Mapping studies of congenital limb anomalies

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Robert-Jan Harmen Galjaard

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Mapping studies of congenital limb anomalies

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General introduction

General introduction

Human congenital limb malformations are supposed to be the second most frequent occurring birth defect after those of heart and vessels (Bamshad *et al.* 1997). Although exact data on the incidence of limb anomalies in liveborns are not yet available it is known that about 1/250 stillborns have a congenital limb malformation (Froster and Baird 1993). A minority of congenital limb malformations occur in association with other anomalies mostly of the craniofacies and the heart (Hennekam 2002). Around 2000 dysmorphological entities that include a limb anomaly have been described in Winter and Baraitser's London Dysmorphology Database (2003).

Parents who are confronted with the birth of a child with a limb malformation, close relatives and sometimes adult patients increasingly ask for genetic counseling to be informed about the cause, the (recurrence) risk in future offspring and possible medical intervention. Accurate information is highly dependent on knowledge about the etiology and pathogenesis of the malformations concerned. Sometimes exogenous factors are involved, the best known are thalidomide and anticonvulsants. In other instances gene defects of Mendelian inheritance explain a malformation syndrome although the exact pathogenesis is yet to be resolved. In many cases the etiology is unknown and believed to be a complex interaction between several genes and environmental factors.

Tetrapod limb development has been the subject of studies in the fields of embryology, anatomy, paleontology, biology, teratology and developmental genetics. Morphologically, the limbs are one of the most diverse organs. Although the limb appears rather late in embryonic development, it has been considered as a model system for patterning/morphogenesis and is a good example for the building of complex three-dimensional structures (Tickle 1994; Cohn and Bright 1999; Hogan 1999; Tamura *et al.* 2001). Cellular and genetic pathways driving morphogenesis of the limb turn out to be remarkably well conserved between different species during evolution (Shubin *et al.* 1997; Zeller 1999). The appendages, used for locomotion, have been evolved from fins to tetrapod limbs.

For about five decades, descriptive and experimental embryologists have contributed a great deal to our current knowledge concerning the embryonic development of limb tissues and the cellular interactions involved. An overview is given in Chapter 1.

The past two decades, thanks to advances in molecular biology, much has been learned about the patterning along the various axes, the interactions between the axes, the role of signaling centers and about the cellular and molecular processes and interactions underlying the embryonic development of various tissues. Most insight has come from studies of the fruit fly and of genetically modified chick and mouse embryos (Chapter 2). Comparison of limb development between different species allows insight in the conservation and changes of developmental pathways.

Additional knowledge is obtained by studying human congenital limb malformations. Clinical descriptions have made use of different classification systems. Swanson's classification is purely based on anatomic bone structures and is frequently used by surgeons, whereas Temtamy and McKusick also include genetic factors (Swanson 1976; Temtamy and McKusick 1978). Therefore the latter is more frequently used by (clinical) geneticists and biologists. Molecular genetic studies in families with several patients and comparative analyses with data from experimental animals have resulted in the identification of an increasing number

of genes causing limb anomalies mostly with a Mendelian pattern of inheritance (Grzeschik 2002). About 120 genes have been mapped and 80 have been identified. Although molecular genetic data increasingly influence existing classifications (Winter and Tickle 1993; Hennekam 2002) our understanding of gene functions is as yet insufficient to define a new classification (Stoll *et al.* 1998). For overview see Chapter 3.
The basis of the experimental work in this thesis is formed by the clinical description and family studies of different types of human congenital limb malformations and the unraveling of their molecular genetic etiology (see Chapter 4 and publications in Chapters 5-8 and discussion in Chapter 9).

Chapter 1

Embryonic limb development

Embryonic limb development

Human limbs are formed between 26 and 54 days of gestation. At specific positions along the main body axis lateral plate mesoderm becomes specified by medially derived signals to form upper and lower limbs (presumptive limb fields) (Johnson and Tabin 1997). Before limb bud formation mesodermal cells obtain information about their position along three axes of asymmetry (fig. 1).

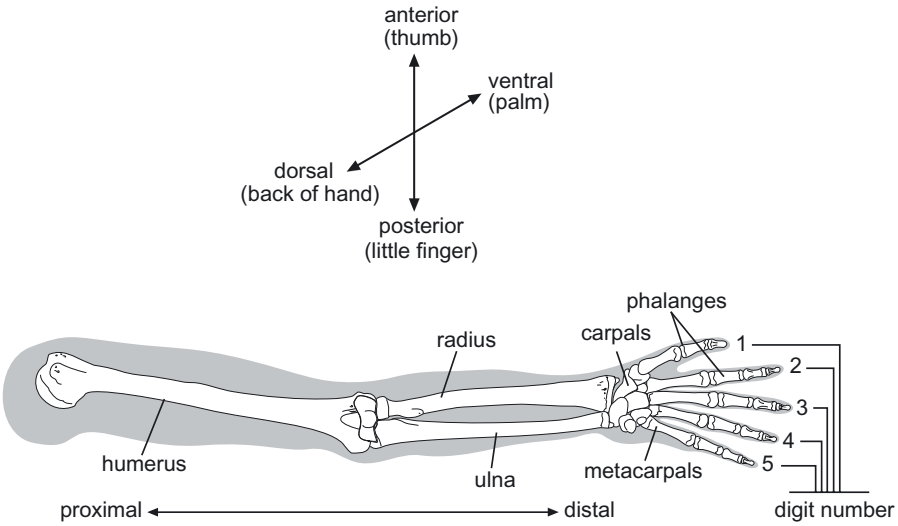

Figure 1. *Skeletal anatomy of the human arm along the three axes of asymmetry. The proximal-distal (P/D) axis runs from the shoulder to the fingers. The anterior-posterior axis (A/P) runs from the thumb to the little finger.The dorsal-ventral (D/V) axis runs from the back of the hand to the palm (Adapted from Gerhart 1997).*

The Proximal to Distal (P/D) axis runs from shoulder to digits and includes three segments after its outgrowth. These segments are the stylopod (humerus/femur), the zeugopod (radius/ulna and tibia/fibula) and the autopod ((meta)carpals/tarsals and the digits). The Anterior to Posterior (A/P) axis runs from the first to the fifth digit in human and mice. The Dorsal to Ventral (D/V) axis runs from the back of the hand (with nails and hair) to the palm.
The shape of the limb results from the coordination of processes that are used repeatedly in different combinations in the build up of different organs during embryogenesis (Johnson and Tabin 1997; Hogan 1999). These processes concern cell proliferation, cell fate determination, cell differentiation and apoptosis (Hogan 1999; Oligny 2001). Other processes like cell adhesion, cell migration, cell-matrix interactions and cytoskeletal organization are coordinated with cell proliferation and cell fate determination during limb bud formation by signaling centers, which are groups of cells regulating the behaviour of surrounding cells by positive or negative intercellular signaling.
The signaling centers act as reference points in a three-dimensional structure and confer a positional identity to the cells of the limb (Mariani and Martin 2003).
This identity determines the nature of the structure that will be formed and determines the overall patterning of the limb.

These signals also determines the differentiation fate and growth together with more localized signals.

1.1 Human limb formation

In humans, the embryo proper develops during the first 8 weeks post fertilization, which has been divided into 23 stages in the Carnegie staging system (O'Rahilly and Gardner 1975).

Before stage 12 (26 days postfertilization) lateral plate mesoderm cells become specified into presumptive limb fields at circumscript rostral and caudal positions along the main body axis of the embryo (Hogan 1999; Niswander 2003). Cells at the rostral presumptive limb fields adopt an identity of upper limbs, whereas those at caudal positions will form the lower limbs. In the presumptive limb fields, the high proliferation rate of the undifferentiated mesenchyme and the overlying ectoderm is maintained, while cells of the interlimb flank show a diminished proliferation rate (Johnson and Tabin 1997). This results into the appearance of paired forelimb buds at stage 12 (ca. 26 days) (O'Rahilly and Gardner 1975). At this stage myogenic precursors populate the early limb mesenchyme. The cells in the early upper limb bud are vascularized by a plexus of vascular channels formed by ingrowth of vascular branches of the dorsal aorta and cardinal vein (Rodriguez-Niedenfuhr *et al.* 2001). Scattered thin walled blood vessels are visible in the upper limbs at stage 13 (ca. 28 days). Simultaneously, the lower limbs appear.

At stage 14 (ca. 32 days) two signaling centers are established. One center, the apical ectodermal ridge (AER), is a specialized epithelial ridge along the distal tip of the limb bud responsible for limb bud outgrowth by keeping the underlying layer of mesenchymal cells (the progress zone or PZ) in a proliferative state (Saunders 1948). The other center consists of mesenchymal cells located at the posterior margin of the limb bud, the zone of polarizing activity (ZPA), which specifies patterning along the Anterior-Posterior (A/P) axis of the limb (Saunders and Fallon 1967). Concomitantly from the nerve plexus at the base of the limb buds, motor and sensory axons project into the limb and join together to form a neural plate, which divides distally into an anterior and posterior branch at stage 15 (ca. 33 days) (O'Rahilly and Gardner 1975; Rodriguez-Niedenfuhr *et al.* 2001). The elongating limb gradually flattens along its Dorsal-Ventral (D/V) axis and takes a paddle shape form during stage 16 (ca. 37 days). At that time, the distal nerves are recognizable up to the future elbow. The anterior nerve will become the radial nerve, whereas the posterior nerve will develop into the median and musculocutaneous nerves. The mesenchymal condensations of the humerus, radius and ulna can be discerned at this stage. The humerus chondrifies shortly afterwards.

At stage 17 (ca. 41 days) the radius starts to chondrify followed by the ulna and the metacarpalia and finger rays are formed. The nerves are now recognizable up to the hand plate. Myoblasts have grouped themselves into a dorsal and ventral muscle mass, from which the proximal mass in the upper arm becomes separated into distinct muscles at stage 18 (ca. 44 days). By then the AER and ZPA have gone into regression. The vascular pattern is well differentiated into ulnar, median and interosseous arteries. The first signs of joint formation in the future metacarpo-phalangeal (MCP) joints are discernable, as well as cellular condensations that form their future ligaments. Carpalia and proximal phalanges chondrify and interdigital apoptosis is started. At stage 19 (ca. 48 days) the middle

phalanges chondrify and interzones are present in the joints of the elbow and the carpalia. The nerves have acquired a definitive pattern. In distal limb the radial artery remains as a capillary plexus. During stage 20 (ca. 51 days) the distal phalanges chondrify and many future ligaments of the hand are discernable as condensations. Digital arteries are present. The distal radial artery acquires its definitive pattern in stage 21 (ca. 52 days). The humerus and the radius start ossification, followed by ossification of the ulna in stage 22 (ca. 54 days). The digits are fully separated at this stage.

1.2 Embryonic development of limb tissues

The human limb is comprised of different structures including skeletal elements, joints, muscles, tendons, an innervating system and a blood vascular network.
An overview of the development of these tissues is given in the following sections. As was mentioned before limb development occurs along three main axes and the asymmetrical patterning results from interactions between tissues, cells and molecules. These interactions, of importance for the understanding of normal and abnormal limb development, have mainly been studied in experimental animal models, which will be reviewed in Chapter 2.

1.2.1 Skeletal elements

The skeletal elements originate from lateral plate mesoderm (fig. 2) (Johnson and Tabin 1997; Niswander 2003 and ref. therein).

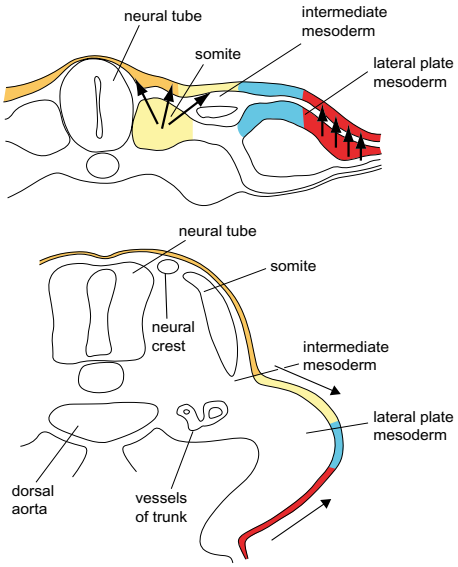

Figure 2. *Anatomy of the early limb bud (chicken). Structures indicated contribute to the formation of the limb structures. Motor axons originate from the neural tube, muscle from the somites, blood vessels sproute from the dorsal aorta and skeletal elements, joints and tendons from the lateral plate mesoderm. Arrows indicate specification by the somites and the lateral plate mesoderm of limb ectoderm (upper figure) into dorsal ectoderm and ventral ectoderm, respectively at a later stage (lower figure) (Adapted from Johnson 1997).*

Skeletal element precursors are first formed by aggregation of mesenchymal cells that forms a cartilage model consisting of chondrocytes and extracellular matrix (ECM). The ECM is mainly composed of water, glycosaminoglycans and fibrous proteins. Most mesenchymal condensations of the limb ossify through enchondral ossification. Only the distal phalanges ossify directly from mesenchymal condensations. Chondrification starts with vascular regression and differentiation of mesenchymal cells into chondroblasts which have a higher degree of cell-cell adhesions and cell-cell interactions (Hall and Miyake 1992; Yin and Pacifici 2001). Dense cell condensations are called centers of chondrification. Within these centers chondroblasts differentiate into chondrocytes that secrete more collagen type 2 for mechanical stability and chondroitine sulfate proteoglycans resisting compressive forces. This increase of the extra cellular matrix results in an increase of intercellular space. In the middle of a chondrifying element chondrocytes start early differentiation and become hypertrophic upon terminal differentiation. The ECM regresses and becomes calcified after which the chondrocytes die (Hall and Miyake 1992; Fawcett 1994). Ossification starts in the middle of a chondrified element (diaphysis) with invasion of blood vessels that bring osteoprogenitor cells. These differentiate into osteoblasts which deposit osteoid that becomes bone after calcification (Stein and Lian 1993; Fawcett 1994). This is called the primary ossification center (Hall and Miyake 1992; Fawcett 1994). Later in bone development, a secondary center of ossification appears at either bone end (epihysis). The epiphyseal or growth plate is located between the diaphysis and both epihyses (Hunziker 1994).
Bone growth is the result of proliferation of chondroblasts and chondrocytes and increase of ECM within the chondrifying elements. In addition, these elements are surrounded by perichondrium that includes an inner mesenchymal cell layer that differentiates into cartilage forming chondrocytes that contribute to appositional growth. Later bone growth is dependent on proliferation in the growth plates that contain five zones: the resting zone of chondrocytes, the proliferating zone, the hypertrophic zone, the calcified zone and the zone of ossification.

1.2.2 Joint formation

Joint formation starts within the chondrifying elements by formation of interzones (Mitrovic 1978; Francis-West *et al.* 1999). These interzones become three-layered. There are two dense chondrogenic outer layers flanking an inner layer that is continuous with the vascular synovial mesenchyme lining the inside of the joint. Cells of the inner layer are lost by apoptosis and subsequently a joint cavity is formed that becomes fluid filled. The outer chondrogenic layers will form the fibrous articular cartilage surfaces.

1.2.3 Muscle formation

Muscle precursor cells originate from the dorsal part of somites (dermomyotome) (fig. 2) (Buckingham 1992; Blagden and Hughes 1999; Buscher and Izpisua Belmonte 1999; Buckingham *et al.* 2003; Francis-West *et al.* 2003). The muscle precursors delaminate from the lateral dermomyotome and migrate into the limb mesenchyme. They differentiate into myoblasts that proliferate and subsequently differentiate into types with a slow- or fast myosin heavy chain isoform. Myoblasts then fuse into a dorsal and ventral muscle mass. These consist of multinucleated myotubes, which assemble and subsequently form musle

fibers or primary fibers (primary myogenesis). Later in development, secondary myoblasts migrate into the developing limb and form secondary fibers that surround the primary fibers (secondary myogenesis). Before the segregation of individual muscle masses, myotubes and their fibers show a clear orientation, related to that of the skeletal elements suggestive for an interaction between muscle and skeletal elements precursors (Kardon 1998).

1.2.4 Tendon formation

Tendons originate from lateral plate mesoderm (fig. 2) (Ros *et al.* 1995; Kardon 1998; Oldfield and Evans 2003). At the dorsal and ventral surface of a chondrogenic element tendon blastemas are formed by subectodermal condensations of mesenchymal cells that proliferate and differentiate into fibroblast-like cells. Comparable with muscle formation tendon blastemas are laid down as three pairs (dorsal/ventral) in a proximal to distal sequence in the outgrowing limb (Ros *et al.* 1995; Kardon 1998; Oldfield and Evans 2003). The blastemas of the upper and lower limb are formed in close association with the muscle masses. Initiation and maintenance of these blastemas are dependent on interactions with these muscle masses. This is not the case for the distal blastema of the hand/leg, which is thought to be dependent on interactions with the skeletal elements.

1.2.5 Innervation of the limb

Limbs are innervated by motor and sensory neurons, projecting their axons to specified targets, like muscles and skin. Motor neurons innervating a single muscle are organized in clusters in the lateral motor columns (LMC) of the spinal cord, derived from the neural tube (fig. 2), at specific positions along the main body axis (Tsuchida *et al.* 1994; Ensini *et al.* 1998; Lin *et al.* 1998; Sharma *et al.* 1998). Motor neurons in the medial and lateral parts of the LMC project their axons eventually in the ventral and dorsal muscle mass, respectively (Tsuchida *et al.* 1994 and ref. therein). Motor neurons project their axons through the rostral side of an adjacent somite. The axons become organized in a segmental pattern of spinal nerves.

