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Intrafamilial variability of the triphalangeal thumb phenotype in a Dutch population: Evidence for phenotypic progression over generations?

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Triphalangeal thumbs (TPTs) are regularly caused by mutations in the ZRS in *LMBR1*. Phenotypic variability can be present in TPT-families. However, recent observations suggest an increased occurrence of severe phenotypes in the Dutch TPT-population. Therefore, the aim of this study is to investigate the progression of the clinical severity of TPT-phenotype through generations. Index patients from a Dutch TPT-population were identified. A 105C>G mutation in the ZRS has previously been confirmed in this population. Questionnaires regarding family occurrence and phenotypes were distributed. Subsequently, families were visited to validate the phenotype. Both occurrence and inheritance patterns of the TPT-phenotype were analyzed through multiple generations. One hundred seventy patients with TPT were identified from 11 families. When considering all 132 segregations (parent-to-child transmission), 54% of the segregations produced a stable phenotype, 38% produced a more severe phenotype while only 8% of the phenotype was less severe when compared to the affected parents. Overall, 71% of the index patients had a more severe phenotype compared to their great-grandparent. Although all family members share an identical mutation in the ZRS (105C>G), it does not explain the wide phenotypic range of anomalies. Our observational study provides better estimations for counseling and provides new insights in the long-range regulation of SHH by the ZRS-enhancer. In the current study, we provide evidence that the assumed variability in TPT-phenotype is not random, but in fact it is more likely that the expression becomes more severe in the next generation. Therefore, we observe a pattern that resembles phenotypic anticipation in TPT-families.

KEYWORDS
genetic enhancer element, genetic variation, hedgehog proteins, polydactyly, thumb abnormalities

Martijn Baas and Jacob W.P. Potuijt contributed equally to this work.

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1 | INTRODUCTION

The triphalangeal thumb (TPT) is a congenital upper limb anomaly in which the thumb has three phalanges. TPT can present as a single isolated malformation (e.g., preaxial polydactyly type II; OMIM:174500), or can be part of a complex hand anomaly accompanied with additional polydactyly and syndactyly (Nicolai & Hamel, 1988; Zguricas et al., 1999). Furthermore, TPTs can also be seen in numerous syndromes, such as Holt-Oram syndrome or Duane-Radial Ray syndrome (Qazi & Kassner, 1988).

In the southern part of the Netherlands, a PPD2 population with an estimated prevalence of 1:1000 has previously been described and studied (Zguricas et al., 1994). These reports already have shown a broad phenotypic variation in PPD2. Multiple studies were performed to elucidate the genetic cause of TPT in these Dutch families. In 1994, Heutink et al. (1994) located the locus for TPT to 7q36 (LOD:12.61). In 2002, Lettice finally identified the 105C>G mutation in the ZPA-Regulatory Sequence (ZRS), that resides in intron 5 of the *LMBR1* gene. The mutation in the ZRS was confirmed in all affected members in a sample of 200 patients from aforementioned southern population in the Netherlands (Heutink et al., 1994; Lettice et al., 2002).

In the first phenotypic description of this population in 1994, most reviewed patients had a TPT, either with or without an additional preaxial ray. However, already a number of cases in the initially described population had a complex phenotype (Zguricas et al., 1994), including multiple preaxial rays, postaxial duplications, and/or syndactyly of digits 3–5. The intra- and inter-familial variability of the phenotype among patients with the same genotype that has been observed in the Dutch TPT-families, has also been described in other families in literature and was accepted to be a natural variation of the phenotype (Balci et al., 1999; Farooq et al., 2010; Lettice et al., 2002; Zguricas et al., 1999).

Recent observations in our clinic, however, suggest an increased familial occurrence of complex hand anomalies in successive generations of TPT families, which could indicate phenotypic progression through generations instead of intra- and inter-familial variability of the phenotype. This alternative hypothesis is supported by family history, which commonly revealed that the affected parent or grandparent of these more complex affected children had a less severe TPT phenotype. This observation is illustrated in Figure 1, by showing the phenotypes of three patients from subsequent generations within one family.

