those with p values <0.05 (i.e. ratio p(normal):p(deleted) ≥20:1 and p(normal):p(duplicated) ≤1:20).

Restricted clinical details (birthweight centile, gestational age and postnatal presentation) were compared using the

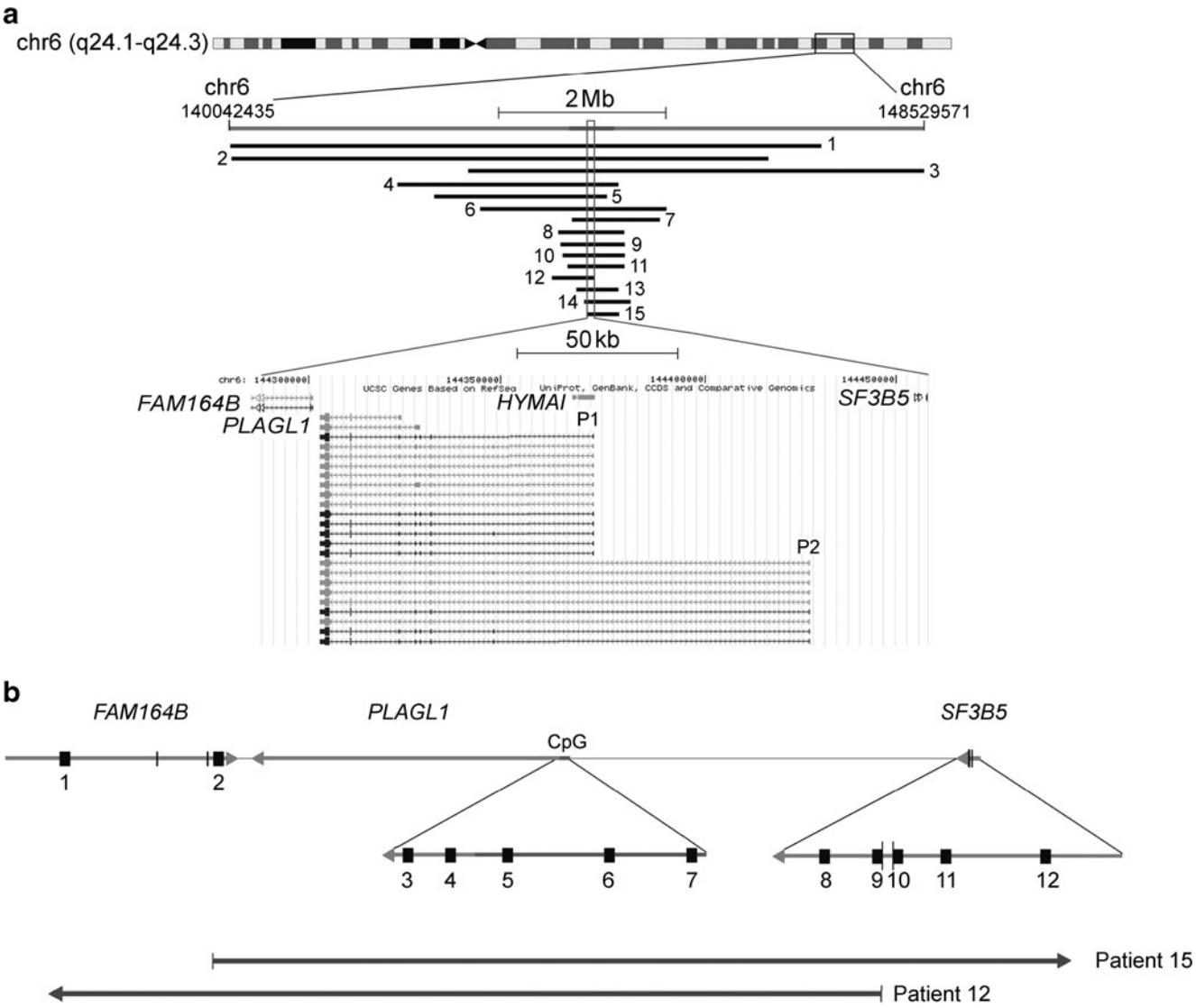

Fig. 1 Schematic representation of the 6q24 duplications in 15 TND patients and positioning of MLPA probes confirming duplication boundaries. (**a**) The black horizontal lines indicate the full extent of each duplication, ordered by duplication size from largest to smallest. Patient numbers included next to each line for identification. Additionally, the previously defined 440 kb minimal region of duplication is represented by the dark section on the expanded chromosomal bar (grey). The refined minimal region of duplication is defined by the smallest region of overlap between patient 15 and 12. This region is highlighted by the downward tram lines. An adapted image taken from the UCSC Genome Browser using the coordinates of the minimal duplication (chr6: 144284985-144458254) shows this region in detail. The imprinted and non-imprinted promoters of *PLAGL1* are markers P1 and P2 respectively. (**b**) The image indicates the region of hybridisation for each of the targeted MLPA probes (1–12), with the DMR CpG island of *PLAGL1* highlighted. The vertical lines border the predicted 5′ and 3′ duplication endpoints obtained from the array CGH analysis of patients 15 and 12 respectively. The MLPA results have been summarised with a solid line spanning the duplicated MLPA probes for patients 15 and 12. Duplicated probes were assessed to have a p value <0.05 by competitive hypotheses testing

boundaries of each duplication, to seek correlations between size of 6q24 duplication and severity of TND phenotype.