The sensory neurons migrate from the neural crest through the somite (fig. 2) and coalesce in dorsal root ganglia located near the neural tube (Hollyday 1995). From there, sensory axons join the motor axons and become intermixed in the spinal nerves.

Spinal nerves from different axial levels join together and form a plexus at the base of a limb. Motor and sensory axons stay in the plexus for about 24 hours, sort and extend in a dorsal or ventral direction into the limb (Wang and Scott 2000). In distal limb the spinal nerves that innervate the same muscle are located in close proximity of each other.

The pathway finding of both motor and sensory axons is guided by interactions from the limb mesenchyme (Lance-Jones and Dias 1991; Tessier-Lavigne and Goodman 1996). However, the target specification of motor and sensory neurons is different, with motorneurons being more rigidly specified (Wang and Scott 2000).

Motor axons that innervate slow muscle fibres at muliple sites travel and branch separately in developing muscle from those that innervate fast muscle fibers at one site only. This is suggestive for an interaction between motor neurons and muscle fibers (Milner *et al.* 1998).

1.2.6 Vascularization of the limb

Vascular endothelial progenitors are named angioblasts that originate from the somitic

mesoderm (fig. 2) (for review see Weinstein 1999; Vargesson 2003). Angioblasts aggregate and form single layered endothelial tubes. A morphologically uniform network of vessels is subsequently formed. This is later remodeled by angiogenesis into a network of mature vessels. New vessels can form from pre-existing ones (sprouting).
The vasculature of the developing limb bud is derived from many asegmental vessels sprouting from the dorsal aorta or the cardinal vein (fig. 2). These anastomose and form a capillary network. This is constantly remodeled and gradually replaced by larger vessels in a proximo to distal sequence along the outgrowing limb. In addition, vascularization occurs through angiogenesis by angioblasts that migrate from the somites into the limb mesenchyme. Throughout limb bud outgrowth a zone of about 100 μm at the tip of the bud remains avascular. Mesenchyme condensations in the limb are preceded by vascular regression, suggestive for a correlation between vascular and limb pattern formation (Yin and Pacifici 2001).

1.3 Conclusions

The present knowledge about human limb malformation and the development of the limb tissues is mainly of a descriptive nature. The timing and the various developmental stages, mainly from weeks 4-8 have been well described and some insight has also been gained into the positioning of specific cell types and the importance of signaling centers for limb outgrowth and patterning along the proximal-distal and the anterior-posterior axes. Since the development of the human embryo is inaccessible to experimentation it is extremely difficult to understand the dynamics of the various developmental processes i.e. the molecular pathways that underlie the differentiation of the various tissues and the interactions between them. Although some investigators believe that the bone formation plays a dominant role also in the development of other tissues there is as yet no evidence for the timing and interactions between the various limb structures, tissues and cell types in humans.

Chapter 2

Animal models for limb development

Animal models for limb development

Most of the knowledge about genes and molecular pathways involved in limb development has been obtained from studies of mice, chicken and the fruit fly.
Chicken limb development is divided into 46 stages of the Hamburger and Hamilton (HH) staging system (1951) at which the limb becomes visible at stage 17. Mouse development can be classified according to the developmental age in postconceptional days (Kaufman 1992).

2.1 Limb positioning, induction, initiation and identity

In chicken embryo, presumptive limb fields are specified in the lateral plate mesoderm (LPM) at the level of somites 15-20 and 26-32 for the upper and lower limbs, respectively. The presumptive limb fields are specified by signals from cells located medially from the LPM (fig. 2). By inserting foil barriers, a gradual transition in the capacity for limb induction was found from medial (Hensen's node/primitive node) to lateral compartments (LPM). Limb induction is possible during stages 6-8 for Hensen's node, during stage 10-11 for the somites, during stages 12-16 for the intermediate mesoderm (IM) and during stages 15-18 for LPM presumptive limb fields (Stephens *et al.* 1991; Geduspan and Solursh 1992; Fernandez-Teran *et al.* 1997; Agarwal *et al.* 2003).

Candidates for the *positioning* of the presumptive limb fields are the homeobox containing transcription factors Hoxb9, Hoxc9 and Hoxd9 (Cohn *et al.* 1997). In mice *Hox* genes are located in four clusters (A-D) on different chromosomes (Dolle *et al.* 1989; Yokouchi *et al.* 1991). Within each cluster, an individual gene has a paralogue in other clusters. From stage 14 onwards these Hox9 encoding genes show differential expression in the upper-, inter- and lower limb region. The borders of *Hox9* expression are identical to the borders separating the upper and lower limb from the interlimb field. These borders are not irreversibly set at this stage since they change upon the position of an ectopic limb induced by implantation of a fibroblast growth factor (FGF) containing bead in the interlimb LPM. *Hoxb5* is implicated in positioning of the forelimbs only. It is expressed before upper limb outgrowth (Wall *et al.* 1992) and homozygous *Hoxb5* knockout mice show an anterior displacement of the position of the upper limbs (Rancourt *et al.* 1995).

A good candidate for a paraxial signal involved in limb *induction* is FGF8 (Vogel *et al.* 1996) as the *Fgf8* expression pattern is in agreement with the timing of limb induction capacity as described above (fig. 3). Beads containing FGF8 implanted in the LPM of the interlimb flank result in the formation of an ectopic limb (Crossley *et al.* 1996; Vogel *et al.* 1996).
Another candidate involved in limb induction is a member of the Wnt family of signaling molecules. In chicken *Wnt2b* in upper limb is expressed in IM at the same time as *Fgf8* (Kawakami *et al.* 2001).

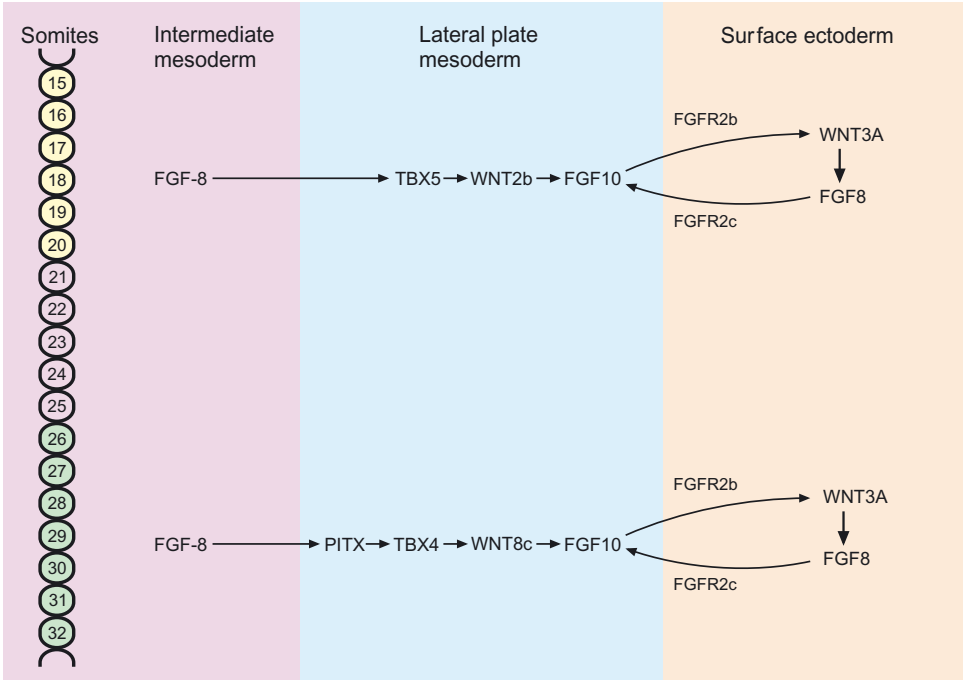

Figure 3. *Molecules involved in limb bud induction, identity, initiation and outgrowth. FGF8 induces WNT2b through TBX5 in upper limb. FGF8 induces WNT8c through TBX4 in lower limb. WNT2b and WNT8c induce FGF10 which signals through FGFR2b and activates FGF8 through WNT3a in ectoderm. FGF8 maintains FGF10 expression through FGFR2c. FGF8-FGF10 signaling stimulates mesenchyme proliferation and limb bud outgrowth (Adapted from Gibson-Brown 1998).*

The genes that are sequentially activated in the *initiation* of the upper limbs are *Tbx5* which encoded T-box containing transcription factor induces *Wnt2b* in mesenchyme (fig. 3) (Naiche and Papaioannou 2003; Rallis *et al.* 2003; Takeuchi *et al.* 2003). Wnt2b signals through the β-catenin signaling pathway and activates mesenchymal *Fgf10.* Fgf10 protein binds to Fgf receptor (Fgfr)2b in overlying ectoderm where it activates *Wnt3a.* Subsequently, Wnt3a activates the *Fgf8* gene in ectoderm (Kengaku *et al.* 1998; Kawakami *et al.* 2001; Barrow *et al.* 2003).

Initiation of the lower limbs results from activation of *Tbx4,* which protein induces *Wnt8c*. Wnt8c activates mesenchymal *Fgf10,* which protein induces ectodermal expressed *Fgf8* through Wnt3a as in the upper limbs.

In *Tbx5* deficient mice, limb outgrowth is not initiated (Agarwal *et al.* 2003) and *Fgf10* expression in LPM of the presumptive limb fields is lost. In addition, there is loss of expression of the *Lef1* and *Tcf1* genes encoding transcription factors, involved in β-catenin WNT signaling. *Fgf10* expression is also downregulated in *Lef1/Tcf1* double homozygous knockout mice that have unaltered expression of *Tbx5* in LPM of the presumptive limb. This demonstrates that *Tbx5* is an upstream regulator of the *Fgf10* gene and signals through WNT (Ng *et al.* 2002; Agarwal *et al.* 2003; Takeuchi *et al.* 2003).

FGF10 protein is both sufficient and necessary for limb bud initiation. Ectopic expression of *FGF10* in interlimb flank LPM of chicken embryo's can induce ectopic limb formation.

Homozygous *Fgf10* knockout mice show either absence or early loss of limb initiation, with absent or truncated limbs (Min *et al.* 1998; Sekine *et al.* 1999).

In addition to the initiation effect, *Tbx5* and *Tbx4* are also involved in *determination of limb identity*. These *Tbx* genes are differentially expressed in the mesenchyme of both limbs, before and during limb outgrowth (Gibson-Brown *et al.* 1996; Isaac *et al.* 1998; Logan *et al.* 1998). Misexpression studies of *Tbx5* in chicken legs lead to transformation of ecto- and mesodermal structures of the leg into those of the wing, whereas *Tbx4* misexpression in wing leads to transformation of these structures into leg characteristics (Logan and Tabin 1999; Rodriguez-Esteban *et al.* 1999; Takeuchi *et al.* 1999). The transcription factor Pitx1 induces Tbx4 gene expression in lower limb fields only (Logan and Tabin 1999; Takeuchi *et al.* 1999). *Pitx1* misexpression in wing leads to transformation of these structures into leg characteristics (Logan and Tabin 1999; Rodriguez-Esteban *et al.* 1999; Takeuchi *et al.* 1999). In homozygous *Pitx1* knockout mice, *Tbx4* gene expression is reduced in lower limbs without transformation of limb identity (Lanctot *et al.* 1999; Marcil *et al.* 2003). Although the exact functions of Tbx5 and Pitx1 through Tbx4 are as yet unknown they seem to be crucial in the determination of limb identity (Saito *et al.* 2002).

2.2 Limb development along three axes

2.2.1 Dorsal -ventral axis

Cellular interactions

Limb ectoderm consists of a dorsal and ventral compartment with the apical ectodermal ridge (AER) at its boundary. Patterning along the dorsal-ventral (D/V) axis of the limb is specified before limb initiation. Cell fate mapping experiments have been conducted in which the origin of cells of dorsal and ventral ectoderm of the presumptive limb regions was determined in chicken. At stage 13, ectoderm covering the somites and the intermediate mesoderm (IM) will migrate and become dorsal ectoderm of the presumptive limb field (fig. 2) (Altabef *et al.* 1997; Michaud *et al.* 1997). Ectoderm that covers the lateral plate mesoderm (LPM) will eventually become the ventral ectoderm, whereas that overlying the medial half of the LPM will become the AER. Possibly, a dorsalizing factor from the somites signals ectodermal cells to occupy the ectoderm of the presumptive limb as grafting presumptive limb tissue in between two rows of somites resulted in bidorsal limbs (Altabef *et al.* 1997; Michaud *et al.* 1997; Chen and Johnson 1999). Likewise, a ventralizing factor is likely to be secreted by the LPM as implantation of a filter between LPM and the presumptive limb region results in bidorsal limbs (Michaud *et al.* 1997). Alternatively, this can be explained by the filter itself blocking migration of ventral ectoderm, leading to dorsal ectoderm only (Chen and Johnson 1999).

Presumptive limb mesoderm can impose its D/V polarity to the overlying ectoderm only between stages 14-16 in chicken (Geduspan and MacCabe 1989). Experiments in which ectoderm, taken at certain developmental stages, was grafted on mesoderm in an inverse D/V orientation, resulted in an inversion of D/V mesodermal structures. It was shown that determination of D/V polarity is gradually established at stage 18, whereafter ectoderm can not be reprogrammed anymore (Geduspan and MacCabe 1989). At later stages, the ecto-

derm imposes the D/V patterning on the mesoderm, as 180 degree rotation of the ectoderm repatterns the developing limb bud (Akita 1996).

Molecules

The earliest genes involved in D/V patterning are the genes encoding Bone morphogenetic proteins BMP4 and BMP7. These genes are expressed in ventral ectoderm and mesoderm in the presumptive limb fields just before limb bud and AER formation (Ahn *et al.* 2001; Pizette *et al.* 2001). These BMP's seem to act as ventralizing factors, regulating the expression of the homeobox containing transcription factor *Engrailed* (*EN)1* in ventral ectoderm (fig. 4). Interruption of Bmp signaling in conditional Bmp receptor (*Bmpr)1a* null mice, or misexpression of the Bmp antagonist Noggin results in loss of *En1* expression in ventral ectoderm (Ahn *et al.* 2001; Pizette *et al.* 2001). In addition, this leads to ectopic expression of the dorsalizing factors *Wnt7a* and homeobox containing transcription factor *Lmx1* in ventral ectoderm and mesoderm, resulting in bidorsal limbs (Ahn *et al.* 2001; Pizette *et al.* 2001).

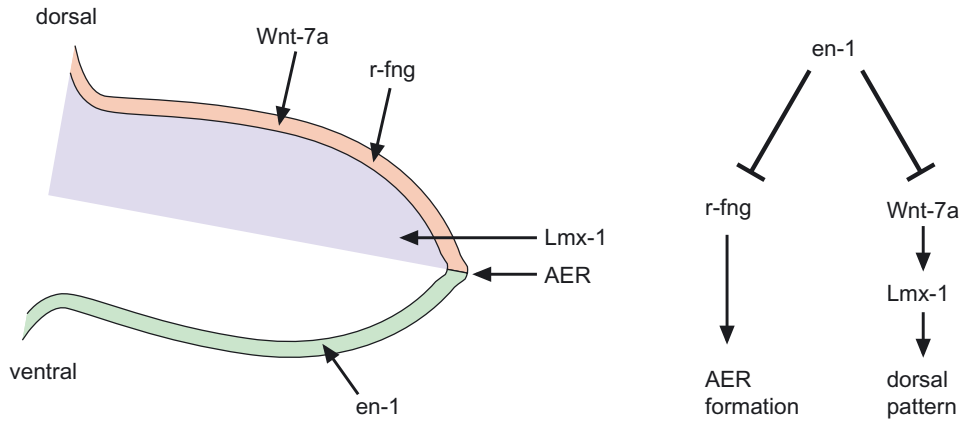

Figure 4. *Dorsal-Ventral patterning of the (chicken) limb. The ventralizing factor En1 is expressed in ventral ectoderm and ventral AER where it inhibits expression of the dorsalizing factors Wnt7a and Rfng. These are expressed in dorsal ectoderm and dorsal AER. Wnt7a induces mesenchymal expression of the dorsalizing factor Lmx1 (Adapted from Johnson 1997).*

The *En1* gene shows a restricted expression pattern in ventral ectoderm only (Loomis *et al.* 1998; Altabef and Tickle 2002). The role of En1 in establishment of dorsal and ventral cell compartments of the limb is still unclear. Misexpression of *En1* does not affect D/V polarity (Altabef *et al.* 2000). However, loss of En1 function results in a loss of ventral limb identity resulting in a bidorsal limb phenotype, due to loss of repression of the dorsalizing factor *Wnt7a,* normally expressed in dorsal ectoderm only (Loomis *et al.* 1996; Cygan *et al.* 1997; Logan *et al.* 1997). This suggests that En1 is necessary but not sufficient for normal D/V patterning.

Wnt7a is normally expressed in dorsal ectoderm before AER formation in chicken (Altabef *et al.* 1997), whereas in mice its expression becomes dorsally restricted after AER forma-

tion (Loomis *et al.* 1998). Wnt7a is a dorsalizing factor, as its loss of function in homozygous knockout mice leads to transformation of dorsal into ventral structures (Parr and McMahon 1995). Wnt7a in dorsal ectoderm subsequently induces the expression of another dorsalizing factor, *Lmx1,* in the underlying dorsal mesenchyme (Riddle *et al.* 1995; Vogel *et al.* 1995; Chen *et al.* 1998; Loomis *et al.* 1998; Altabef and Tickle 2002). However as *Lmx1* expression is unaffected in *Wnt7a* knockout mice, other, yet unknown factors must be involved in regulation of its expression (Vogel *et al.* 1995; Altabef and Tickle 2002).

