A variant in the sonic hedgehog regulatory sequence (ZRS) is associated with triphalangeal thumb and deregulates expression in the developing limb

Dominic Furniss1,2, Laura A. Lettice3, Indira B. Taylor1, Paul S. Critchley2, Henk Giele2, Robert E. Hill3 and Andrew O.M. Wilkie1,*

1Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DS, UK, 2Department of Plastic and Reconstructive Surgery, Oxford Radcliffe Hospitals NHS Trust, Oxford OX3 9DU, UK and 3MRC Human Genetics Unit, Western General Hospital, Edinburgh EH4 2XU, UK

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INTRODUCTION

Triphalangeal thumb is characterized by the presence of three phalanges within the thumb. The extra middle phalanx may be fully formed, trapezoidal in shape, or a small triangular ‘delta’ phalanx. In addition the thumb may be normally opposable, or non-opposable and in the plane of the fingers, and the first web space may be tight. These abnormalities often lead to progressive thumb deformity with functional limitation requiring surgical intervention (1). The extra middle phalanx may be fully formed, trapezoidal in shape, or a small triangular ‘delta’ phalanx. In addition the thumb may be normally opposable, or non-opposable and in the plane of the fingers, and the first web space may be tight. These abnormalities often lead to progressive thumb deformity with functional limitation requiring surgical intervention (1). A frequently associated feature is pre-axial polydactyly; in combination, this clinical entity is described as pre-axial polydactyly type II [PPD2, MIM 174500].

Pre-axial polydactyly has been shown in several mouse models to be caused by perturbation of the normal antero-posterior axis of the developing limb (2). The signalling molecule Sonic Hedgehog (Shh) is normally expressed in a spatially and temporally restricted fashion in the posterior part of the developing limb bud, termed the zone of polarizing activity (ZPA). This restriction, combined with the effects of other molecules such as the processed repressor form (Gli3R) of Gli3 (GLI-Kruppel family member 3), leads to patterning of the developing autopod (3,4). Ectopic mis-expression of Shh in the anterior part of the limb bud in mice results in a secondary ZPA, leading to disturbance of the antero-posterior axis, and

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the anterior formation of polydactylous digits or a more ‘finger-like’ triphalangeal thumb (2).

The initial evidence that mutations affecting the regulation of the human SHH gene might cause limb malformation came from the study of the transgenic mouse mutant Susqueatch (Ssq). The Ssq mouse has pre-axial polydactyly and displays ectopic Shh expression at the anterior margin of the developing limb bud (5). The transgene insertion responsible for the phenotype was shown to be linked to Shh, but resided in intron 5 of a neighbouring gene, Lmbr1 (limb-region homolog 1), located ~0.8 Mb away (6). To demonstrate that the phenotype was caused by disruption of a Shh regulator, rather than an effect on Lmbr1, a genetic cis-trans test was designed which showed that the Ssq mutation acted in cis to affect directly expression of the Shh allele (6).

In mice, transgenic analysis using a construct containing ~1.7 kb of murine sequence from Lmbr1 intron 5 [linked to the Hbb (haemoglobin, beta) promoter, LacZ reporter and SV40 polyA signal], showed expression of β-galactosidase in the posterior margin of the developing limb bud in a spatiotemporal manner analogous to normal Shh expression (7). Multi-species comparison of this `ZPA regulatory sequence’ (ZRS) utilizing mouse, human, chick and fugu sequences demonstrated a highly conserved ~800 bp region within it (7); here we refer to these murine and human DNA sequences as Zrs and ZRS, respectively. The ZRS is highly conserved among cartilaginous and bony fishes and in the tetrapod lineage (8), but is absent from limbless reptiles and amphibians (9). Furthermore, homozygous deletion of the Zrs in the mouse (10) leads to loss of Shh expression in the limb associated with a limb truncation phenotype similar to that seen in the Shh knockout mouse (11). Interestingly, the severe midline central nervous system and craniofacial defects present in Shh knockout mice and leading to embryonic lethality (11) were not seen in the Zrs knockout mice, which were viable and survived until at least 3 months after birth (10). Taken together, these data demonstrate that the Zrs is an important limb-specific regulator of Shh expression.