In order to determine whether recent observations of more complex phenotypes in newborns are incidental events or a structural pattern of phenotypic anticipation with progressive complexity among multiple families, the Dutch TPT population was revisited. The aim of this study is to investigate the progression of the severity of the TPT phenotype through generations. Secondarily, we aim to provide better estimates for phenotypic differences within families to determine the risk of a less severe, stable, and more severe phenotypes for future children.

2 | METHODS

Index patients were identified from the database of patients with a congenital upper limb anomaly in the Sophia Children's Hospital who visited the clinic between 1972 and 2014. All medical records of patients with either a registered TPT or preaxial polydactyly were reviewed for normal preoperative photo's as well as X-rays. Patients with at least one preaxial ray or with a triphalangeal component, with or without family history at referral, were eligible for inclusion. Eligible patients were contacted and subsequently a questionnaire was distributed among the index patients or their parents. The aim of the questionnaire was to create a pedigree by identifying other affected family members of the participant.

It is important that all families that were included in this study, have an identical mutation in the ZRS, as various mutations in the ZRS causes different TPT-phenotypes. For example, 295C>T mutations provide a mild phenotype (Furniss et al., 2008), whereas mutations at position 404 of the ZRS cause a severe phenotype (Lettice et al., 2003; Wieczorek et al., 2010; Zguricas et al., 1999). To ensure a genetic homogenous population, only patients whom ancestors originate from the small region in the South-West of the Netherlands (in which the 105C>G mutation is highly prevalent) were included. Genetic homogeneity has previously been established in families in this population (Heutink et al., 1994; Zguricas et al., 1999). In order to confirm the genetic homogeneity in this population, genealogical research was performed by reviewing the municipal archives to identify a common ancestor that connects all individual families with each other.

Additionally, local general practitioner's records were reviewed to identify patients that were not referred to the Sophia Children's Hospital. If affected family members were operated on in other hospitals, consent to acquire their medical records was requested. Furthermore, families were

FIGURE 1 TPT-phenotype of three subsequent generations from the TPT-population. (a) Post-operative image of grandmother of index-patient. During the operation, an additional thumb on both hands was removed. (b) Pre-operative image of mother of index-patient, presenting with a triplication of the thumb on the left hand and a quadruplication of the thumb on the right hand. (c) Pre-operative image of the index patient. The index patient has a symmetrical phenotype on both hands; with a triplication of the thumb, syndactyly between digits 4 and 5. All patients were born with a postaxial polydactyly, but were removed prior to the photographs in grandmother and mother of index patient. [Color figure can be viewed at wileyonlinelibrary.com]

visited to confirm the phenotype of the affected family members (using family/birth photos) and to gather additional familial information. Relatedness of the family members was investigated through genealogical research. Sub-pedigrees were established using only family members with confirmed TPT phenotypes. Although multiple sub-pedigrees were produced, all family members originated from one larger pedigree with one common ancestor. In order to analyze the phenotypic pattern of TPT among patients, the phenotype of every patient in the different sub-pedigrees was categorized among six types in consecutive order of complexity of phenotype (Figure 2). Patients have phenotype type I when only an isolated TPT is present. Type VI encompasses an extensive phenotype, with both additional preaxial and postaxial malformations combined with another aberration, for example, polydactyly of the feet. The intermediate types of TPT were categorized based on the hypotheses of increasing levels of ectopic SHH-signaling in the anterior margin of the limb bud and subsequently disruption of SHH-signaling in the posterior margin of the limb bud.

According to the initial prevalence of phenotypes in the investigated population (Zguricas et al., 1994), type I (isolated TPT) and II (TPT with preaxial polydactyly) were considered "classic" phenotypes (preaxial polydactyly type II, OMIM:174500). Phenotypes type III and higher were regarded as complex TPT phenotypes.

Patients have been arranged in three birth cohorts: from 1890 to 1940, from 1940 to 1990, and from 1990 to 2015. These cohorts were selected in order to compare affected patients of different generations with each other. Family members of the first and second generation were born before 1940, whereas patients of the third and fourth generation were born between 1940 and 1990. The youngest cohort consisted of affected family members who were born after 1990.

After all phenotypes in the sub-pedigrees were established, the variation of the phenotype over the different generations in the family was obtained. The transmission of the phenotype was regarded "stable" when the phenotype is the same in the analyzed ancestor (parent, grandparent, and great-grandparent) and child. When a child has a more complex phenotype than their affected ancestor, the transmission is considered "more severe." In the opposite case, when a less complex phenotype of TPT is observed in the child in comparison with their parent, the inheritance pattern is described as a "less severe" transmission of the phenotype.