2.2.2 Anterior-posterior axis

Cellular interactions

The zone of polarizing activity (ZPA) or polarizing region is located in posterior early limb mesenchyme. This is inferred from grafting posterior mesenchyme to the anterior mesenchyme in chicken limb which resulted in mirror-image duplications of digits and extra anteriorly located digits with a posterior identity (Saunders and Gasseling 1968). When few ZPA cells were transplanted the ectopic digits obtain a more anterior identity, so it was postulated that ZPA cells produce a morphogen that determines the identity of cells along the A/P axis by responding to its concentration (Wolpert *et al.* 1969). When exposed to high concentrations nearby the ZPA, cells obtain a more posterior identity.

Molecules

Sonic hedgehog (SHH) is known to be the morphogen produced by the ZPA cells that mimics the polarizing activity (Riddle *et al.* 1993). *Shh* is highly expressed posteriorly at the time of polarizing activity of the ZPA and its protein can act over a distance of about 30 cell diameters, or 300 μm. This is possible because of an autoprocessing reaction that links cholesterol to the N terminal signaling moiety of Shh (Lewis *et al.* 2001).

Moreover, beads soaked in Shh that are implanted in mouse anterior limb mesenchyme can induce the mirror-like duplications similar to those found in the ZPA grafting experiments (Riddle *et al.* 1993).

The polarizing activity of the ZPA is preceded by that along the main body axis long before limb bud initiation (Hornbruch and Wolpert 1991; Ros *et al.* 1996), indicating that the ZPA and Shh are not the first initiators of A/P polarity. Polarizing activity becomes gradually restricted to the presumptive posterior upper limb fields (Hornbruch and Wolpert 1991). This is further corroborated by the observation that ectopic limbs, induced by FGF, show an anterior location of the ZPA and reversed polarizing activity (Cohn *et al.* 1995; Ohuchi and Noji 1999)

Positioning of the ZPA and/or Shh is mainly determined by the transcription factors dHand, Gli3, Alx4 and by the Bmp antagonists Formin and Gremlin (fig. 5).

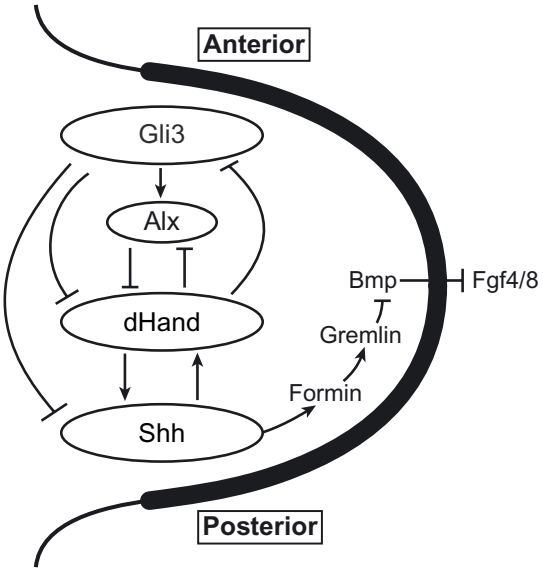

Figure 5. *The molecular cascade involved in the coordinated signaling of Shh. Gli3 and dHand restrict each others expression probably through Alx4. The expression of Shh is maintained by dHand and the Shh-Fgf positive feedback loop in which Shh activates Gremlin through Formin. Gremlin cancels the suppression by Bmp's on Fgf's expression. Fgf's maintain Shh expression thus completing the feedback loop.*

One of the earliest genes involved in A/P patterning is *dHand* which is expressed in the LPM. Its expression becomes gradually posteriorly restricted prior to limb initiation (Charite *et al.* 2000; Fernandez-Teran *et al.* 2000). This posterior restriction is caused by Gli3, which is expressed in the anterior mouse limb (Charite *et al.* 2000; te Welscher *et al.* 2002). Dhand is essential for the induction of *Shh* expression, as *dHand* homozygous null mice lack *Shh* expression in the limb (Charite *et al.* 1994). Ectopic expression of *dHand*, as found in *Gli3* deficient mice, also results in ectopic expression of *Shh* (Litingtung *et al.* 2002; te Welscher *et al.* 2002). The ectopic *Shh* expression in *Gli3* null mice is not only preceded by that of *dHand*, but also by *Formin, Gremlin, 5'Hoxd* genes and *Fgf4*, suggesting these are involved in *Shh* induction (Zuniga *et al.* 1999; Zuniga and Zeller 1999; te Welscher *et al.* 2002). Alx4 is probably also involved in positioning of Shh expression since *Alx4* deficient mice show ectopic *Shh* expression in anterior limb mesenchyme (Qu *et al.* 1997).

Finally, the *Hoxb8* and the 5'*Hoxd* genes also play a role in the induction of *Shh* expression (Zuniga *et al.* 1999; Zuniga and Zeller 1999) as ectopic expression of either *Hoxb8* or *Hoxd12* leads to anterior expression of *Shh* (Charite *et al.* 1994; Knezevic *et al.* 1997).

2.2.3 Proximal-distal axis

Cellular interactions

The apical ectodermal ridge (AER) is a ridge of pseudostratified columnar epithelial cells, located at the D/V boundary of the tip of the outgrowing limb. As mentioned before, the AER cell precursors are derived from a broad ectodermal domain and gradually converge at a border at the tip of the distal limb ectoderm (Kimmel *et al.* 2000).

In mice at E9.5, AER precursor cells are derived from ventral ectoderm and assemble along a dorsal border (Kimmel *et al.* 2000). This border separates pre-AER from dorsal ectoderm.

An additional border is formed within the pre-AER that separates the dorsal pre-AER from the ventral ectoderm. At E10.5, a ventral border is formed between pre-AER and ventral ectoderm. At E11.5, there is a loss of the D/V border within the now mature AER.

After the AER is established it permits outgrowth of the limb bud. The AER expresses many genes, from which the *FGF's* are crucial to its function. They keep undifferentiated mesenchyme cells located in the so-called progress zone (PZ), 200-300 µm underneath the AER, in a proliferative state (Dudley *et al.* 2002). According to the progress zone model the differentiation fate of a cell is determined by the time spent in the PZ under the influence of the AER (fig. 6) (Summerbell *et al.* 1973).

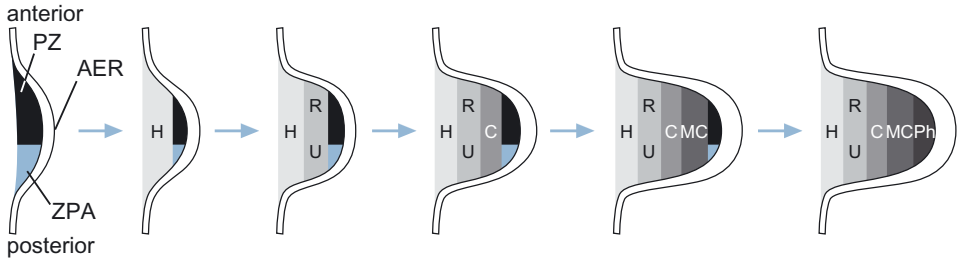

Figure 6
Schematic overview of the signaling centers directing patterning and outgrowth of the limb. The apical ectodermal ridge (AER) promotes outgrowth of the limb along the P/D axis through sustaining of proliferation of the undifferentiated mesenchyme in the progress zone (PZ). The zone of polarizing activity (ZPA) directs patterning along the A/P axis. According to the progress zone model skeletal elements are specified in a proximal to distal sequence. H=humerus, R=radius, U=ulna, C=carpals, MC=metacarpals, Ph=phalanges (Adapted from Gerhart 1997).

A cell that leaves the PZ early in limb development adopts a proximal fate and differentiates into a proximal element. A cell leaving the PZ at a later time point will adopt a more distal fate and differentiates into a distal element. This model was supported by experiments in which AER removal at successive stages of chicken limb development results in progressive distal truncations of the limb (Summerbell 1974).
Dudley *et al.* (2002) suggest a different model for limb bud patterning along the P/D axis: the early specification model. According to this model PZ cells are specified into progenitors of the three limb segments during early limb bud outgrowth. These cells proliferate up to a limit necessary for a mesenchymal condensation. They start differentiation after they escape the influence of the AER and become subject to intercellular signaling of the mesenchyme underlying the PZ.
An argument used in favor of the early specification model is the observation that descendants of early stage labeled limb mesenchyme cells at different positions along the P/D axis are conferred to one segment only, suggestive for early specification of the skeletal elements (Dudley *et al.* 2002). However, this result of cell fate mapping does not corroborate with those found by others (Vargesson *et al.* 1997; Wolpert 2002).
In either model, the outgrowth of the limb is mediated through the FGF's since implantation of FGF soaked beads immediately after AER removal completely rescues limb development,

albeit with size reductions of skeletal elements (Niswander *et al.* 1994; Cohn *et al.* 1995; Crossley *et al.* 1996; Vogel *et al.* 1996; Ohuchi *et al.* 1997).

Molecules
Many signaling molecules involved in AER formation show differential expression patterns along the D/V axis. Alternatively, expression patterns are or become restricted to the AER. Differences of expression of some of these genes in chicken and mice could reflect differences in AER formation between these species.
Fgf8 is involved in AER induction (Crossley and Martin 1995; Loomis *et al.* 1998; Altabef and Tickle 2002). Loss of its upstream regulator gene *Wnt3* in conditional knockout mice, results in (partial) absence of *Fgf8* expression and loss of AER induction (Galceran *et al.* 1999; Barrow *et al.* 2003).
Loss of AER induction is also seen in *Bmpr1a* knockout mice (Ahn *et al.* 2001; Pizette *et al.* 2001). Bmpr1a binds Bmp4 and Bmp7 expressed in AER. *En1* and *Wnt7a* are not only involved in positioning of the D/V border, but also in positioning and formation of the AER (Kimmel *et al.* 2000). En1 seems to be involved in condensation of pre AER cells along the ventral border. In *En1* homozygous knockout mice, the ventral midline border of the AER is extended in ventral ectoderm and the AER occasionally bifurcates into two ridges (Loomis *et al.* 1996; Cygan *et al.* 1997; Kimmel *et al.* 2000). *Wnt7a* knockout mice have a slightly dorsally broadened AER without bifurcations (Loomis *et al.* 1998). In *En1/Wnt7a* double knockout mice, the AER is slightly broadened, however without bifurcations, so Wnt7a seems necessary for normal formation of a ridge at the dorsal border (Cygan *et al.* 1997; Loomis *et al.* 1998; Kimmel *et al.* 2000). Since *En1/Wnt7a* double mouse mutants have an AER other genes must also be involved in establishing the ventral border of the AER (Kimmel *et al.* 2000).

Radical fringe (Rfng), a signaling protein, has been implicated in AER formation and AER positioning in chicken. It is first expressed in dorsal ectoderm of the presumptive limb fields at stage 15/16, with highest expression in the AER at later stages (Laufer *et al.* 1997; Rodriguez-Esteban *et al.* 1997). Rfng is postulated to be involved in positioning of the AER, since the ventral boundary of the AER is coincident with the boundary of *Rfng* expression in chicken limb development. In mice, however, it is expressed at low levels and its loss of function does not result in limb anomalies, which can either be explained by functional redundancy of other fringe genes or Rfng is not involved in AER formation in mice (Moran *et al.* 1999).

Patterning along the P/D axis is also regulated by differential expression of the *Meis1* and *Meis2* genes encoding homeobox containing transcription factors. In chicken, *Meis1*- and *Meis2* gene expression becomes restricted to the proximal limb only from stage 19 onwards (Capdevila *et al.* 1999; Mercader *et al.* 1999). Endogenous high concentrations of retinoic acid (RA), upregulate *Meis* gene expression in the proximal limb bud, whereas Fgf signaling represses RA levels distally. Therefore, the *Meis* genes differentially regulate other genes involved in patterning and outgrowth of proximal and distal limb.

2.2.4. Interactions between the axes.

The patterning along the three axes must be coordinated for proper limb bud outgrowth. As far as the interaction between the P/D and A/P axis is concerned coordinated signaling between several molecules of the AER and ZPA is required to maintain each other's function. Both signaling centers are in close proximity to each other throughout limb bud outgrowth. Removal of the AER results in loss of polarizing activity of the ZPA (Niswander *et al.* 1993; Vogel and Tickle 1993). Homozygous conditional *Fgf8* knockout mice have reduced *Shh* expression, whereas *Shh* deficient mice show loss of *Fgf8* expression (Lewandoski *et al.* 2000; Moon and Capecchi 2000; Chiang *et al.* 2001).
The molecular cascade involved in the coordinated signaling mentioned above consists of a number of steps. Shh induces Formin, which subsequently activates Gremlin. Gremlin cancels the suppression by Bmp's on *Fgf's* expression (Zuniga *et al.* 1999). Fgf's maintain *Shh* expression thus completing the feedback loop (Mahmood *et al.* 1995; Vogel *et al.* 1996; Zuniga *et al.* 1999).
Likewise, A/P patterning interacts with D/V patterning. This is inferred from maintenance of *Shh* expression by Wnt7a (Parr and McMahon 1995; Yang and Niswander 1995). So the P/D, A/P and D/V axis are interconnected since Fgf's and Wnt7a maintain expression of Shh.

2.3 Molecules involved in the formation of limb structures

2.3.1 Formation of skeletal elements

Mesenchyme condensations
Skeletal element formation starts with mesenchymal condensations. The number or size of limb skeletal elements can already be abnormal from the early stages of mesenchyme condensations. The chick mutant *talpid* displays limb shortening and polydactyly (Hinchcliffe and Ede 1967). Already at stage 20, their mesenchymal condensations appear abnormal in shape, possibly due to perturbations of cell adhesion. In addition, absence of cell death was noted in the anterior necrotic zone (ANZ) that is located at the anterior border of the AER, as well as in the posterior necrotic zone (PNZ) located at the posterior border of the AER. This results in lengthening of the AER anteriorly which could explain the polydactyly phenotype.
When the number of mesenchymal cells remains below the threshold required to enter chondrogenesis this may result in size reductions of skeletal elements (Hall and Miyake 1992).
Fgf's secreted from the AER are postulated to determine the size and shape of the mesenchymal progenitors of the skeletal elements. Loss or hypoplasia of skeletal precursor populations correlate with missing or hypoplastic skeletal elements in single or double *Fgf* knockout mice (Lewandoski *et al.* 2000; Moon *et al.* 2000; Moon and Capecchi 2000; Sun *et al.* 2002). Apart from reduction of proliferation these precursor populations might also remain too small because of diminished cell adhesion or insufficient cell migration into condensation areas (Li and Muneoka 1999; Tanaka *et al.* 2000; Sun *et al.* 2002).
Molecules implicated in the initiation of mesenchyme condensations are Bmp2, Bmp4, Bmp5 and Bmp7 (Kingsley *et al.* 1992; King *et al.* 1994; Pizette and Niswander 2000). In *BmprIA* knockout mice, no mesenchyme condensations are initiated (Zou *et al.* 1997).

Likewise, *BmprIB* knockout mice, miss certain phalanges, due to a decrease of proliferation and mesenchymal cell condensation (Baur *et al.* 2000).
The *Hox* genes are involved in the timing of condensations as well as in the determination of their growth (Davis *et al.* 1995; Yokouchi *et al.* 1995; Davis and Capecchi 1996; Stadler *et al.* 2001). This is inferred from loss of bones or bone shortening in *Hox* knockout mice. The *Hoxa* genes show successive expression along the P/D axis (fig. 7) (Nelson *et al.* 1996).

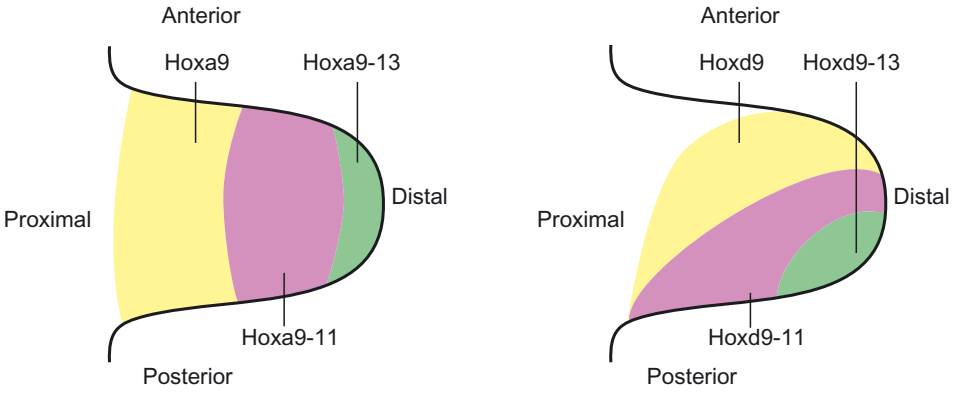

Figure 7. *Expression of 5'Hoxa and Hoxd genes in early limb. The more 5'Hoxa genes show a more distal expression. Hoxa9 is expressed in the entire limb, whereas Hoxa13 is expressed in distal limb only. Similarly, Hoxd9 is expressed in anterior and posterior limb whereas Hoxd13 is only expressed posteriorly (Adapted from Wolpert 1999).*

In general *Hoxa9* contributes to morphogenesis of the upper limb whereas *Hoxa10* contributes predominantly to that of the distal upper limb and proximal middle limb. *Hoxa11* contributes to morphogenesis of the middle limb and *Hoxa13* to that of the distal limb. Before the start of digit morphogenesis *Hoxd* genes are expressed in a posterior domain (fig. 7). Subsequently they become more anteriorly expressed, starting with *Hoxd13* and ending with *Hoxd10* (Dolle *et al.* 1989; Yokouchi *et al.* 1991; Nelson *et al.* 1996; Rijli and Chambon 1997; Kmita *et al.* 2002).