Further evidence for the importance of the ZRS in regulating limb development has been obtained from the identification of point mutations in mice, cats and humans. In mice and cats, six different single nucleotide substitutions in the Zrs (three in each species) are associated with pre-axial polydactyly (7,12,13), and in humans seven point mutations (14–16). One of the latter reports, four additional polymorphisms in the ZRS, with frequencies of 10–30% in both Dutch controls and families, were thought to be neutral variants (7). Here we provide genetic and functional evidence that one of these, 295T>C, is in fact a dominant mutation with reduced penetrance that is a common cause of triphalangeal thumb in southern England.

RESULTS

Screening for ZRS mutations in congenital limb malformation

We recruited a cohort of 187 patients with a congenital limb malformation requiring reconstructive surgery. Of these, 5 had triphalangeal thumb (2 unilateral, 3 bilateral; one unilateral and one bilateral case were associated with unilateral pre-axial polydactyly) and 34 had pre-axial polydactyly (24 unilateral, including the two patients with triphalangeal thumb, and 10 bilateral). We previously identified a SALL1 mutation in the patient with unilateral triphalangeal thumb and pre-axial polydactyly (17). Two patients in the cohort (neither of whom had either pre-axial polydactyly or triphalangeal thumb) had previously undescribed heterozygous ZRS variants (18) (718T>A and 775G>C) that were considered unlikely to be pathogenic (see Materials and Methods). However, three additional patients (OX1925, OX3159 and OX3601), all of whom had bilateral triphalangeal thumb, were heterozygous for an identical 295T>C variant detected on denaturing high performance liquid chromatography (dHPLC) screening and DNA sequencing (Fig. 1A and B), and are the subject of this report.

Patient OX1925 presented as a 7-week-old female with isolated bilateral triphalangeal thumbs (Fig. 2A). There was no family history of congenital malformation, although the patient’s mother was adopted and had no knowledge of her birth family (Fig. 3A). Patient OX3159 presented as a male infant with bilateral triphalangeal thumb and right hand pre-axial polydactyly (Fig. 2B). There was an extensive family history of similar malformations (Fig. 3B). Patient OX3601 presented as a 2-year-old female with isolated bilateral triphalangeal thumb. There was a family history of similar malformations (Fig. 3C). None of the affected individuals in any of the three families had additional malformations, including foot malformations. In particular, the mother of patient OX3601 was affected with bilateral triphalangeal thumb, and had no additional phenotypic features.

Linkage and haplotype analysis

To assess the pathogenic significance of the 295C variant, we genotyped additional family members and healthy controls using a primer-mismatch generated AhvNI restriction digest. DNA was available from 15 relatives of patient OX3159, including 7 affected by triphalangeal thumb and/or pre-axial polydactyly, 4 clinically unaffected obligate carriers and 4 unaffected individuals at 50% prior risk. DNA was also available from the affected mother and aunt of patient OX3601. The 295C allele was present in heterozygous state in all affected individuals except the mother of OX3601, in whom it was homozygous (Figs. 1B and C, 3B and C). In addition, the 295TC genotype was found in the four unaffected obligate carriers and one unaffected individual at 50% prior risk (Fig. 3B). The 295C allele was absent in 762 normal chromosomes originating from ethnically matched north European Caucasian populations. The difference in occurrence of the 295C allele in probands with triphalangeal thumb (3/5), compared with controls (0/381), was highly significant ($P = 1.05 \times 10^{-5}$, Fisher’s exact test).

Before undertaking linkage analysis to test the significance of the association in the family of OX3159, we estimated the penetrance of the 295C allele by comparing the observed number of affected offspring born to known carriers (=9) with that expected if penetrance was complete (38/2 = 19), yielding a value of 0.47. However, this figure may be an overestimate as it does not take into account any occult carriers who had only unaffected offspring. As a more direct estimate
based on a smaller sample size, the proportion of obligate carriers who are affected is 3/10 = 0.3. We obtained a LOD score of 4.01 between the mutant allele and the phenotype in this family assuming a penetrance of the heterozygous mutant allele of 0.3 and a mutant allele frequency of 1/10000; this was robust to variation in either the penetrance (between 0.2 and 0.5), or mutant allele frequency (up to 1/150), yielding LOD scores in the range 3.92–3.96. This provides statistical support that the phenotype in family OX3159 is linked to the 295C allele.