The transmission of the TPT phenotypes was obtained for all individual segregations as well as the sum of consecutive transmissions (up to five generations). Analyzing phenotypical inheritance through multiple generations may display a more distinct inheritance pattern of TPT-phenotype in these families and might provide a better estimation of the risk of developing a more complex phenotype in subsequent offspring for clinical consultation.

2.1 | Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 21. Proportions were tested using a Chi-square test, unless specified differently. Variability was defined as an equal chance of non-stable phenotypes being more severe or less severe as compared to the ancestor, therefore to statistically test deviation from this theory the H_0 hypothesis of 50% contribution (of either subgroup of non-stable phenotypes) was assumed for the Student's t-test.

3 | ETHICS

Our research proposal (MEC-2015-278) has been approved by the Medical Ethics Committee of the Erasmus University Medical Centre in Rotterdam, The Netherlands.

Categorization of Triphalangeal Thumb Phenotypes
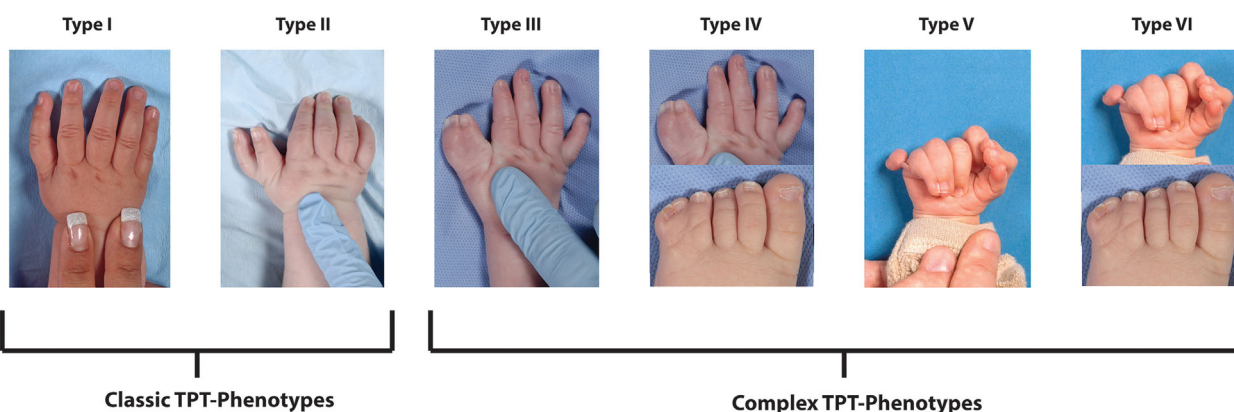
Type I	Isolated TPT
Type II	TPT + 1 additional preaxial ray
Type III	TPT + >1 additional preaxial ray
Type IV	TPT + ≥ 1 additional preaxial ray + other aberration
Type V	TPT + ≥ 1 additional preaxial ray + postaxial syndactyly/polydactyly
Type VI	TPT + ≥ 1 additional preaxial ray + postaxial syndactyly/polydactyly + other aberration

FIGURE 2 Categorization of TPT-phenotypes according to complexity. Type I and II are considered a classic phenotype. Type III or higher have additional aberrations and therefore are categorized as a complex TPT-phenotype. [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Overview of included sub-pedigrees

	Number of lineages (N)	Patients with TPT (N)	Generations per lineage	More severe	Less severe
Sub-pedigree 1	13	38	4.23 (3.00–5.00)	8	0
Sub-pedigree 2	9	31	4.44 (3.00–5.00)	7	0
Sub-pedigree 3	6	25	3.33 (2.00–4.00)	5	0
Sub-pedigree 4	4	19	4.25 (3.00–5.00)	1	2
Sub-pedigree 5	4	11	3.50 (3.00–4.00)	2	0
Sub-pedigree 6	3	10	3.00	1	0
Sub-pedigree 7	3	10	3.66 (3.00–4.00)	3	0
Sub-pedigree 8	3	8	3.33 (3.00–4.00)	1	0
Sub-pedigree 9	1	7	4.00	1	0
Sub-pedigree 10	1	6	4.00	0	0
Sub-pedigree 11	1	5	3.00	0	0
Total	**48**	**170**	**3.90**	**29**	**2**