Generation of *Hoxa* and *Hoxd* knockout mice has shown that the various genes can take over each other's functions especially when they are closely related physically or evolutionary. This and the fact that 5' *Hoxa* and *Hoxd* genes are involved in limb formation explains the large diversity in phenotypes of the knock out mice (Davis and Capecchi 1994; Favier *et al.* 1996; Fromental-Ramain *et al.* 1996).

Apoptosis in the limb
Apoptosis or programmed cell death plays an important role in cell number, histiogenesis and morphogenesis. Soon after the appearance of the limb buds, an anterior necrotic zone (ANZ) is present in the anterior limb mesenchyme adjoining the body wall (fig. 8). The ANZ borders the anterior AER (Hinchliffe and Ede 1973).

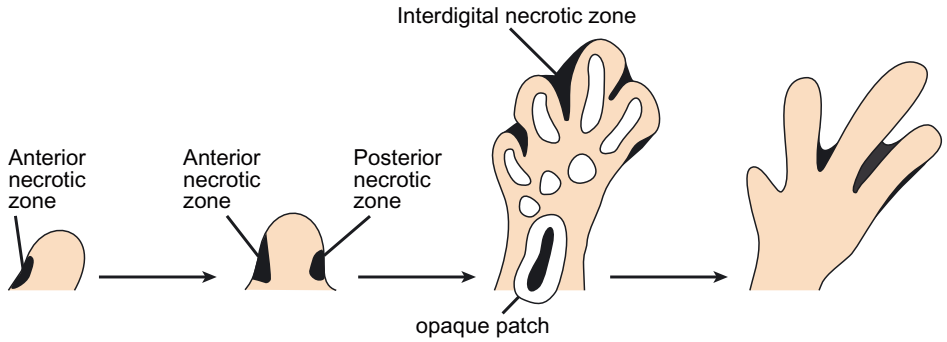

Figure 8 *Apoptosis in the developing chicken hind limb. First an anterior necrotic zone appears followed by that posteriorly that borders the AER. The opaque patch is a necrotic zone located between the precursors of the radius and ulna. Apoptosis in the interdigital necrotic zone results in separation of the fingers (Adapted from Gilbert SF 1997).*

Shortly thereafter, a posterior necrotic zone (PNZ) appears in the posterior limb mesenchyme, located at the posterior border of the AER (Hurle and Hinchcliffe 1978; Brewton and MacCabe 1988). The ANZ and PNZ are probably functional in limiting the length of the AER, thereby influencing anterior-posterior patterning of the limb (Salas-Vidal *et al.* 2001 and ref. therein). Apoptosis is also present in the central core of the mesenchyme, in the so-called opaque patch which is presumably necessary to separate the radius from the ulna (Dawd and Hinchliffe 1971).
At the tip of the limb bud interdigital apoptosis restricts the number of cells available for proliferation during outgrowth of the digits and removes interdigital tissue (fig. 8) (Mori *et al.* 1995; Salas-Vidal *et al.* 2001). The interdigital apoptosis starts after regression of the AER and ZPA (Salas-Vidal *et al.* 2001). It is preceded by restriction of *Fgf8* expression from the entire AER to the AER covering the digits and by downregulation of the anti-apoptotic molecule Bag1 and the Bmp antagonist Gremlin (Mori *et al.* 1995; Merino *et al.* 1999; Salas-Vidal *et al.* 2001; Crocoll *et al.* 2002).

Both *Hoxa* and *Hoxd* genes are involved in apoptosis, as several single or double mouse mutants of 5'*Hoxa* and *Hoxd* genes show fusions of specific carpal and/or skeletal precursors (Dolle *et al.* 1993; Davis and Capecchi 1994; Davis *et al.* 1995; Davis and Capecchi 1996; Fromental-Ramain *et al.* 1996; Zakany and Duboule 1996; Stadler *et al.* 2001).
Bmp2, Bmp4 and Bmp7 are also involved in the induction of interdigital apoptosis. They are expressed in interdigital mesenchyme before onset of apoptosis (Francis *et al.* 1994; Lyons *et al.* 1995; Laufer *et al.* 1997; Macias *et al.* 1997). Ectopic expression of these Bmp's in interdigital mesenchyme induces apoptosis (Ganan *et al.* 1996; Macias *et al.* 1997). Blocking Bmp signaling by expression of a dominant negative Bmpr1A- or Bmpr1B, results in interdigital webbing/syndactyly (Yokouchi *et al.* 1996; Zou and Niswander 1996).
Based on expression of the homeobox containing transcription factor encoding *Msx1* and *Msx2* genes in interdigital mesenchyme and induction of apoptosis after *Msx2* overexpression, a stimulatory role was inferred for these genes in the interdigital mesenchyme (Coelho *et al.* 1992; Ferrari *et al.* 1998).

Finally, apoptosis also delineates the pathways for grow cones of axons and the remodeling the vascular patterning (Tosney *et al.* 1988; Vaux and Korsmeyer 1999). However, no specific molecular mechanisms for these processes have been identified.

Chondrification

Several signaling molecules are implicated in the proliferation and/or differentiation of chondrocytes. Chondrocytes express *Indian hedgehog* (*Ihh*) after their differentiation from proliferating into prehypertrophic chondrocytes until terminal differentiation into hypertrophic chondrocytes (Vortkamp *et al.* 1996; St-Jacques *et al.* 1999; Chung *et al.* 2001; Kobayashi *et al.* 2002). Ihh protein induces proliferation of chondrocytes but delays chondrocyte differentiation through induction of expression of parathyroid hormone related peptide (PTHrP) in the periarticular proliferating chondrocytes of the growth plates. PTHrP delays differentiation of the proliferating periarticular chondrocytes into columnar chondrocytes and subsequently into prehypertrophic chondrocytes through binding to the receptor PTHrPR. Ihh also delays differentiation of chondrocytes, but independent of PTHrP.

Fibroblast growth factor receptor (FGFR)3 is expressed in proliferating and prehypertrophic chondrocytes and Ffg18 is expressed in perichondrium. Both repress *Ihh* expression as can be inferred from loss of function mutant mice (Naski and Ornitz 1998; Ohbayashi *et al.* 2002).

BmprIB null mice, show a reduction of chondrocyte proliferation in the phalangeal region (Baur *et al.* 2000; Yi *et al.* 2000). Misexpression of *BmprIA*, delays chondrocyte differentiation, with loss of expression of *Ihh* but normal induction of PTHrP, suggestive for a role in regulation of *Ihh* expression (Zou *et al.* 1997).

Growth and differentiation factor (Gdf)5 stimulates chondrocyte proliferation and differentiation (Tsumaki *et al.* 1999). *BmprIB/Gdf5* double mutant mice show the same digit phenotypes as the *Gdf5* null mice, which suggest that *Gdf5* signals through Bmpr1B (Baur *et al.* 2000; Yi *et al.* 2000).

The 5'*Hoxa* and the *Hoxd* genes have a role in chondrification of long bones in the limbs. *Hoxa13* or *Hoxd13* null mutants, as well as, *Hoxa13*/*Hoxd13* or *Hoxd12*/*Hoxd13* transheterozygotes show absence or delay of chondrification (Morgan and Tabin 1994; Davis and Capecchi 1996; Fromental-Ramain *et al.* 1996; Mortlock *et al.* 1996; Goff and Tabin 1997).

Several Wnt's and the Wnt antagonist Frizbee 1 are also involved in the regulation of chondrogenesis. Misexpression or overexpression of *Wnt1*, *Wnt5a*, *Wnt7a* in chicken limb in vitro leads to inhibition of chondrogenesis of long bones. In contrast, overexpression of *Wnt4* results in its acceleration (Zakany and Duboule 1993; Rudnicki and Brown 1997; Hartmann and Tabin 2000; Tufan and Tuan 2001; Chimal-Monroy *et al.* 2002). Misexpression of *Lef/Tcf* (β-catenin signaling pathway), leads to accelerated chondrogenesis, whereas misexpression of dominant negative Wnt receptors Frizzled (Fz)1-and Fz7 leads to its delay, indicative for differential effects of Wnt signaling pathways on chondrogenesis (Hartmann and Tabin 2000).

It is striking that most loss of function mutants of the signaling molecules involved in chondrogenesis show size reductions of a limited number of skeletal elements. This suggests a limited role of the genes mentioned above in the formation in the skeletal elements.

However, loss of function mutant mice for genes involved in extra cellular matrix components like *Collagen* (*Col*)*2a1*, *Col9a2, Col10* and *Aggrecan* show size reductions of all skeletal elements (Warman *et al.* 1993; Watanabe *et al.* 1994; Li and Schwartz 1995; Muragaki *et al.* 1996).

Ossification
Little is known about the genes involved in ossification of the limb skeletal elements. *Ihh* promotes differentiation of perichondrial cells, committed to the osteoblastic cell lineage, into mature osteoblasts (Chung *et al.* 2001). Fgf18 in periost promotes proliferation and differentiation of osteoblasts and replacement of hypertrophic chondrocytes by osteoblasts (Liu *et al.* 2002; Ohbayashi *et al.* 2002). *Fgf18* null mice show a delay in ossification and, in the fibula, incomplete ossification.
Msx2 is expressed in periost and articular preosteoblasts. In *Msx2* deficient mice a reduction of expression of several genes involved in bone differentiation has been observed (Satokata *et al.* 2000). *Msx1* is expressed in differentiating and in differentiated cells of the osteoblastic- and clastic cell lineage and is postulated to be involved in the regulation during several stages of osteogenesis although its precise function is not yet known (Orestes-Cardoso *et al.* 2001).

2.3.2 Joint formation
Wnt14 is involved in the initiation of joint formation (Hartmann and Tabin 2001). It is expressed transversely at sites of future joints as examined in the distal limb. In mature joints, it is expressed in fibrous connective tissue of joint capsules and the synovial membrane. Misexpression of *Wnt14* leads to expression of genes normally restricted to joints like *Gdf5* and to downregulation of genes normally excluded from the future joint region. In addition, Wnt14 can repress chondrogenesis *in vitro* (Hartmann and Tabin 2001).
Gdf5 is expressed in areas to become joints and in fibroarticular joint cartilage. *Gdf5* null mice show loss of joint formation in distal skeletal elements (Storm *et al.* 1994; Storm and Kingsley 1996; Hartmann and Tabin 2001).

Another Bmp family member, namely Bmp7 has been implicated in joint formation, since its ectopic expression in digits leads to absence of joints (Macias *et al.* 1997). In addition, excess of Bmp signaling in mice deficient for the Bmp antagonist *Noggin* results in failure of joint formation (Brunet *et al.* 1998).
In 5' *Hoxa* and *Hoxd* (double) mutants, fusions of carpals and/or digits are present, indicative for a role in joint specification (Francis-West *et al.* 1999 and ref. therein).

2.3.3 Muscle and tendon formation
The transcription factor Pax3 is essential in the determination of myogenic precursor cells in the lateral dermomyotome and their migration into the limb (Francis-West *et al.* 2003 and ref. therein). The same is true for Hepatocyte growth factor (Hgf) signaling which expression is regulated by Pax3 (Dietrich 1999). Migration of myogenic precursor cells is also regulated by extracellular matrix and cell surface molecules (Burkin and Kaufman 1999; Schafer and Braun 1999). It is believed that inhibition of their differentiation by mesenchymal Bmp4, Hgf, Fgf's and the transcription factor Twist allows migration of

the myogenic precursor cells (Brand-Saberi and Christ 1999; Francis-West *et al.* 2003). After entering the limb bud, the precursors become committed to the myogenic cell fate, differentiate into myoblasts and start to express the myoblast specific transcription factor encoding genes *Myf5*, *MyoD*, *Myogin* and *Mrf4* (Buckingham *et al.* 1992). Fgf, insulin-like growth factor 1 and presumably Msx1 stimulate their subsequent proliferation. The transcription factor encoding *Eya1* and *Eya2* gene expression in ventral and dorsal muscle mass, respectively, are likely to be involved in D/V patterning (Xu *et al.* 1998). Terminal differentiation into primary slow-or fast fibers, is possibly mediated by differential expression of Wnt's and MyoD (Francis-West *et al.* 1999).
In chicken limb, scleraxis expression colocalizes with tendon precursors and it is also expressed in ligaments (Schweitzer *et al.* 2001). Its expression can be induced by ectodermal signals, can be maintained by Fgf4 and be inhibited by Bmp2, Bmp4 and Bmp7. Tendon precursors coalesce forming dorsal and ventral masses with differential expression of the transcription factor encoding genes *Eya2* and *Six1* in dorsal mass and *Eya1* and *Six2* in ventral mass (Edom-Vovard *et al.* 2002). Likewise, the *Eph4* gene encoding a receptor, the transforming growth factor (*Tgf)β2* and *Fgf8* genes are expressed in distal tendons only (Patel *et al.* 1996; Merino *et al.* 1998; D'Souza and Patel 1999; Edom-Vovard *et al.* 2002). Proximal specific genes involved in tendon formation have not been identified.

2.3.4 Innervation and vascularization

Motor neuron differentiation is induced by Shh, expressed in the notochord and floorplate of the neural tube (Sharma *et al.* 1998 and ref. therein). Differential gene expression encoding transcription factors has been noted, with coexpression of *Islet1* and *Islet2* by the motor neurons which will innervate the ventral muscle mass and *Lim1* and *Islet2* expression by those innervating the dorsal muscle mass (Tsuchida *et al.* 1994).
Likewise, motor neurons expressing Er81 innervate anteriorly derived muscles and those expressing Pea3 innervate intermediate-and posteriorly derived muscles (Lin *et al.* 1998). Sensory neurons express both Er81 and Pea3 but after connection with motor neurons in the same muscle mass they also exhibit a differential expression pattern in ventral and dorsal muscle, respectively.

Little is known about the molecular processes involved in the vascularization during limb formation. Vascular endothelial growth factors (VEGF)'s and its receptors are essential in initial angiogenesis and vasculogenesis in the embryo. VEGF's promote endothelial cell proliferation, migration and formation of vascular tubes until maturation of the vasculature (Vargesson 2003 and ref. therein). Loss of VEGF signaling in mice results in deficient blood vessel formation and embryonic death before limb outgrowth (Weinstein 1999 and ref. therein). In the embryo several other molecules have been implicated in angiogenesis, but specifically for the limb it has been shown that TGFβ signaling controls vessel calibre (Vargesson 2003 and ref. therein).

2.3.5 Digit identity

Recently, more insight has been gained in the specification of digit identity. Digit identity becomes specified after joints are formed and is determined by the interdigital (ID) mesenchyme (Dahn and Fallon 2000). Removal of ID mesenchyme in chicken results in

a more anterior digit phenotype (Dahn and Fallon 2000). For example, mesenchyme located between digits 3 and 4 specifies the digit 3. Its removal results in a digit 2 instead of a digit 3, whereas digit 4 remains unaltered.
Several genes play a role in this digit identity specification. When Bmp signaling is blocked, digit 3 has the identity of digit 2. This is corroborated by the observation that ectopic anterior digits, induced through Bmp2 loose their identity after blocking Bmp signaling (Drossopoulou *et al.* 2000).
Loss of *Gli3* gene expression leads to polydactyly with loss of normal digit identity (Litingtung *et al.* 2002; te Welscher *et al.* 2002). Loss of *Shh* expression leads to a loss of all but digit 1 which has a normal identity (Chiang *et al.* 2001; Kraus *et al.* 2001). *Shh*/*Gli3* double homozygote knockout mice are phenotypically similar to *Gli3* deficient mice (Litingtung *et al.* 2002; te Welscher *et al.* 2002). In *Shh* null mice with one functional *Gli3* allele, a variable number of digits are formed but all have the same identity. This is suggestive for a role of Shh and Gli3 in the specification of digit identity.

2.4 Conclusions

The use of experimental animals, mainly the chicken and mouse has enabled the removal or grafting of specific tissues at various stages of development and to follow cell migration. At the molecular level homology studies and inactivation of specific genes have contributed to the identification of genes that play a role in the positioning, induction and identity of limbs. Advances have also been made in the characterization of genes/proteins that are important in the formation of the limb tissues, their interaction and the patterning along three axes. In some instances a cell biological explanation of specific limb anomalies like polydactyly could be derived from animal experiments. Up to now the identified genes playing a crucial role in limb development belong to a relatively small number of gene families such as Fgf's, Tgfß's, Wnt's and Hedgehogs. The encoded proteins may exert different functions by interaction with other proteins at various stages of development even within the same tissue (von Bubnoff and Cho 2001). A major challenge ahead is the unraveling of the structure and function(s) of the encoded proteins and to relate specific biochemical pathways to the major steps in the development of limb structures and tissues.