We used genotyping of four microsatellite loci (MS-A, -B, -C, -D) spanning a 1.1 Mb region of chromosome 7q36 flanking the ZRS to determine whether the three apparently unrelated families shared a common haplotype around this region. In the family of OX3601, the homozygote mother (295CC) was also homozygous at all four flanking microsatellite loci, and analysis of OX3159 and his mother showed an identical haplotype (allele sizes 253-274-233-303) on the disease chromosome (Fig. 4). In 30 English parent–child trios, this haplotype was not present in any of the 120 chromosomes, indicating that it is not common in this population. We could not obtain DNA from the parents of OX1925, however one copy of these same four alleles is also present in this individual; the probability of this occurring by chance is $8.5 \times 10^{-4}$, indicating that the same disease haplotype is likely to be present in OX1925 (Fig. 4). The demonstration of a common haplotype at these four multiallelic loci indicates a likely founder effect for the 295C allele, showing that it has segregated with the phenotype over many generations. This further strengthens the genetic evidence that either the 295C allele itself, or a nearby variant in linkage disequilibrium with it, is causally responsible for the abnormal phenotype.

**Analysis of transgenic mice**

To investigate whether the 295C allele might itself be pathogenic, we analysed ZRS reporter constructs in transgenic mice. In total eight embryos containing the 295C transgene showed robust staining of the ZPA in both forelimbs and
hindlimbs, indicating that the construct produced reporter expression in the normal pattern of Shh (Fig. 5A). Of these, three embryos showed ectopic β-galactosidase staining in the anterior part of the developing forelimb bud (1 bilateral and 2 unilateral staining) at embryonic day (E)11.5 (Fig. 5B; range of expression patterns shown in Supplementary Material, Fig. S1B and D). No ectopic staining was present in any hindlimb, giving a total of 4/32 limb buds showing ectopic staining. Although the anterior limb bud staining was weak, it was never observed with the wild-type construct (n = 9 mice, corresponding to 0/36 limbs, Supplementary Material, Fig. S1A), indicating a specific pathogenic effect of the 295C allele (P = 0.044, Fisher’s exact test).

DISCUSSION

In this work, we have demonstrated that 295T>C in the ZRS is not a neutral polymorphism as previously reported (7), but represents the major cause of triphalangeal thumb (3 of 5 cases) in our patient cohort from southern England. In contrast, only one of 34 patients (case OX3159 described here) with pre-axial polydactyly had a ZRS change, indicating that ZRS point mutations are overall a rare cause of pre-axial polydactyly. We failed to detect the 295C variant in 381 ethnically matched controls, indicating that it is rare in this population; neither is it listed in dbSNP, indicating that it has not been detected in any surveys of genetic variation. Prompted by these findings, the data originally suggesting that the 295C variant was frequent in the Dutch population (7) were reassessed, and could not be replicated (E. de Graaff, personal communication). Given the microsatellite evidence for a single founder mutation in our three families, it will be interesting to determine whether this variant is present at low frequencies in other geographic areas, in which case it is likely to contribute substantially to cases of triphalangeal thumb.

The 295C variant is the 14th pathogenic single nucleotide change in the most highly conserved part of the ZRS/Zrs sequence to be identified in humans, mice or cats. Like all except two of the previously described changes, it affects a nucleotide that is conserved between human, mouse, chicken and fugu, being located at the last position in a sequence of 19 fully conserved residues (Fig. 1A). The ZRS provides one of the best examples for studying the biology and pathophysiology of regulatory sequence mutations acting at a distance, currently a major challenge in the molecular analysis of development.

The inconsistent and weak ectopic reporter staining compared with other transgenic constructs (13), and restriction to the forelimb, imply that the pathogenic effect of the 295C variant is mild. This reflects the clinical picture of this mutation, the most
common presentation being triphalangeal thumb without pre-axial polydactyly (including in the homozygous case), with a majority of heterozygotes being non-penetrant. The results extend further the previously observed correlation between the extent of β-galactosidase staining using the transgenic assay, and the severity of the phenotype associated with the corresponding mutation (13). Moreover the 295T>C mutation, which exclusively affects the forelimbs, was correspondingly shown by the transgenic assay to direct ectopic reporter gene expression only in the forelimb buds.