4 | RESULTS

In total, 46 index patients from the southern part of the Netherlands were suitable for inclusion, eight patients were not included due to expired contact information. Furthermore, three patients were not willing to participate in this study. Therefore, 35 index patients were included in the study from 11 branches of the family. Genealogical research confirmed relatedness of the 11 branches of the family by identifying a common ancestor who was born in 1731. All index patients showed triphalangism of at least one preaxial ray on the preoperative X-ray. Through questionnaires and provided information of the index patients, we identified 135 additional family members with TPT. In total, 170 patients were included in this study. The 105C>G mutation in the ZRS was confirmed in multiple different branches in this pedigree (Supplementary Figure S1).

Table 1 demonstrates an overview of characteristics of the included sub-pedigrees. Each pedigree is formed by of a certain number of lineages. Lineages consist of subsequent affected family members of different generations. A lineage of three generations contains three subsequent patients with TPT: a child, a parent, and a grandparent. The average number of relatives contributing to a lineage was 3.90. Therefore, the average analysis of the inheritance pattern is between child and their great-grandparent. The number of patients with a TPT within these sub-pedigrees varies from 5 to 38 patients.

In total, the TPT phenotype segregated 132 times. Segregation is defined as a transmission of the TPT-phenotype from a parent to child. As we want to analyze the pattern of segregation in these sub-pedigrees, only segregations were included in the analysis when the phenotype of both the parent and child were known.

The distribution of classic and complex TPT phenotypes is displayed over the three different birth-cohorts (from 1890 to 1940, from 1940 to 1990, and from 1990 to 2015) in Figure 3. The observed percentage of complex phenotypes among these groups was 6%, 21%,
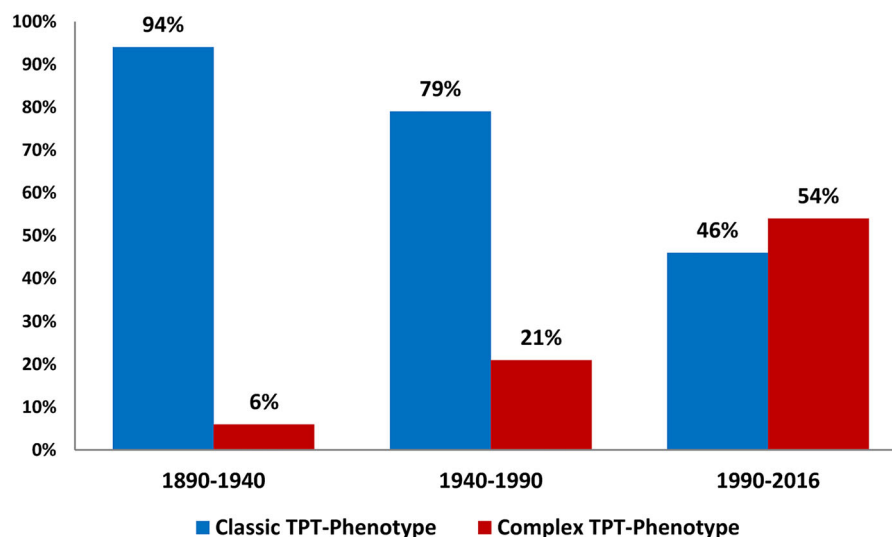