Chapter 3

Genes involved in human congenital limb malformations

Genes involved in congenital limb malformations

Normal limb development can be disturbed by (a combination of) many different factors, including chromosomal anomalies, multigenic or triallelic inheritance, single gene disorders, amniotic bands, vascular disruption and teratogens.
Clinical descriptions and family studies of limb anomalies preceded histological and biochemical studies (Warman 2000). Along with studies of spontaneously occurring or experimentally induced murine and chicken mutants, clinical, histological and biochemical data were used for mapping and identification of genes from the early nineties of the twentieth century on. This resulted in a rapidly growing list of mapped and identified genes, causing mostly syndromes with a Mendelian pattern of inheritance (Grzeschik 2002). The association of anomalies of limb development with anomalies in other organ systems can be inferred from the knowledge that genes involved in limb development are often not limb specific (Stoll *et al.* 1998).
In addition to what has been mentioned about classification systems of congenital limb malformations in Chapter 1, a classification system has been formulated based on limb deficiencies, supernumerary structures and fusion/separation defects (Stoll *et al.* 1998). This classification system is nowadays often used by clinicians. However, most syndromic cases show a combination of limb defects and cannot readily be classified into one category only. In addition, bone overgrowth is not included in this classification.
Thanks to the convergence of human and mouse genetics a start has been made with an explanation of several human congenital limb malformations. A summary of the molecular etiology of human limb malformations and the molecular pathogenesis in mice homologues is presented in the table (see next page).

Table MOLECULAR ETIOLOGY AND PATHOGENESIS OF HUMAN LIMB MALFORMATIONS

Disorders	Mode of inheritance	Gene mutated in humans	Protein mutated in humans	Molecular pathogenesis in mice homologues
Polydactyly				
Greig cephalopolysyndactyly syndr	AD	*GLI3*	transcription factor	inhibition of *Bmp* through Gremlin, enhanced Fgf8 signaling
Pallister-Hall syndr	AD	*GLI3*	idem	idem
Post axial polydactyly	AD	*GLI3*	idem	idem
Rubinstein-Taybi syndr	AD	*CBP*	transcription co-factor	downregulation Gli3 and Twist, enhanced Fgf signaling
Triphalangeal thumb syndr	AD	*LMBR1*	regulatory element of SHH	misregulation Shh
Smith-Lemli-Opitz syndr	AR	*DHCR7*	enzyme Δ7 sterol reductase	downregulation Shh, impairment Fgf-Bmp signaling balance
Pelger-Huet syndr	AD	*LBR*	enzyme Δ14 sterol reductase	idem
Greenberg syndr	AR	*LBR*	idem	idem
Chondrodysplasia punctata	X-LD	*EBP or NSDHL*	enzyme Δ8 sterol isomerase or	idem
CHILD syndr	X-LD	*EBP or NSDHL*	3β-OHsteroid dehydrogenase	idem
Bloom syndr	AR	*RECQ3*	DNA helicase	unknown
Syndactyly				
Apert syndr	AD	*FGFR2*	growth factor receptor	enhanced Fgf signaling
Pfeiffer syndr	AD	*FGFR1 or 2*	idem	idem
Saethre-Chotzen syndr	AD	*TWIST, FGFR2 or 3*	transcription factor, idem	idem
Synpolydactyly	AD	*HOXD13*	transcription factor	misregulation target genes (Bmp2/7, Ihh, PTHR and Gdf5)
Brachydactyly				
Brachydactyly A1	AD	*IHH*	signaling molecule	downregulation PTHrP, impairment chondrogenesis
Brachydactyly B	AD	*ROR2*	ROR2 kinase receptor	idem
Brachydactyly C	AD	*CDMP1*	growth/differentiation factor	impairment chondrogenesis
DuPan syndr	AR	*CDMP1*	idem	idem
Hunter-Thompson syndr	AR	*CDMP1*	idem	idem
Grebe syndr	AR	*CDMP1*	idem	idem
Brachydactyly similar to types D/E	AD	*HOXD13*	transcription factor	misregulation target genes (Bmp2/7, Ihh, PTHR and Gdf5)
Hand-Foot-Genital syndr	AD	*HOXA13*	transcription factor	impairment chondrogenesis

Preaxial deficiency				
Fanconi anemia syndr	AR	*FANCA-H, BRCA2*	DNA damage response protein	unknown
Rothmund-Thomsen syndr	AR	*RECQ4*	DNA helicase	unknown
Holt-Oram syndr	AD	*TBX5*	transcription factor	impairment Fgf signaling
Post axial deficiency				
Ulnar-mammary syndr	AD	*TBX3*	transcription factor	impairment Shh signaling
Central deficiency				
Split-hand-split-foot malf.	AD	*P63*	transcription factor	impairment AER maintenance
Robinow syndr	AR	*ROR2*	ROR2 kinase receptor	downregulation PTHrP, impairment chondrogenesis
Chondrodysplasia				
Thanatophoric dysplasia	AD	*FGFR3*	growth factor receptor	downregulation Ihh/PTHrPR, impairment chondrogenesis
Achondroplasia	AD	*FGFR3*	idem	downregulation Ihh, impairment chondrogenesis
Hypochondroplasia	AD	*FGFR3*	idem	idem
Blomstrand chondrodysplasia	AR	*PTHPR*	PTHP receptor	impairment chondrogenesis
Achondrogenesis type 1B	AR	*DTDST*	sulphate transporter	undersulfation proteoglycans, impairment Fgf signaling
Diastrophic dysplasia	AR	*DTDST*	idem	idem
Atelosteogenesis type 2	AR	*DTDST*	idem	idem
Dyssegmental dysplasia (Silverman-Handmaker type)	AR	*HSPG2*	proteoglycan	impairment Fgf signaling
Simpson-Golabi-Behmel syndr	X-LR	*GPC3*	proteoglycan	unknown
Campomelic dysplasia	AD	*SOX9*	transcription factor	downregulation Col2a1, Ihh, PTHrPR, impairment chondrogenesis
Achondrogenesis type 2	AD	*COL2A1*	extracellular matrix protein	impairment cartilage formation
Kniest dysplasia	AD	*COL2A1*	idem	idem
Osteochondrodysplasia				
Pycnodysostosis	AR	*CTSK*	lysosomal enzyme	impairment bone resorption
Osteodysplasia				
Cleidocranial dysplasia	AD	*CBFA1*	transcription factor	impairment osteogenesis
Oto-Palato-Digital syndr type 2	X-LD	*FLNA*	cytoskeletal protein	impairment ossification

3.1 Supernumerary structures: polydactyly

Polydactyly or extra digit(s) have a prevalence of 1.4-1.9/1000 from which 85% are isolated anomalies (Castilla *et al.* 1996; Castilla *et al.* 1997; Castilla *et al.* 1998). Polydactyly is usually subdivided into an extra first (preaxial), middle (central) and fifth (postaxial) digit (Temtamy and McKusick 1978).

Interestingly, these different kinds of polydactyly are features of patients with a mutation in the transcription factor GLI3. *GLI3* mutations can cause three different autosomal dominant phenotypes, namely Greig cephalopolysyndactyly syndrome (MIM 175700), Pallister-Hall syndrome (MIM 146510), as well as isolated pre- or postaxial polydactyly (MIM 147200, MIM174700) (Vortkamp *et al.* 1991; Kang *et al.* 1997a; Kang *et al.* 1997b; Radhakrishna *et al.* 1997b; Radhakrishna *et al.* 1999). In Greig syndrome, the polydactyly is either pre- or postaxial (Gollop and Fontes 1985), whereas in Pallister-Hall syndrome it is central or postaxial (Biesecker and Graham 1996; Biesecker *et al.* 1996). The phenotypes can not be predicted from the position of a mutation in the *GLI3* gene (Radhakrishna *et al.* 1999).

In addition to polydactyly, syndactyly can be present in these phenotypes, which is suggestive for a role of GLI3 in the determination of the number of digits and in interdigital apoptosis. In *Gli3* null and *Shh/Gli3* double null mice, the phenotypes are strikingly similar (Hui and Joyner 1993; Litingtung *et al.* 2002; te Welscher *et al.* 2002). Both show polydactyly and syndactyly with digits with one identity only, namely that of the first digit. When one allele of *Gli3* is restored, more digits develop compared with *Shh* null mice but with syndactyly and loss of their normal identity. This indicates that *Gli3* constrains the number of digits and that different pathogenic mechanisms lead to polydactyly, syndactyly and digit identity (Litingtung *et al.* 2002).

Preaxial polydactyly and syndactyly can be explained by the effect of a *GLI3* mutation on its target genes. Full length GLI3 can act as a transcriptional activator (GLI3A) of target genes and is readily cleaved posttranslationally in a repressor form (GLI3R), which is predominant (Biesecker 1997; Dai *et al.* 1999; Ruiz i Altaba 1999; Shin *et al.* 1999; Litingtung *et al.* 2002). High concentrations of SHH in the posterior limb mesenchyme repress transcription of GLI3A, which lowers subsequently the amount of its cleaved repressor form (Wang and Scott 2000; Litingtung *et al.* 2002). In addition, SHH represses the cleavage of GLI3A resulting in a lower concentration of GLI3R posteriorly. In anterior limb mesenchyme, the SHH concentration is lower than posteriorly and does not repress transcription of GLI3A and its conversion into GLI3R, so there is a high concentration of GLI3R since it is predominant over GLI3A.

This explains the loss of repression of target genes as seen in *Gli3* deficient mice. This results in enhanced *Fgf8* expression in an anteriorly lengthened AER and that could explain preaxial polydactyly. In interdigital mesenchyme of normal mice Gli3 inhibits *Gremlin.* Gremlin binds Bmp's thus preventing Bmp signaling. Bmp's induce interdigital apoptosis, resulting in separation of the digits. Therefore, Gli3 is indirectly involved in interdigital apoptosis in normal mice. In *Gli3* mutants, loss of repression of Bmp antagonist *Gremlin* in interdigital mesenchyme explains inhibition of apoptosis and syndactyly (Aoto *et al.* 2002).

Patients with the autosomal dominant Greig syndrome or Rubinstein-Taybi syndrome (RTS) share some hand anomalies but these occur in different frequencies (MIM 180849, Hennekam *et al.* 1990; Rubinstein 1990). Broad first digits are a rare occurrence in Greig

syndrome patients, whereas preaxial or postaxial polydactyly are rare features in RTS patients. In about 20% of RTS cases, a mutation in the CREB Binding Protein (CBP) has been found (Petrij *et al.* 1995; Petrij *et al.* 2000). GLI3 uses CBP as a coactivator, so CBP deficiency could result in downregulation of GLI3 (Dai *et al.* 1999). If so, preaxial polydactyly could be explained by mesenchymal/AER widening of the anterior limb and upregulation of FGF expression as in *Gli3* deficient mice. If this widening is less outspoken it leads to a broadened digit, which might be considered as a less severe clinical expression of polydactyly. This minor expression of preaxial polydactyly in RTS patients could be explained by the combined effect of enhanced FGF expression due to lower *GLI3* activation and reduced FGF expression by downregulation of TWIST. Downregulation of *Twist* is found in fruit fly mutants *dCbp* (Akimaru *et al.* 1997).
It is striking that the limb skeletal phenotypes in human *GLI3* mutations and in *Cbp* mutant mice mainly concern anomalies of the distal limbs. *GLI3* is one of the earliest genes involved in determination of limb asymmetry along the A/P axis before limb outgrowth. It is one of the molecules that positions and induces *SHH* expression in the ZPA. GLI3 and SHH restrict each other's expression domain along the A/P axis during patterning and initial outgrowth of the limbs. Patients heterozygous for *SHH* mutations have holoprosencephaly (MIM 142945) but normal limbs (Roessler *et al.* 1996).
Shh null mice, however, have a predominant distally shortened proximal limb that is fused to a single severely shortened middle limb without A/P identity. This middle limb is also fused to one digit, with a digit 1 identity (Chiang *et al.* 2001; Kraus *et al.* 2001). This suggests a predominant role for SHH in A/P patterning in distal compared to proximal limbs.

Recently, patients have been described with different limb phenotypes caused by mutations in the Limb region (*LMBR)1* gene, which is located at a distance of about 1 Mb from *SHH* (Lettice *et al.* 2002). Loss of LMBR1 results from mutations in the coding region of this gene. It causes autosomal recessive Acheiropodia (MIM 200500) in which patients have a loss of skeletal elements except the upper arm/leg (Ianakiev *et al.* 2001). This limb phenotype is similar to that of *Shh* null mice. Interestingly, mutations in intron 5 of the *LMBR1* gene cause autosomal dominant triphalangeal thumb polysyndactyly syndrome (MIM 190605; Lettice *et al.* 2003). The *LMBR1* gene contains a regulatory element of the *SHH* gene. The preaxial polydactyly phenotype can be explained by ectopic anterior *SHH* expression. Preaxial polydactyly due to ectopic *Shh* expression has been described before in *Alx4* deficient mice (Litingtung *et al.* 2002; te Welscher *et al.* 2002).
Postaxial polydactyly and syndactyly in patients with autosomal recessive Smith-Lemli-Opitz (SLO) syndrome (MIM 270400) can be explained by inhibition of cholesterol biosynthesis. Patients have a low serum cholesterol and elevated levels of 7-dehydrocholesterol (7-DHC), a precursor in the cholesterol biosynthesis pathway. This is due to mutations in the gene encoding the enzyme 7-dehydrocholesterolreductase (DHCR7) that is involved in the synthesis of cholesterol from 7-DHC (Wassif *et al.* 1998). Cholesterol deficiency is supposed to impair SHH function. It was shown that inhibition of cholesterol biosynthesis in rats at the level of 24-DHC results in a limb phenotype of postaxial polydactyly, syndactyly between digit 1-2 or 2-3 and loss of digit 1 (Gofflot *et al.* 2003). Reduced apoptosis of interdigital mesenchyme and enlargement of the posterior autopod is due to a reduced range of SHH signaling. This results in a lower level of SHH in the anterior limb, which is speculated

to disrupt the FGF-BMP signaling balance. In interdigital mesenchyme loss of *Bmp2* and *Bmp7* expression explains inhibition of apoptosis and syndactyly. It has been postulated that SHH induces apoptosis in the posterior necrotic zone (PNZ) (Sanz-Ezquerro and Tickle 2000; Gofflot *et al.* 2003). If so, disruption of SHH signaling could lead to an increase of posterior limb mesenchyme and AER, explaining postaxial polydactyly (Gofflot *et al.* 2003).

Patients with the autosomal dominant Pelger-Huet syndrome (MIM 169400) have polydactyly and brachydactyly caused by heterozygosity of the gene encoding the lamin B receptor (*LBR*) gene, another cholesterol biosynthetic enzyme. On the other hand patients homozygous for *LBR1* gene mutations have lethal Greenberg skeletal dysplasia (MIM 215140). Polydactyly is a rare occurrence in them and could be explained by reduced SHH signaling. Patients with Greenberg dysplasia have shortening of the upper limbs (rhizomelia) and middle limbs (mesomelia). The proximal long bone shortening in patients with Greenberg dysplasia differs from the distally affected bones in *Shh* null mice that also have loss of identity along the A/P axis, but do not have residual SHH signaling. Patients with Greenberg dysplasia have irregular growth plates with mesenchyme invasion and disorganized cartilage with loss of chondrocyte columns and calcified deposits (Waterham *et al.* 2003). There is also a loss of chondrocytes in reserve cartilage and a delay in ossification in patients with mutations in two other genes encoding the Emopamil-binding protein (EBP) and the NAD(P)H steroid dehydrogenase-like (NSDHL) protein, that are involved in successive steps in cholesterol biosynthesis. Mutations in the *EBP* or *NSDHL* genes can cause either X-linked dominant chondrodysplasia punctata (CDPX2) characterized by rhizomelia (MIM 302960), or congenital hemidysplasia, ichthyosis and limb defects syndrome (MIM 308050) characterized by a unilateral CDPX2 phenotype (Braverman *et al.* 1999; Derry *et al.* 1999; Grange *et al.* 2000; Konig *et al.* 2000). Polydactyly is not a feature in these patients.
An alternative explanation for these phenotypes is the presence of enhanced levels of cholesterol precursors due to enzyme deficiencies that could also affect the limb phenotype. A correlation between the 7-DHC concentration in patients with SLO and the severity of their phenotype has been observed (Witsch-Baumgartner *et al.* 2000; Yu *et al.* 2000a).

Pre-or postaxial polydactyly and syndactyly can be features of patients with autosomal recessive Bloom syndrome (MIM 21900). This phenotype is caused by mutations in the *RECQ3* gene encoding the Bloom protein (Ellis *et al.* 1995). This protein is involved in suppression of the hyperrecombination and participates in unwinding DNA duplexes during DNA replication, DNA recombination and DNA repair (Walpita *et al.* 1999; Brosh *et al.* 2000; Karow *et al.* 2000). It is hypothesized to be a sensor of abnormal double stranded DNA structures during replication (Chakraverty and Hickson 1999; Wang and Scott 2000; Mohaghegh and Hickson 2001).

3.2 Fusion defect: syndactyly

Syndactyly has been mentioned before in combination with other limb anomalies. It is a common feature of several autosomal dominant craniosynostosis syndromes due to mutations in one of the genes encoding fibroblast growth factor receptors (FGFR).