Results

Among the 15 patients studied, a range of duplications was observed using custom aCGH (Fig. 1a; full data available in Electronic supplementary material [ESM] Table 1). The minimal 6q24 TND duplication was defined by patients 15 and 12 as having a 5′ breakpoint between chr6:144284985-144298365 (patient 15) and a 3′ breakpoint between chr6:144458190-144458254 (patient 12). These results indicate the minimal 6q24 TND duplication to be 160–173 kb in size. As a result *PLAGL1* and *HYMAI* are the only genes wholly contained within the refined region of minimal duplication (Fig. 1).

The array results were confirmed by targeted MLPA analysis (Fig. 1b; ESM Table 2). MLPA probes targeting exon 7 and exon 8 of *FAM164B* confirmed the inclusion of only its final exon and 3′UTR in the revised minimal duplication (patient 15). MLPA probes spanning *SF3B5* confirmed the 3′ breakpoint to exclude 238 nt of the 737 nt single exon gene (patient 12). All other patients were shown to be duplicated at the minimal 5′ and 3′ breakpoints by MLPA (supplementary material 2), with the exception of patient 14, whose 3′ breakpoint lies within intron 7 of *FAM164B*.

Available clinical information linked to each duplication is shown (Table 1). It was observed that two patients (14 and 15) with 5′ breakpoints in intron 7 of *FAM164B* had higher birthweight centiles (33 and 19.5 respectively) than those typically observed in 6q24 TND duplication patients. The duplication of patient 13 is similar in size to those of patients 14 and 15, but encompasses the whole of *FAM164B*; and patient 13 had a typical 6q24 TND birthweight centile (0.008).

Discussion

This study has reduced the minimal region of duplication for 6q24 TND from 440 kb to 160–175 kb, containing only the genes *PLAGL1* and *HYMAI*. This region is very similar in size and gene content to that used to recapitulate the key features of TND in transgenic mice [10]. It is also noteworthy that all the duplications identified include both the imprinted and upstream promoters of *PLAGL1*. The minimal duplication excludes the promoters and a significant portion of the coding sequences of *FAM164B* and *SF3B5*, and it is therefore unlikely that these genes are involved in the diabetic presentation of 6q24 TND.

Due to the overlapping monoallelic promoters of *PLAGL1* and *HYMAI*, it will be difficult to distinguish whether overexpression of one gene, or both, is necessary to cause 6q24 TND. *PLAGL1* remains the most likely

Table 1 Clinical and duplication details for each patient

Patient	Duplication details			Clinical details		
	Size (Mb)	Start	End	Gestation (weeks)	Birthweight (centile)	Presentation (days)
1	7.2	140042435	147222964	n/a	n/a	n/a
2	6.5	140042435	146544145	35	6.97	2
3	5.5	142934075	148529630	40	0.11	19
4	2.6	142242116	144842682	40	0.21	3
5	2.0	142552212	144587021	40	0.001	4
6	1.3	143874017	145220083	36	0.064	5
7	1.0	144175518	145156996	40	0.02	11
8	1.0	143922363	144902205	40	0.002	1
9	0.8	143960512	144785129	38	1.27	15
10	0.7	144070080	144768641	40	>0.001	60
11	0.7	144118859	144768641	40	0.009	9
12	0.6	143879154	144458190	39	0.13	9
13	0.5	144219934	144737194	40	0.008	9
14	0.5	144282782	144789340	40	33	33
15	0.2	144298365	144458190	37	19.5	1

Patients 1–15 are ordered by duplication size from largest to smallest as in Fig. 1a. The duplication details provide the size (Size Mb) and coordinates (Start and Stop) of each chromosome 6 duplication according to the 2006 human genome assembly 18. Restricted clinical details of gestation in weeks, IUGR (birthweight centile) and recorded presentation in days after birth are also included

n/a, not available

candidate gene for 6q24 TND. However, despite the current lack of evidence of function, *HYMAI* cannot be eliminated as a candidate gene for 6q24 TND. This is particularly true in light of the growing recognition of the role of non-coding RNA, in both imprinting and general gene regulation. Identifying the molecular aetiology of the 6q24 TND phenotype may be the only way to truly identify the critical gene or isoforms in 6q24 TND.

In addition, this study shows that individuals whose duplications encompass *FAM164B* are affected more severely than others by intrauterine growth retardation (IUGR). This observation provides the first evidence that *FAM164B* may be imprinted, since paternal duplications including *FAM164B* are associated with IUGR, whereas maternal duplications are not. Alternatively, sequences around *FAM164B* may contain tissue-specific regulatory elements for *PLAGL1*/*HYMAI* that are required for full presentation of the TND phenotype.

Unfortunately it is not currently possible to confirm the imprinting status of *FAM164B* due to lack of informative SNPs, and the function of the gene remains unknown. However, further study of these rare patients may yield valuable insight into the imprinted regulation of *PLAGL1* and *HYMAI*, the influence of the *FAM164B* region, and the roles of their gene products in neonatal diabetes.

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