FIGURE 3 Distribution of phenotypes among three birth cohorts. A significant increase of complex TPT-phenotypes is observed when comparing the cohort "1990–2016" with the other cohorts (chisquared, $p < 0,001$). [Color figure can be viewed at wileyonlinelibrary.com]
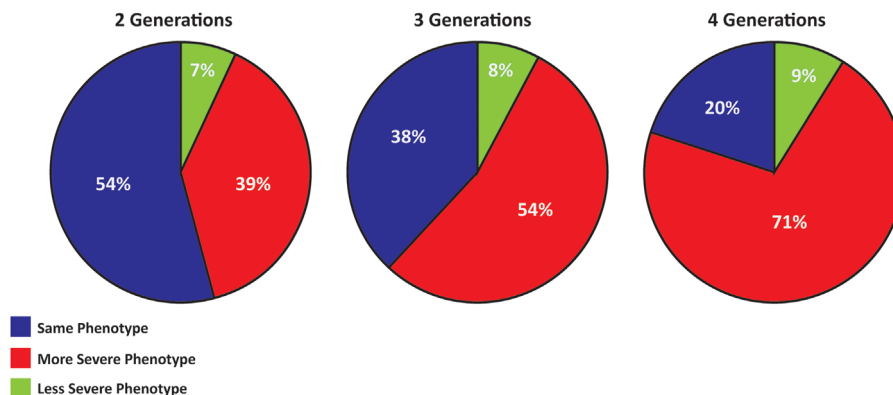
FIGURE 4 Distribution of phenotypic transmission in TPT families when taking multiple consecutive generations into account. Two consecutive generations; $n = 132$, three consecutive generations; $n = 90$, four consecutive generations; $n = 45$. [Color figure can be viewed at wileyonlinelibrary.com]

and 54% respectively. The distribution between the groups was significantly different ($p < 0,001$).

If the phenotype of the youngest generation is compared to their oldest identified ancestor, the phenotype was more severe in 29 patients, the phenotype remained stable in 17 patients while the phenotype became less severe in only two patients, as illustrated in Table 1. Assuming the presence of intrafamilial variability as null hypothesis (the probability of a more severe phenotype equals the probability of a less severe phenotype), the differences found are significantly different ($p < 0,001$, Student's t-test, $H_0 = 0.5$).

Considering, all 132 segregations of the phenotype, we observed that 54% of the segregations produced a stable phenotype in the next generation, whereas in 38%, the phenotype was more severe, and in 8% the phenotype was less severe when compared to the affected parent. In the cases in which the phenotype was not stable, the chance of a more complex phenotype is 5.6 times higher than the chance of a less severe phenotypes. When the analysis is expanded to two and three subsequent segregations we observed that the subsequent segregations produce a more severe phenotype in respectively 54% and 71% of the observed cases (Figure 4).

The result of segregation, if the phenotype of the parent was taken into account, is displayed in Figures 5a and 5c. Given that the parent has an isolated TPT (Figure 5a), the child had a more severe phenotype in 50% of cases. Most of these children had an additional preaxial polydactyly. However, when the parent already had an additional preaxial polydactyly (Figure 5b), the children had a complex phenotype in 28% of the cases, like additional triplications and/or postaxial (syn) polydactyly. Moreover, once the parent already had a complex phenotype, all children had complex phenotypes as well. No regression to either isolated TPT or TPT with preaxial polydactyly was observed within this offspring.

5 | DISCUSSION

In this study, we illustrate that the severity of an inherited phenotype of affected patients in TPT families is not random, but rather shows a

pattern of increased severity over subsequent generations. We found that in 39% of the cases, the phenotype increased in severity in one generation whereas only 7% decreased in severity. As a result, we found that in consecutive generations the phenotype of the youngest patients was more severe that the phenotype of their ancestor in 71% of the evaluated segregation lines. The probability of progression depends on the phenotype of the parent and varies from 28% to 50% in classic TPT-phenotypes. If the parent presents with a complex phenotype (type III–VII), no regression to a milder phenotype can be expected. Counselors can use this information to prepare future parents for the possible increase in severity of the phenotype in TPT-families.

Our observations are based on the Dutch TPT-population previously described by Zguricas et al. (1994) interestingly the same mutation in the ZRS found in this Dutch TPT family has been described in a Chinese Han Family. Although this Chinese family shared the
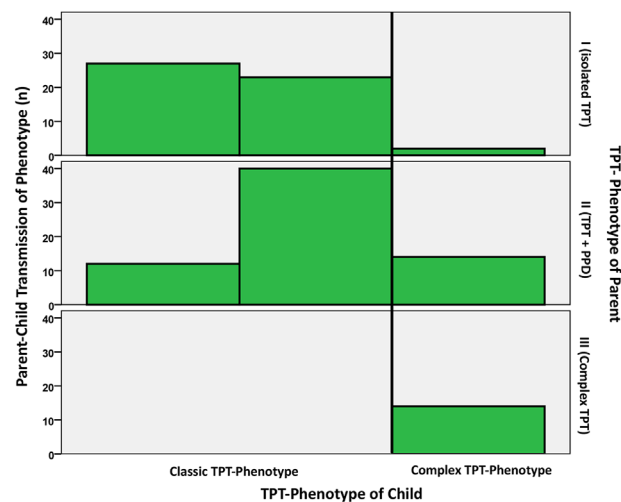