FGFR's are composed of three extracellularly located immunoglobulin (Ig)-like domains, a transmembrane domain and a tyrosine kinase domain (for reviews see Ornitz 2000; Wilkie *et al.* 2002). There are two isoforms of FGFR2 due to alternative splicing of the third Ig-like domain (IgIII). IgIII consists of IgIIIa, IgIIIb and IgIIIc. The FGFR2b isoform contains IgIIIa/IIIb, whereas FGFR2c contains IgIIIa/IIIc. FGFR2b and FGFR2c are differentially expressed in ectoderm and mesenchyme, respectively. Both isoforms are involved in FGF signaling between limb ectoderm and mesenchyme. The ligand binding specificity is (partially) determined by the Ig-like domains with ectodermally expressed FGFR2b mainly binding mesenchymal FGF7 and FGF10, whereas mesenchymally expressed FGFR2c binds ectodermally expressed FGF2 and FGF4.

Patients with Apert syndrome (MIM 101200) show the most severe limb phenotype in the clinically related craniosynostosis syndromes. They have severe osseous syndactyly, symphalangism (phalangeal fusion), broad first digits and synostosis (osseous fusion) between the humerus and radius or ulna. In many cases, this phenotype is caused by mutations in the linker region between IgII and IgIII of FGFR2 (Cohen and Kreiborg 1995; Wilkie *et al.* 1995). Severe syndactyly is explained by enhanced FGF signaling in limb mesoderm (Yu *et al.* 2000b). This is due to loss of ligand binding specificity of the FGFR2 resulting in ectopic ligand dependent activation. Mesenchymal expressed FGFR2c can then be activated by mesenchymal expressed FGF7 and FGF10. This leads to enhanced mesenchymal FGF signaling, which prevents (interdigital) apoptosis and explains syndactyly. Apert syndrome is in rare cases caused by an Alu element insertion upstream or within *FGFR2*. This probably disrupts normal splicing and results in ectopic expression of the isoform FGFR2b in interdigital mesenchyme. FGFR2b can then be activated by mesenchymal FGF7 and FGF10 explaining the syndactyly (Oldridge *et al.* 1999; Wilkie *et al.* 2002).

Patients with Pfeiffer syndrome (MIM 101600) have a variable limb phenotype ranging from broad first digits to broad first digits in combination with variable syndactyly, brachydactyly and synostosis of the skeletal elements of the elbow joints (Cohen 1993; Plomp *et al.* 1998; Robin *et al.* 1998). The phenotype can be caused by splice site mutations in the IgIIIc region of the *FGFR2* gene, resulting in ectopic expression of FGFRb, as seen in the Alu insertion mutation in Apert syndrome (Oldridge *et al.* 1999). Mutations in IgIIIa and in IgIIIc of the *FGFR2* gene lead to a milder Pfeiffer syndrome phenotype. Mutations result in enhanced activation of FGFR2c in interdigital mesenchyme leading to enhanced FGF signaling and syndactyly (Wilkie *et al.* 2002). Pfeiffer syndrome can also be caused by missense mutations in the linker region between IgII and IgIII of the *FGFR1* gene (Muenke *et al.* 1994).

Jackson-Weiss syndrome (MIM 123150) has been featured as a different clinical entity for a long time and includes a variable limb phenotype. The most consistent limb anomalies are the presence of broad toes, mild cutaneous syndactyly between the second and third toe and fusion between metatarsals. In the original kindred described by Jackson *et al.* (1976), it was already noticed that the variability of this phenotype encompasses the whole spectrum of clinically related craniosynostosis syndromes except for Apert syndrome. This observation was later confirmed when a mutation in Jackson-Weiss syndrome patients

was found to be identical as in patients with either Pfeiffer or Crouzon syndrome. These Jackson-Weiss syndrome patients are currently rediagnosed as one of the other craniosynostosis syndromes (Cohen 2001).

FGFR2 and/or *FGFR3* mutations have also been described in Saethre-Chotzen (SCS) syndrome (MIM 101400) (Paznekas *et al.* 1998). The main limb phenotype is cutaneous syndactyly, brachydactyly and broad great toes. Interestingly, mutations causing SCS were first identified in the *TWIST* gene (El Ghouzzi *et al.* 1997; Howard *et al.* 1997; El Ghouzzi *et al.* 2000). Mutations in either the *TWIST, FGFR2* or *FGFR3* genes result in one clinical Saethre-Chotzen phenotype. This confirms a role of TWIST in stimulating FGF signaling (Chen and Behringer 1995; Paznekas *et al.* 1998; Loebel *et al.* 2002; O'Rourke *et al.* 2002; Zuniga *et al.* 2002).

Brachydactyly is present in *Twist* null mice, which show limb shortening at E 10.5 (O'Rourke *et al.* 2002). These mice also show reduced Fgf signaling which is not compatible with inhibition of apoptosis in interdigital mesenchyme, so the pathogenesis of the limb phenotype remains to be understood.

3.3 Combined fusion defects and supernumerary structures: synpolydactyly

The classical phenotype of autosomal dominant synpolydactyly (SPD) (MIM 186000) is syndactyly between digits 3-4 with an extra digit in the syndactylous web (Goodman 2002). Syndactyly of digits 3-5, brachydactyly and postaxial polydactyly can also be present in these patients. In most cases, the phenotype is caused by a polyalanine stretch expansion of 7-10 alanine's in the amino terminal part of the HOXD13 protein (Akarsu *et al.* 1996; Muragaki *et al.* 1996). This polyalanine expansion is caused by an unequal crossover event (Warren 1997). There seems to be a correlation between the size of these expansions and the penetrance and severity of the phenotype (Goodman *et al.* 1997). The classical SPD phenotype cannot be explained by the loss of function of the HOXD13 protein only. Functional haploinsufficiency of HOXD13 due to (micro)deletions/missense mutations in the *HOXD13* gene, results in an atypical form of SPD with a different foot phenotype (Goodman *et al.* 1998; Debeer *et al.* 2002; Goodman 2002). In addition, *Hoxd13* null mice do not show a SPD like phenotype, in contrast to mice homozygous for a loss of the *Hoxd11-Hoxd13* cluster (Dolle *et al.* 1993; Zakany and Duboule 1996). The *spdh* mouse is a mouse homologue of human classical SPD with a polyalanine stretch expansion of the HOXD13 protein (Bruneau *et al.* 2001). These mice have normal expression patterns of 5' *Hoxd* genes, so the mutation does not affect other *Hoxd* genes but more likely other Hox proteins.

Taking these data into account one can postulate that the classical SPD phenotype in humans results from a loss of function of several HOX proteins (Bruneau *et al.* 2001). This might explain an altered expression of various target genes (Albrecht *et al.* 2002).

In interdigital mesenchyme of *spdh* mouse mutants, expression of two members of the apoptosis inducing Bmp family *Bmp2* and *Bmp7* was abnormal and this can explain the loss of apoptosis resulting in syndactyly. In their growth plates, loss of expression of genes

involved in chondrocyte proliferation and differentiation including *Ihh* and *PTHR* can explain the reduced chondrocyte proliferation observed, probably leading to the brachydactyly. The polydactyly remains unexplained but seems SHH independent, as *Shh* expression is normal in *spdh* mutants. In developing joints, several genes including *Gdf5*, normally restricted around the future joint in wild type mice, were not expressed in *spdh* mutant mice and this could account for their joint fusions.

Recently, mutations were reported in the human *HOXD13* homeodomain, resulting in central polydactyly and brachydactyly (Caronia *et al.* 2003; Johnson *et al.* 2003). One of the mutations occurs in a DNA binding domain and alters DNA binding specificity. The phenotype could either be explained by loss of this DNA binding site or by a higher affinity for a new binding site, leading to misregulation of HOXD13 target genes (Johnson *et al.* 2003).

3.4 Deficiencies

3.4.1 Brachydactyly

Brachydactyly (Bd) or shortening of hand bones has been classified by Bell (1951) into five types (A-E) including subtypes and by Fitch (1979) into 11 types on the basis of clinical phenotype. Bell's classification is most commonly used.
In autosomal dominant isolated brachydactyly type A1 (MIM 112500), all hand bones can be shortened resulting in a short normally shaped hand. The most common hand phenotype presents as hypoplasia of the middle phalanges and proximal phalanx of the first digit. Symphalangism can also be part of the phenotype. BdA1 can be caused by dominant mutations in the *IHH* gene (Gao *et al.* 2001; Armour *et al.* 2002; Kirkpatrick *et al.* 2003). Patients homozygous for *IHH* mutations have brachydactyly as part of autosomal recessive acrocapitofemoral dysplasia (MIM 607778). The bone shortening including brachydactyly can be explained by premature epimetaphyseal fusion in hand tubular bones and in proximal femur (Mortier *et al.* 2003). Interestingly, heterozygous parents of these patients do not have BdA1, in contrast to heterozygotes of the dominant form. This suggests that the clinical phenotype is not related to a doses effect of IHH but rather to the type of mutation and its effect on protein structure/function (Hellemans *et al.* 2003).

Ihh null mice show reduced chondrocyte proliferation and accelerated chondrocyte differentiation through stimulation of PTHrP. This results in size reduction of long bones (St-Jacques *et al.* 1999) and can explain brachydactyly. In addition, IHH controls the site where epihyseal chondrocytes differentiate and the site of bone collar formation (Chung *et al.* 2001).
In isolated autosomal dominant brachydactyly type B, the distal phalanges of digits 2-5 are shortened, sometimes seen together with syndactyly/symphalangism (MIM 113000). The phenotype is caused by dominant mutations in the *ROR2* gene (Oldridge *et al.* 2000).

In normal mice, *Ror2* is expressed in chondrocytes of condensing mesenchyme and proliferating chondrocytes of mature growth plates. In mice homozygous for an insertion in *Ror2*, predominant shortening of distal bones is present like in patients with BdB caused by heterozygous *ROR2*

mutations (DeChiara *et al.* 2000). The mutant mice show shortened mesenchyme condensations and abnormal growth plates with a decrease of the proliferating zone and an increase of the hypertrophic zone. Therefore ROR2 must have an analogous role to that of IHH, although in a distinct pathway (DeChiara *et al.* 2000). This can explain brachydactyly by a defect of chondrogenesis in patients with BdB.

Autosomal dominant isolated brachydactyly type C is mainly characterized by shortening of the first metacarpal and middle phalanges of digits 2-4 with relative sparing of the fourth digit (MIM 113100). In addition, hypersegmentation as well as symphalangism can be present. The phenotype is caused by mutations of the Cartilage derived morphogenetic protein *CDMP1* gene (Polinkovsky *et al.* 1997).

CDMP1 transgenic mice with overexpression of human *CDMP1* have thick enlarged skeletal elements (Tsumaki *et al.* 1999). These mice show an increased number of cells in the mesenchymal condensations and a reduction of the proliferative zone due to accelerated differentiation in the primordial cartilage (Tsumaki *et al.* 1999). This is suggestive for a role of CDMP1 in recruitment of mesenchymal cells into condensations and stimulation of chondrocyte differentiation into hypertrophy, resulting in enhanced enchondral ossification and bone growth. This stimulatory role of CDMP1 in limb chondrogenesis explains bone shortening/brachydactyly in C*DMP1* mutant mice. The symphalangism can be explained by the role of CDMP1 in induction of joint formation. When deficient, joint formation does not occur.

Patients homozygous for recessive mutations in the *CDMP1* gene show either of the following three limb phenotypes. Autosomal recessive Du Pan syndrome (MIM 228900) is characterized by complex brachydactyly and fibular shortening. Hunter-Thompson syndrome (MIM 201250) is characterized by a more severe shortening of the hand/foot and forearm/leg (Hunter and Thompson 1976). Grebe syndrome (MIM 200700) is the most severe form with dwarfism caused by extreme shortening of limbs (Kumar *et al.* 1984; Langer *et al.* 1989). All three phenotypes are caused by homozygosity for mutations in the *CDMP1* gene (Thomas *et al.* 1996; Thomas *et al.* 1997; Faiyaz-Ul-Haque *et al.* 2002a; Faiyaz-Ul-Haque *et al.* 2002b). The severe phenotype of patients with Grebe syndrome has been attributed to a dominant negative effect of the mutant CDMP1 which forms dimers with other BMP proteins thereby preventing their secretion (Thomas *et al.* 1997).
However, in other families with Grebe syndrome mutations have been found that result in a loss of CDMP1 (Costa *et al.* 1998; Faiyaz-Ul-Haque *et al.* 2002a). In this instance no dimers can be formed and the severe phenotype must be explained by a dose dependent effect.

The brachydactyly found in patients with a mutation in the *HOXD13* homeodomain resemble clinically the brachydactyly types D and E (Caronia *et al.* 2003; Johnson *et al.* 2003). Brachydactyly type D is characterized by a short distal phalanx of the thumb, whereas one or more metacarpals/metatarsals are shortened in type E.
Patients with brachydactyly as part of the autosomal dominant Hand-Foot-Genital syndrome (MIM 140000) have mutations in *HOXA13* (Mortlock and Innis 1997). The muta-

tions include nonsense mutations, which result in non-functional proteins and include polyalanine expansions (Utsch *et al.* 2002). *Hoxa13* deficient mice have smaller or absent precartilagenous condensations (Stadler *et al.* 2001). This points to an early role of this gene in the formation and growth of mesenchymal condensations, which could explain the brachydactyly. Involvement of the hand is in agreement with the predominant expression of *Hoxa13* in the distal limbs (Nelson *et al.* 1996).
Brachydactyly also occurs as part of syndromes caused by a defect in chondrogenesis or osteogenesis but these will be discussed in sections 3.5.5-3.5.7.

3.4.2 Preaxial deficiency
Preaxial absence or hypoplasia of the radius and thumbs are common features of patients with autosomal recessive Fanconi anemia (FANC) syndrome (MIM 227650; Glanz and Fraser 1982). Sometimes duplication of the thumbs is seen in these patients as well as in patients with other preaxial ray deficiencies, to be mentioned later. Cells of Fanconi anemia patients show an increased sensitivity for chromosome breakage, which can be induced by exposure to an alkylating agent (Glanz and Fraser 1982). There are currently eight different complementation groups FANCA-H. Six Fanconi anemia genes have been identified until now (Strathdee *et al.* 1992; Fanconi anaemia/breast cancer consortium 1996; Foe *et al.* 1996; de Winter *et al.* 1998; de Winter *et al.* 2000a; de Winter *et al.* 2000b; Timmers *et al.* 2001). In addition, biallelic mutations in the breast cancer gene *BRCA2* can also lead to the FANCB and FANCD1 phenotypes (Howlett *et al.* 2002). There is no clear genotype-phenotype correlation (Demuth *et al.* 2000).

In mice the *Fanca* and *Fancc* genes are expressed in undifferentiated chondro- and osteoprogenitor cells of developing long bones, perichondrium, periosteum and diaphysis (Krasnoshtein and Buchwald 1996). Mice with loss of the most prevalent *Fanca* gene have a normal skeletal phenotype (Cheng *et al.* 2000). The role of the *FANC* genes neither in limb formation nor in its pathology is yet understood.

Patients with the autosomal recessive Rothmund-Thomsen syndrome (MIM 268400) have radial ray absence or hypoplasia. Mutations in the *RECQ4* have been found (Kitao *et al.* 1998). This gene encodes a DNA helicase which is an enzyme involved in the maintenance of DNA stability (Kitao *et al.* 1998). Mutations in *RECQ* family members can cause genome instability with a frequent occurrence of DNA deletions, translocations, replication defects resulting in an extended cell cycle and chromosome breaks (Kitao *et al.* 1998; Mohaghegh and Hickson 2001). The pathogenic mechanism of the limb anomalies in Rothmund-Thomsen syndrome patients is currently unknown. However, one wonders whether transient inhibition of the cell cycle as described by Ohsugi *et al.* (1997) in early embryonic chick limb development might explain the radial ray deficiency. Lengthening of the cell cycle was induced by implantation of beads containing an inhibitor of DNA polymerase α. Beads implanted in anterior limb mesenchyme results in absence or size reduction of the radius and preaxial polydactyly. When the cell cycle is lengthened in the posterior limb, *Shh* and *Hoxd13* gene expression is reduced or absent. This could explain loss of preaxial ray elements since reduced SHH levels in anterior limb mesenchyme results in a loss of anterior skeletal elements as shown by Gofflot *et al.* (2003). In the same experi-

ment there is ectopic expression of B*mp2* and *Hoxd11* in anterior mesenchyme as well as ectopic expression of *Fgf4* in anteriorly expanded AER. Earlier, ectopic *Bmp2* expression in anterior chick limb mesenchyme was shown to induce *Fgf4* in anterior AER and *Hoxd11* expression in anterior mesenchyme resulting in preaxial polydactyly (Duprez *et al.* 1996). Lengthening of the cell cycle in experiments on chicken revealed ectopic expression of *Bmp2* and *Hoxd11* as well as preaxial polydactyly (Ohsugi *et al.* 1997). These experiments seem to offer a reasonable pathogenic mechanism.

Predominant preaxial ray defects of the upper limbs only are features in patients with the autosomal dominant Holt-Oram syndrome (MIM 142900). The phenotype is caused by mutations in the *TBX5* gene which is only expressed in the upper limbs (Basson *et al.* 1997; Li *et al.* 1997). *Tbx5* null mice have a Holt-Oram syndrome like phenotype and TBX5 was shown to be necessary for maintenance of *FGF10* expression in mesenchyme and *FGF8* expression in AER (Rallis *et al.* 2003). This explains why *TBX5* mutations result in a loss of skeletal elements.