Recent evidence indicates that the ZRS acts as a target for transcription factor binding, specifically for Hoxd proteins (18); the identification of a signalling centre for another class of transcription factors, Tbx2/Tbx3, symmetrically present on both the anterior and posterior sides of the limb bud (19), raises the possibility that the point mutations in the ZRS confer, in a dominant fashion, competence for Shh expression at the ectopic site. This is predicted to alter the identity of the first digit from a thumb to a finger, as development of the thumb depends on expression of Gli3R in the absence of Shh (20). The predominant triphalangeal thumb phenotype described here (as opposed to more extensive pre-axial polydactyly) is consistent with weak and localized anterior SHH expression as observed in the transgenic analysis (Fig. 5).

**MATERIALS AND METHODS**

**Clinical cohort and control samples**

Following approval of this work from the Oxfordshire Research Ethics Committee C (C99.181), patients were recruited from the paediatric hand surgery clinic at Oxford Radcliffe Hospitals NHS Trust between 1999 and 2006. All patients and their parents who presented to the clinic with a congenital limb malformation requiring reconstructive surgery, and who gave informed consent, entered the study. At operation, a maximum of 5 ml of venous blood was collected, from which genomic DNA was isolated using
phenol/chloroform extraction. The three families described in this report all came from southern England and were of northern European Caucasian origin. Unrelated, ethnically matched control samples were obtained from three sources: (i) ECACC Human Control DNA panels HRC-1, HRC-2, HRC-4 (n = 267); (ii) Molecularly proven unaffected individuals from the UK and Ireland recruited into various genetic studies (n = 96); and CEPH Caucasian samples (n = 18). The parent–child trio samples for microsatellite genotyping were also of northern European Caucasian origin.

**Molecular genetic analysis**

The human ZRS, for which we use the numbering originally adopted by Lettice et al. (7), was screened in a total of 187 patients. Genomic DNA from the ZRS was amplified in two overlapping fragments (F3R3, F4R4) by PCR on a DNA Engine Dyad Peltier Thermal Cycler (Bio-Rad) using the primers and annealing temperatures shown in Supplementary Material, Table S1 and other reaction conditions as in Johnson et al. (21). The products were sequenced for heterozygous mutations using WAVE dHPLC (Transgenomic) at the temperatures indicated in Supplementary Material, Table S1, and any abnormally eluting fragments were subjected to DNA sequencing as previously described (21). The 295T/C genotype was obtained by digestion of a primer-mismatched amplimer (FSR5) with AlwNI (Supplementary Material, Table S1). Multiplex-ligation-dependent probe amplification was performed using synthetic oligonucleotide probes to the ZRS and six previously characterized control loci (MRC-Holland: http://www.mrc-holland.com/pages/index-pag.html) to exclude heterozygous deletion of the ZRS in the apparently homozygous individual (Supplementary Material, Table S2). Microsatellite genotyping was performed according to Johnson et al. (21) on four loci spanning a 1.1 Mb region of chromosome 7q36 flanking the ZRS (Supplementary Material, Table S1). Three of these (MS-A, MS-C, MS-D) were previously uncharacterized, whereas MS-B corresponds to marker HING1 in Heus et al. (22).

In addition to 295T>C we detected two other previously undescribed heterozygous ZRS variants (318T>A and 775G>C) in single patients. These were considered unlikely to be causative because the respective clinical presentations (unilateral post-axial polydactyly of the hands in the patient with 318T/A and bilateral first and second web syndactyly of the feet with tarso-talar coalition in the patient with 775G/C) fall outside the range of described phenotypes for ZRS mutations. In addition, we demonstrated that the same 318T/A genotype was also present in that patient’s unaffected mother and maternal grandfather.

**Transgenic mice**

Construction of 1.7 kb wild-type human clones (plasmid vector pBGZ40 #1230) containing the ZRS has been described previously (13). The 295T>C mutation was introduced using PCR-based site directed mutagenesis and verified by DNA sequencing of the complete construct. Constructs were excised from the vector by restriction endonuclease digestion with SalI and NotI, and purified by electrophoresis using an Elutrap (Schleicher & Schuell). Transgenic mice were generated by pronuclear injection using standard techniques (7). G0 embryos were harvested at E11.5, fixed in 4% paraformaldehyde and stained as previously described (23).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.

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