FIGURE 5 Distribution of offspring phenotype based on parental phenotype. (a) Distribution of the child's phenotype if the parent had an isolated TPT. (b) Distribution of the child's phenotype if the parent had TPT with an additional thumb. (c) Distribution of the child's phenotype if the parent had a complex phenotype. [Color figure can be viewed at wileyonlinelibrary.com]

105C>G mutation (Zhao, Yang, Sun, & Zhang, 2016), they predominantly have isolated TPT and there was no indication for progression of the phenotypic among this four-generation family. The results presented in this study must therefore be interpreted with care as the inclination towards complex phenotypes in the Dutch population might not be generalizable to all TPT populations and could be induced by factors that are specific to the Dutch TPT-population.

By retrospectively studying a formally isolated TPT-population, bias might be introduced affecting the validity and generalizability of the presented results. First, the validity might be affected due to recall bias in the obtained phenotypes, especially in the cohort born between 1890 and 1940. Therefore, we repeated analysis in the sub-pedigrees, disregarding the oldest birth cohort (thus including only those patients born between 1940 and 2015) and found similar phenotypic progression rates that correspond with the primary reported analysis. Second, inbreeding has been reported in other studies that have investigated this population, which could affect generalizability. We reviewed the presence of inbreeding in our cohort and found that inbreeding was predominantly present in the founding population (birth cohort 1750–1850). Additionally, our pedigree data did not reveal the presence of inbreeding in the included TPT-families. Third, the generalizability of increasing severity of the phenotype over generations can be questioned due to the isolated population and their rare 105C>G mutation. However, phenotypic progression over generations can also be observed in several other families that have been reported in literature (Furniss et al., 2008; Gurnett et al., 2007; VanderMeer et al., 2014). Considering the repeated analysis when disregarding the oldest cohort, the lack of evidence for inbreeding and the presence of other families in which the phenotype seems to progress, we conclude that although these biases might be present, they are unlikely to contribute much to our observations.

To be able to hypothesize on the pathophysiology of our findings, we must understand the molecular mechanism of the ZRS in relation to the observed phenotypes. If the ZRS is properly folded and bound with transcription factors that are spatiotemporally unique to the Zone of Polarizing Activity (ZPA), the ZRS is functionally active and upregulates SHH expression to specify digit number and morphology. The specification of digit number and identity has been predominantly attributed to a morphogenic SHH-gradient across the limb bud, hypothesized by Wolpert's morphogen gradient model (Wolpert, 1969). Higher concentrations and longer duration of SHH-expression at the posterior side of the limb bud result in the development of digits IV and V. Absence of SHH-concentration in the anterior side of the limb bud will conversely create a biphalangeal thumb. Point mutations in the ZRS disrupt normal SHH-patterning in the limb and cause ectopic SHH-expression on the anterior margin of the limb bud, which can result in a TPT (Lettice et al., 2003). Advancing on the SHH-gradient model, triplications, and quadruplications of the thumb will presumably be caused by increasing concentrations of ectopic SHH expression in the anterior limb bud. Furthermore, loss of SHH function in the ZPA causes postaxial polydactyly in animal models (Bouldin, Gritli-Linde, Ahn, & Harfe, 2010). The observed postaxial polydactyly in the Dutch TPT-population could therefore be caused by

reduced SHH expression in the posterior limb bud. Besides the SHH-gradient model, an analogy can be made to the pathophysiology of Greig Cephalopolysyndactyly syndrome. Patients with Greig syndrome commonly present with combined pre-axial, post-axial polydactyly, and syndactyly which is caused by GLI3 mutations. SHH is an important mediator for GLI3 to active GLI3A transition in the limb bud. Point mutations in the ZRS therefore might influence the same pathway as GLI3 mutations in the posterior margin of the limb bud (Al-Qattan & Al-Motairi, 2013).