3.4.3 Postaxial deficiency

Patients with the autosomal dominant Ulnar-Mammary syndrome (UMS) (MIM 181450) have predominant postaxial ray defects. UMS is caused by mutations of the *TBX3* gene (Bamshad *et al.* 1997). Tbx3 is involved in the initiation and/or maintenance of *Shh* expression (Davenport *et al.* 2003). As mentioned before, *Shh* null mice have predominant loss of postaxial autopod structures which explains the postaxial deficiencies, but does not explain the occurrence of postaxial polydactyly (Chiang *et al.* 2001; Kraus *et al.* 2001).

3.4.4 Central deficiency

Split-hand/split-foot malformation (SHFM) is characterized by the absence of central digits of the hand/foot (MIM 183600). SHFM presents either as an isolated malformation, or is combined with syndactyly. Most cases show a dominant pattern of inheritance with reduced or non-penetrance in about one third of the cases (Chiang *et al.* 2001; Kraus *et al.* 2001). Strikingly there is large inter/intra-familial variability in expression of the phenotype. SHFM is a genetically heterogeneous disorder with currently five known loci of which one gene (*p63*) is identified in SHFM type 4.

SHFM is caused by mutations in the gene encoding the transcription factor P63 (Ianakiev *et al.* 2000). P63 stimulates differentiation and maintenance of the AER and other ectodermal derived structures (Mills *et al.* 1999; Yang *et al.* 1999). In the mouse mutant Dactylaplasia, which is currently a model for SHFM, a defect in maintenance of central AER was observed which is in accordance with the absence of central digits (Crackower *et al.* 1998).
Mutations in one of the six isoforms of P63 may also cause syndromes involving malformations of the hands. Two mutations in the DNA binding domain of P63 cause isolated SHFM4 (MIM 6005289) or the Ectrodactyly-Ectodermal dysplasia-Cleft lip/palate (EEC)3 syndrome (MIM 604292). Distinct mutations in the DNA binding domain cause isolated SHFM4 and EEC syndrome (Celli *et al.* 1999; Ianakiev *et al.* 2000; Brunner *et al.* 2002). EEC syndrome can also be caused by a mutation in the P63α isoform. The mutated P63α isoform could exert a dominant negative effect on transactivation of the other P63 iso-

forms, which mechanism is also seen in patients with Limb-Mammary syndrome (LMS) (MIM 603543; van Bokhoven *et al.* 1999; van Bokhoven *et al.* 2001). LMS patients have a variable limb phenotype that includes ectrodactyly and also preaxial polydactyly, brachydactyly and syndactyly. This variable limb phenotype cannot yet be explained. Ectrodactyly as part of the Acro-Dermato-Ungual-Lacrimal-Tooth syndrome (MIM 103285) is caused by mutations in P63 isoforms normally lacking transactivation activity resulting in the novo transactivation (Amiel *et al.* 2001; Duijf *et al.* 2002).

Split-hand/split-foot is a rare feature in patients with the autosomal recessive Robinow syndrome (MIM 268310; Afzal *et al.* 2000; van Bokhoven *et al.* 2000). Robinow syndrome only occurs in homozygotes for *ROR2* gene mutations (Duijf *et al.* 2003).

3.4.5 Chondrodysplasia

Different kinds of *FGFR3* gene mutations cause more general shortening of limb skeletal elements resulting in clinically related forms of dwarfism/chondrodysplasias.
Micromelia or shortening of limb bones is seen in three autosomal dominant phenotypes with different severity of bone shortening: thanatophoric dysplasia (TD) (MIM 187600), achondroplasia (ACH) (MIM 100800) and hypochondroplasia (MIM 146000).

The difference in clinical severity between lethal TD and ACH is mainly explained by experiments in mice. *Fgfr3* mutant mice with a similar mutation as in human ACH show inhibition of chondrocyte proliferation (Chen *et al.* 1999). This is not only due to inhibition of *Ihh*, which normally activates chondrocyte proliferation, but also to activation of inhibitors of chondocyte proliferation (Chen *et al.* 1999). The same was found in *Fgfr3* mutants with a similar mutation as in human TD, but in addition, there is a reduction of expression of *PTHrPR* (Chen *et al.* 2001). This leads to premature chondrocyte differentiation as is also seen in *PTHrPR* loss of function mutant mice.

Another group of patients with a lethal form of dwarfism is those with autosomal recessive Blomstrand chondrodysplasia (MIM 215045). They were found to have inactivating mutations in the human *PTHPR* gene resulting in extremely premature skeletal maturation due to enhanced chondrocyte differentiation (Loshkajian *et al.* 1997; Jobert *et al.* 1998). This results in bone growth impairment and premature fusion of the epiphyses. This supports the idea that in TD patients the severity of clinical features is also explained by a reduced expression of PTHrPR.

Three autosomal recessive chondrodysplasia phenotypes, namely diastrophic dysplasia (MIM 222600), achondrogenesis type 1B (MIM 600972) and atelosteogenesis type 2 (MIM 256050) are caused by mutations in the Diastrophic dysplasia sulphate transporter (*DTDST*) gene (Hastbacka *et al.* 1994; Hastbacka *et al.* 1996; Superti-Furga *et al.* 1996). This gene encodes a transmembrane protein, mainly expressed in cartilage, which is involved in the flux of pericellular sulphate into the chondrocytes. Mutations in *DTDST* results in undersulfation of cell surface heparan sulphate proteoglycans. These together with the glycosaminoglycan heparin are necessary for FGF dimerization and binding of FGF to their receptors (Rapraeger *et al.* 1991; Ornitz *et al.* 1996; Schlessinger *et al.* 2000).

Undersulfation of cell surface heparan sulphate proteoglycans results in impairment of a growth response of chondrocytes to FGF in vitro, which explains growth deficiency of limb bones (Satoh *et al.* 1998).

There is a correlation between the severity of the chondrodysplasia phenotype and the residual activity/function of the DTDST protein (Superti-Furga *et al.* 1996; Rossi and Superti-Furga 2001). The correlation is, however, partial and suggests involvement of additional factors (Karniski 2001).

The clinical phenotypes of patients with mutations in genes encoding two different cell surface heparan sulphate proteoglycans (hspg) are quite different from each other. Patients with mutations in the *HSPG2* gene encoding Perlecan have a severe autosomal recessive lethal chondrodysplasia with short bowed bones named dyssegmental dysplasia, Silverman-Handmaker type (MIM 224410). In contrast, patients with mutations in the gene *GPC3* encoding Glypican have X-linked recessive Simpson-Golabi-Behmel syndrome (MIM 312870) and display overgrowth with tall stature, brachydactyly, postaxial polydactyly and syndactyly (Neri *et al.* 1998).

Limb shortening in *hspg2* null mice is explained by reduction of chondrocyte proliferation with fibrous invasion and ectopic calcification of perichondrium, similar to *Fgfr3* mutant mice (Arikawa-Hirasawa *et al.* 1999; Arikawa-Hirasawa *et al.* 2001). *Fgfr3* expression is upregulated in *hspg2* deficient mice, which suggests a role for Perlecan in regulating *Fgfr3* expression.

Patients with autosomal dominant Campomelic dysplasia (MIM 114290) show bowing of long bones and brachydactyly. It is caused by mutations in the *SOX9* gene (Foster *et al.* 1994; Wagner *et al.* 1994). SOX9 is coexpressed with and activates *COL2A1* in all chondroprogenitor cells, except hypertrophic chondrocytes (Bell *et al.* 1997; Ng *et al.* 1997; Zhao *et al.* 1997; Lefebvre *et al.* 1998).

Mouse *Sox9* null limb mesenchyme cells do not differentiate into chondrocytes (Bi *et al.* 1999; Bi *et al.* 2001; Akiyama *et al.* 2002). Heterozygous *Sox9* mice have small skeletal element precursors, which is supposed to be the result of recruitment of fewer cells into the mesenchyme condensations. When *Sox9* is inactivated after the condensations have differentiated, chondrocyte proliferation was reduced. Also the expression of the genes *Ihh* and *PTHrPR*, which normally play a role in chondrocyte proliferation/differentiation was reduced. This can explain the hand bone shortening in Campomelic dysplasia patients.

Other chondrodysplasias caused by mutations in genes encoding extracellular matrix components include autosomal dominant achondrogenesis type 2 (MIM 200610) and the clinically less severe autosomal dominant Kniest dysplasia (MIM 156550). Both are caused by mutations in the *COL2A1* gene (Winterpacht *et al.* 1993; Ritvaniemi *et al.* 1995). There is no genotype-phenotype correlation (Wilkin *et al.* 1999; Korkko *et al.* 2000). The *Col2a1* gene is the predominant protein of cartilage and is essential for the formation of collagen

fibrils and the normal organization and differentiation of growth plate chondrocytes (Li *et al.* 1995). Collagen type 2 is one of 19 distinct types each composed of three procollagen chains. Mutations in genes encoding several types of collagens have been described in humans and mice showing a spectrum of (osteo)chondrodysplasias indicating that defects of collagen encoding genes affect chondrogenesis and sometimes osteogenesis, which explains bone shortening (Kuivaniemi *et al.* 1997; Wilkin *et al.* 1999).

3.4.6 Osteochondrodysplasia

Autosomal recessive Pycnodysostosis (MIM 265800) is an osteochondrodysplasia characterized by the presence of short stature and brachydactyly. It is caused by mutations in a gene *CTSK* encoding a lysosomal cysteine protease Cathepsin K. This enzyme is abundant in osteoclasts where it is believed to play a vital role in the resorption and remodeling of bone (Gelb *et al.* 1996; Saftig *et al.* 1998). Long bone shortening can be explained by a disturbed metabolism of extracellular matrix, which is important in longitudinal bone growth. Bone growth is mainly dependent on synthesis of new matrix by hypertrophic chondrocytes, but the hypertrophy is dependent on resorption of the rigid cartilaginous matrix, rich in collagens. When this resorption is affected bone growth is impaired.

3.4.7 Osteodysplasia

The autosomal dominant Cleidocranial dysplasia (CCD) phenotype includes brachydactyly and is caused by mutations in the *CBFA1* gene (MIM 119600; Lee *et al.* 1997; Mundlos *et al.* 1997). The *CBFA1* gene is expressed in mesenchyme condensations and cells of the osteoblast lineage (Ducy *et al.* 1997). The CBFA1 protein binds to and induces expression of osteoblast specific genes. This is confirmed by impaired osteoblast development in homozygous *Cbfa1* null mice that have a human CCD like phenotype (Ducy *et al.* 1997; Komori *et al.* 1997; Otto *et al.* 1997). Therefore, brachydactyly in CCD patients can be explained by a defective osteogenesis.

Patients with X-linked dominant Oto-Palato-Digital type 2 syndrome (MIM 304120) exhibit bowing of the long bones with loss of the fibula, brachydactyly and syndactyly. This is caused by mutations in the *FLNA* gene encoding Filamin A, a cytoskeletal protein involved in the organization and cross linking of filamentous actin into bundles (van der Flier and Sonnenberg 2001; Robertson *et al.* 2003). Mutations in this gene cause impairment of periostal ossification with abnormalities of periostal and trabecular bone. Bone shortening resulting from a defect in osteogenesis has been described before but the pathogenesis of syndactyly remains to be explained. However, it could be caused by altered intercellular signaling due to disorganization of extracellular microfibrils. This disorganization might lead to defective bone deposition as supported by experiments with fibrilline 2 deficient mice that have osseous and cutaneous syndactyly (Arteaga-Solis *et al.* 2001; Chaudhry *et al.* 2001).

3.5 Conclusions

As in many other instances studies of abnormal limb development not only give insight in the etiology and pathogenesis of abnormalities but also form an important means to understand normal development. To explain in molecular terms the nearly 2000 clinical entities with limb malformations is an enormous task. In gene linkage studies it is important to deal

with clinically well defined abnormalities which is not easy because of varying combinations of limb deformities and clinical heterogeneity even within families. This overview shows, however, that progress is being made in defining responsible gene defects for a variety of syndromes with polydactyly, syndactyly, combined fusion defects, deficiencies including chondrodysplasias and osteodysplasias. In most instances it concerns limb anomalies with a Mendelian inheritance pattern. Sometimes the genes involved have unanticipated functions like DNA repair in the absence of the radius and thumb. Up to now relatively few genotype-phenotype correlations could be established.
Homology between humans and mouse models has in some cases enabled an explanation of the pathogenesis of human limb anomalies. In the great majority of human gene defects however, very little is known about the nature and function(s) of the proteins involved let alone about the molecular/biochemical pathways that underlie specific abnormalities during the formation of limb structures and tissues. Such knowledge is required to explain inter-and intraindividual variability.

Chapter 4

Introduction to the experimental work

Introduction to the experimental work

Studies on cellular and molecular interactions during limb development in different species contribute to our knowledge about normal and abnormal limb morphogenesis. Several human limb anomalies have been studied in order to unravel their molecular pathogenesis. In most cases these studies start with gene mapping in families with several patients with limb anomalies, without any previous knowledge about the pathogenesis of their congenital defects. In some of these studies the disease causing mutation could be identified.

Isolated human limb anomalies occur more often than limb anomalies as part of a syndrome. In the latter case the phenotype is more severe and has therefore been described more frequently by clinicians. Since familial syndromal cases often follow Mendelian inheritance patterns they have been used in gene mapping studies. Presently many more genes have been identified that cause syndromal limb anomalies of Mendelian inheritance than gene defects causing isolated limb anomalies.
Although the mortality of most patients with limb anomalies is low, the functional limitations can be substantial. The need for information about a (recurrence) risk for offspring with a congenital limb anomaly can be derived from the increasing requests for genetic counseling by relatives or the patients themselves. Precise knowledge of the molecular pathogenesis of abnormal limb development provides new perspectives for genetic counseling. This includes information about recurrence risks, genotype-phenotype correlations and possibilities of carrier detection and prenatal diagnosis.
For this thesis we have studied several families with patients with limb anomalies representing examples of deficiency-, duplication- and fusion/separation defects according to the classification by Stoll *et al.* (1998). They represent patterning/growth defects along the proximal-distal (P/D)- and/or the anterior-posterior (A/P) axis during embryonic limb development. The aim of this thesis was to identify the gene loci involved and their relation to the clinical phenotypes.

We have studied two families with isolated complex brachydactyly type C (BdC) for which no responsible gene locus had been described (Chapter 5). The clinical BdC phenotype is genetically heterogeneous and two loci had been described at the start of our study (Polymeropoulos *et al.* 1996; Polinkovsky *et al.* 1997). Mutations in the *CDMP1* gene on chromosome 20q11.2 had been found in patients with a non-complex BdC phenotype (Polinkovsky *et al.* 1997). A second locus on chromosome 12q24 was associated with complex BdC (Polymeropoulos *et al.* 1996). We investigated whether our families mapped to the *CDMP1* gene or to the other known locus. In view of the high variability of the BdC phenotype in our patients, we hypothesized that molecular diagnostics could contribute to a better understanding of the genotype-phenotype correlation.

We also studied a family with a radial ray defect, consisting of bilateral absence of the radius in the presence of hypoplastic thumbs. This radial ray defect is suggestive for a patterning defect along both the A/P and P/D axis during embryonic limb development. We have investigated whether this patterning defect fits the model proposed by Shubin and Alberch (1986) in which they explain the formation of limb skeletal elements by branching and segmentation processes during limb development. Radial ray deficiencies have an incidence of

about 1/1000 live births in humans (Froster and Baird 1992). X-linked inheritance has only been described in patients with a combination of several congenital malformations classified as VACTERL-H association (MIM 314390, MIM 312190). We considered our patients to have a new entity and hypothesized that the radial ray defect is caused by a mutation in an X-linked recessive gene (Chapter 6).

Postaxial polydactyly (PAP) (MIM 174200) is probably a result of a patterning defect along the A/P limb axis during embryonic limb development. Clinically, it is divided into PAP-A with an extra fifth digit and PAP-B in which this extra digit is rudimentary (Temtamy and McKusick 1978). PAP is genetically heterogeneous with four loci including one containing the *GLI3* gene. As mentioned before mutations in *GLI3* can also cause a phenotype with PAP-A in one hand and PAP-B in the other (PAP-A/B) (Radhakrishna *et al.* 1997a). We studied a family with patients with all three clinical phenotypes. Such a family had never been subject of a genetic study. Since many patients with PAP have cutaneous syndactyly (Castilla *et al.* 1998) we also investigated whether syndactyly and PAP are caused by the same gene (Chapter 7).

Finally we studied an interesting nuclear family consisting of a female patient with PAP-B, minor facial dysmorphisms and mental retardation who carries a cytogenetic balanced translocation (4;7)(p15.2)(q35). Strikingly we discovered that her father has PAP-B and shows mosaicism for this translocation in blood lymphocytes and skin fibroblasts. We hypothesized that the chromosome translocation causes the PAP-B phenotype and assumed that the absence of additional anomalies and mental retardation in the father can be explained by the presence of normal cells in for example his brain. The chromosomal regions containing the breakpoints had never been implicated in the PAP phenotype and we therefore focused on narrowing down the breakpoints to identify the disrupted genes (Chapter 8).