The progression of the phenotype could be explained by the many differences the Dutch population might have with the Chinese 105C>G population. We hypothesize two different causes for the observed phenotypic progression. First, phenotypic progression might be attributable to genetic or molecular factors that influence SHH-expression in the ZPA in the limb bud additional to the 105C>G mutation. Second, generation specific environmental or parental factors could be present and might interfere with normal limb development through epigenetic modulation of the genome.

The first genetic factor could be somatic mosaicism. Increasing polydactyly phenotypes have been described as a result of somatic mosaicism of a point mutation in the ZRS (Vanlerberghe et al., 2015). Subsequently, somatic mosaicism could explain a more severe phenotype of the child than the phenotype of the parent. However, somatic mosaicism fails to explain phenotypic anticipation in multiple subsequent generations.

An alternative genetic hypothesis is that the progressive phenotypes in TPT-families are due to a second genetic locus introduced by the primarily non-affected parent which modifies the effect of the original 105C>G mutation. Although the presence of an additional influencing locus in an isolated population is a valid hypothesis, the extent of observations among different segregation lines in different sub-pedigrees and an example of two half siblings sharing the same affected father with both the same increasing phenotype all devaluate this hypothesis.

The last genetic hypothesis is based upon the fact that the observed phenotypic progression could be regarded as phenotypic anticipation. Phenotypic anticipation is a well-described phenomenon in repeat disorders such as Huntington disease, which is genetically caused by increasing CAG repeat sequences in the HTT gene. Although phenotypic anticipation is not known within the field of congenital upper limb malformations, repeat sequence disorders have been described in both HOXD13 and HOXA13 related synpolydactyly phenotypes (Goodman et al., 1997, 2000; Muragaki, Mundlos, Upton, & Olsen, 1996). However, synpolydactyly phenotypes in HOXD13 and HOXA13 repeat sequence disorders show limited intrafamilial variability with an exception for consanguineous families, in which patients with homozygous mutations do show far more severe hand anomalies. (Al-Qattan, 2011) Many subsequent research groups evaluated the TPT genotype in the past, resulting in the identification of various point mutations in the ZRS. Although repeat sequences were not found in the linkage analysis by Heutink et al. (1994) exon-trapping analysis performed in later studies (Heus et al., 1999) of the candidate region would have identified exonic repeats in the REPEATMASKER analysis. The presence of intronic

repeats outside the ZRS, however, cannot be excluded and should be further investigated.

There are several arguments that could support environmental or parental specific factors as a cause for phenotypic progression in TPT-families. First of all, generation specific environmental factors, such as the stimulated use of folic acid during pregnancy from the early 90's onwards, could have explained the higher occurrence of severe phenotypes in the youngest birth cohort, however fails to explain the earlier progression as observed in the pedigree in Supplementary Figure S2. In this pedigree, both classic and complex phenotypes have been observed in the same generation. It is therefore unlikely that such generation specific factors are causative to the changing phenotypes.

A second environmental hypothesis might be the increasing paternal age. Also, the large changes in family planning over the last century could lead to a change in parental age at conception. As Zhu, Madsen, Vestergaard, Basso, and Olsen (2005) have suggested, high paternal age might increase the overall incidence of congenital malformations. We performed a preliminary analysis on parental age in a limited number of segregation lines, but did not find enough support for the influence of the age of the parents.

The last environmental hypothesis is the significant increase of obesity in the western population over the past 25 years. Parental obesity has been widely associated with a higher risk of birth defects in newborns. The notion that parental obesity might also play a role in phenotypic progression of the TPT phenotype cannot be disregarded (Stothard, Tennant, Bell, & Rankin, 2009).

This study underlines the importance of thorough phenotypical assessment in studies on familial disease. In order to explore the causality of the structural evidence of phenotypic progression in these TPT-families, additional molecular genetic research and revisiting the Dutch population is required. Expression assays in transgenic animal models still remain the standard in molecular genetic research on limb development. We encourage future collaborations between clinicians, geneticists, and developmental biologists that will lead to a more comprehensive understanding of the role of SHH and its regulatory elements on congenital limb anomalies like TPT and polydactyly.

6 | CONFLICTS OF INTEREST

None.

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SUPPORTING INFORMATION

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