Chapter 5

Differences in complexity of isolated brachydactyly type C cannot be attributed to locus heterogeneity alone

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Differences in Complexity of Isolated Brachydactyly Type C Cannot Be Attributed to Locus Heterogeneity Alone

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Hereditary isolated brachydactyly type C (OMIM 113100) mostly follows an autosomal dominant pattern of inheritance with a marked variability in expression. This phenotype has been mapped to two different loci on chromosomes 12q24 and 20q11.2. The latter locus contains the cartilage-derived morphogenetic protein (*CDMP)1* gene, in which a null mutation has been found in patients with malformations restricted to the upper limbs. A more complex brachydactyly type C phenotype has been mapped to chromosome 12q24. Differences in complexity of these phenotypes have been attributed to locus heterogeneity. Clinical subclassification based on the degree of complexity of the phenotype has therefore been suggested. We present patients with a complex brachydactyly type C phenotype in whom there is considerable intra- and interfamilial variability in expression. We show that clinical subclassification based on the complexity of the brachydactyly type C phenotype related to the genetic defect is not feasible. We present evidence that differences in complexity are not only due to locus heterogeneity, but that genetic modifiers and/or environmental factors must also play a role.

KEY WORDS: Brachydactyly type C; clinical heterogeneity; CDMP1 mutation; genetic heterogeneity

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INTRODUCTION

Inherited isolated brachydactylies (shortening of digits) were classified by Bell [1951] into five different types (A–E), including three subtypes (A1–A3). This classification is based on anatomical observations. Fitch [1979] extended this classification, also taking into account the complexity of the phenotype of patients with brachydactylies.

Brachydactyly type C (OMIM 113100) is distinguished from the other brachydactyly types by: the presence of short middle phalanges with relative sparing of the fourth phalanx; the usual presence of a shortened first metacarpal; and hypersegmentation of a digit, mostly involving the proximal phalanges of the second and third digits [Bell, 1951; Fitch, 1979; Wood, 1988]. The isolated brachydactyly type C phenotype mostly follows an autosomal dominant mode of inheritance [Bell, 1951; Haws, 1963], with marked variability in expression [Haws, 1963; Sanz and Gilgenkrantz, 1988; Camera et al., 1994] and penetrance [Haws, 1963; Sanz and Gilgenkrantz, 1988].

Locus heterogeneity has been described. Brachydactyly type C with anomalies of the upper and lower limbs [Haws, 1963] was mapped to chromosome 12q24 [Polymeropoulos et al., 1996]. Brachydactyly type C with anomalies restricted to the upper limbs [Robin et al., 1997] was mapped to chromosome 20q11.2 [Lin et al., 1996]. A disease-causing mutation was found in this family in the cartilage-derived morphogenetic protein (*CDMP)1* gene, which belongs to the transforming growth factor (TGF)-β superfamily [Polinkovsky et al., 1997]. It has been suggested that the interfamilial variability of brachydactyly type C could be explained by this locus heterogeneity [Robin et al., 1997; OMIM].

In this study we describe two new families with brachydactyly type C. We used these families for haplotype and mutation analysis. We detected a new mutation causing brachydactyly type C in one family, with involvement of both the upper and lower limbs,

clearly showing that differences in complexity cannot be attributed to locus heterogeneity alone.

SUBJECTS AND METHODS

Patients

The index patient of the first family, named hereafter family 1, was seen for short hands. An interview and physical examination was done of 13 available family members, shown in the pedigree diagram in which the order of birth was changed for the sake of privacy (Fig. 1). Posterioranterior (PA) radiographs of the hands and feet were made of a subset of these individuals. PA radiographs of the hands were made from all cases except II_7, III_{10}, and III_{11}. Radiographs of the feet were made of the individuals last mentioned, except for cases I_2, II_2, and II_5. The length of the 19 tubular bones of the hand was measured. The results were compared to normative data and graphically presented as a Z-score metacarpophalangeal pattern (MCPP) profile [Garn et al., 1972; Poznanski, 1984].

The index patient of the second family, named hereafter as family 2, was seen for further evaluation of a Mallet finger. A pedigree of seven generations was constructed (Fig. 3). Fifteen individuals, thirteen of whom are affected with brachydactyly, were evaluated by interview, physical examination, and PA radiographs of the hands. The length of the 19 tubular bones of the left hand were measured, and all were given a Z-score MCPP.

Molecular Studies

Blood was obtained with informed consent from thirteen members of family 1, nine of whom are affected with brachydactyly type C; and from twenty-seven members of family 2, sixteen of whom are affected. Genomic DNA was isolated as described before [Miller et al., 1988]. For genotyping individuals of family 1, the following five microsatellite markers, located on chromosome 12q24, were chosen with the order and distances in cM according to the CLHC) database: D12S342-6.7-D12S2078-12.6-D12S97-0.6-D12S1045-8.5-D12S392. Twenty-three members of family 2 were genotyped using the microsatellite markers D12S97 and D12S1045. For chromosome 20q11.2, the following markers were selected with the order and distances according to Généthon: D20S112-10.9-D20S106-30.9-D20S120. Analysis of polymorphisms was performed essentially as described by Weber and May [1989]. PCR conditions were: 10 min denaturation at 94°C, followed by 25 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 90 sec, and final extension at 72°C for 5 min. PCR products were separated on denaturing polyacrylamide gels. Alleles were visualized by autoradiography.

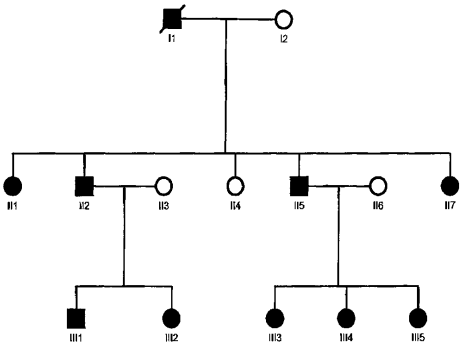

Fig. 1. Pedigree diagram of family 1.

For mutation analysis, the sequence of the *CDMP1* gene (also named the growth/differentiation factor (*GDF*)*5* gene) was obtained from Hötten et al. [1994] and Genbank X80915. Four sets of primers were used to amplify the ORF and the intron-exon boundaries. The reverse primers for amplification of the second part of exons 1 and 2 were obtained from Polinkovsky et al. [1997]. The forward primer for amplification of the first part of exon 2 was obtained from Thomas et al. [1997]. The other primers were based on published sequences. Set 1, forward primer: GCTGCTGCCGCTGTTCT-CTTTGGTGTCATTCAGC; reverse primer: GGTGCC-TTGCCTCCGGGAAGCTGTCCT. Set 2, forward primer: CTGAACCCAAGCCAGGACA. Set 3, reverse primer: ACAGGTACTCATACACGG. Set 4, forward primer: AGAAGGCCCTGTTC CTGGTG. PCR products from genomic DNA were analyzed by automated sequencing (ABI Prism 377 DNA sequencer, Perkins-Elmer, Foster City, CA), using big dye chemistry.

Allele-specific oligohybridization (ASO) was used on all members of family 1 and on 100 control chromosomes (25 normal Dutch males and 25 normal Dutch females from the general population). The mutation found in family 2 creates a novel Bgl I restriction site. The first part of exon 1 of the *CDMP1* gene was amplified by PCR, using primerset 1, using genomic DNA from 27 members of family 2 and from 100 control chromosomes. Bgl I was added to the purified PCR products, and the digested products were visualized by ethidiumbromide staining on a 2% agarose gel.

RESULTS

Patients

Family 1. The medical history of the index patient (number III_5 in the pedigree diagram, Fig. 1, age 10 at the time of evaluation) is unremarkable, except for congenital luxation of the left hip and postaxial polydactyly of her right foot. Results of physical examination are normal except for short hands (Fig. 2, left upper photo) and feet. Radiographs of her right hand show short proximal phalanges (PPh) I–III; middle phalanges (MPh) II–V, with relative sparing of IV; and of PPh II, III, and MPh II-V of her left hand (Fig. 2, left middle photo). Pseudoepiphyses of metacarpal (MC) I, II, and PPh IV, and V were also noted. Radiographs of her feet show short middle phalanges of digits II–V and short distal phalanges (Fig. 2, left lower photo).

Her younger sister (number III_3 in the pedigree diagram, Fig. 1), originally considered to be unaffected,

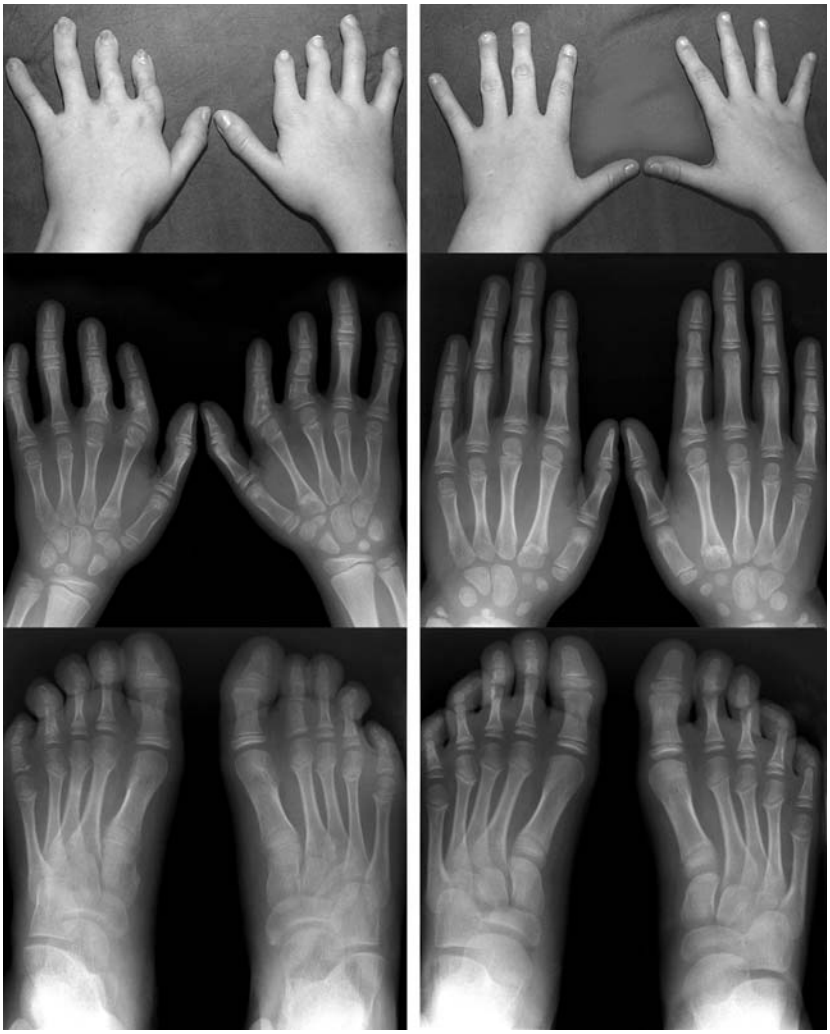

Fig. 2. Left upper photo: hands of the index patient (III_5 in the pedigree diagram) of family 1, showing short digits with relative sparing of the fourth digits. Right upper photo: hands of patient III_4 of family 1, showing relatively short thumbs. Left middle photo: radiograph of the hands of patient III_5 of family 1. (MC, metacarpal; PPh, proximal phalanx; MPh, middle phalanx.) Right hand: short PPh I–III, MPh II–V with relative sparing of MPh IV. Left hand: short PPh II, III, MPh II–V. Pseudoepiphyses of MC I, II, PPh IV,V. Right middle photo: radiograph of the hands of patient III_4 of family 1. Short MC I. Pseudoepihyses of MC I, II, PPh III, IV. Left lower photo: radiograph of the feet of patient III_5 of family 1. Short middle (II–V) and distal phalanges. Right lower photo: radiograph of the feet of patient III_4 of family 1. Short middle phalanges.

was noted to have relatively short thumbs (Fig. 2, right upper photo) due to a short MC I, a slightly short MC V, and MPh II on a MCPP plot. There are pseudoepiphyses of MC I, II, and PPh III, IV (Fig. 2, right middle photo). Radiographs of her feet show short middle phalanges (Fig. 2, right lower photo). On earlier radiographs, bilateral non-osseous postaxial polydactyly was seen.

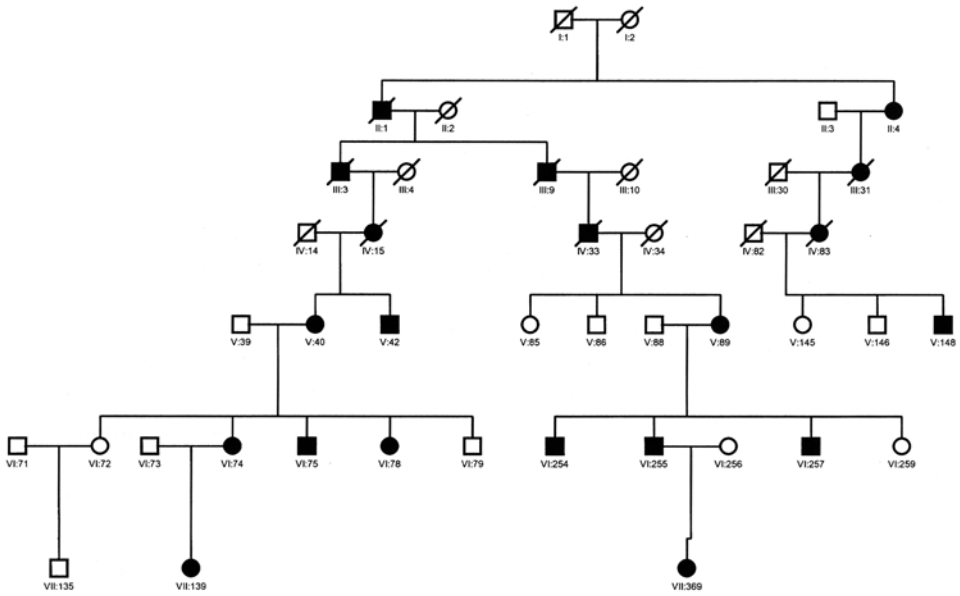

Fig. 3. Pedigree diagram of family 2.

Short hands and feet are present in the other affected members of this family who have been examined.

Family 2. The index patient (VI-74 in the pedigree diagram, Fig. 3) has no congenital malformations except for short hands. Radiographs of her hands show short MC I–IV, MPh II, III, and PPh III, as well as hypersegmentation of PPh II and III (Fig. 5, Table I).

The results of measurements of the 19 tubular bones of the left hand of this patient and other examined affected members of this family are given in Table I.

Haplotype Analysis

Haplotype analysis shows segregation of the disease phenotype with markers on chromosome 20q11.2, but not on chromosome 12q24, in both families.

Mutation Analysis

Subsequent mutation analysis by sequencing showed a deletion of a single base (ΔC493) in exon 1 of the *CDMP1* gene (Fig. 4) in all patients of family 1. The mutation was found in all patients, but not in 100 normal control chromosomes. The mutation results in a frameshift and leads to a premature stop codon. This is expected to result in a truncated protein. This mutation has not been described before. Another mutation ΔG121 mutation was found in exon 1 of the patients' *CDMP1* gene (Fig. 4), but not in unaffected individuals of family 2. The mutation was found in all patients, but not in 100 normal chromosomes. It also results in a frameshift, a premature stop codon, and presumably a truncated protein as described before.

DISCUSSION

Considerable variation in expression of the brachydactyly type C phenotype is clearly present in the families, and has also been described previously in the literature [Haws, 1963; Sanz and Gilgenkrantz, 1988; Camera et al., 1994]. We do not find that one particular middle phalanx is consistently the shortest one in our patients (Table I). This agrees with the studies of Herrmann [1974] and Walbaum [1983], which give conflicting data concerning the most severely affected middle phalanx. Therefore, we also do not consider it feasible to subtype brachydactyly type C based on clinical criteria alone.

Locus heterogeneity has been proposed to explain the differences in complexity of brachydactyly type C [Robin et al., 1997]. The theory is that there could be a very restricted time in embryonic development during which a *CDMP1* gene defect might cause malformations of only the upper limbs, since the upper limbs develop one to two days prior to the lower limbs [Robin

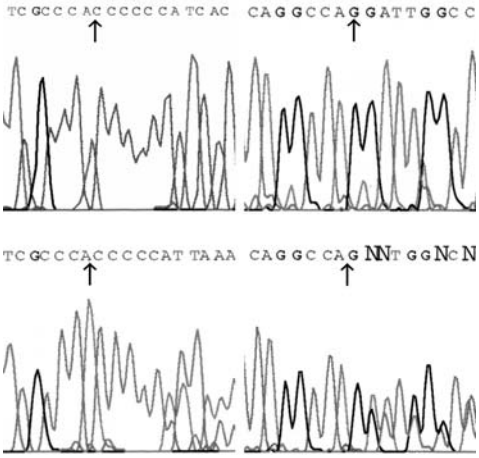

Fig. 4. Left upper photo: normal sequence of exon 1 of the *CDMP1* gene around base 493, indicated by an arrow. Left lower photo: single base (ΔC493) deletion, indicated by an arrow, in exon 1 of the *CDMP1* gene in a patient of family 1. The sequences of the two alleles that are now out of frame are visible following the deletion. Right upper photo: normal sequence of exon 1 of the *CDMP1* gene around base 121 which is indicated by an arrow. Right lower photo: single base ΔG121) deletion, indicated by an arrow, in exon 1 of the *CDMP1* gene in a patient of family 2. The sequences of the two alleles that are now out of frame are visible following the deletion.

et al., 1997]. We reject this hypothesis because we have found that null mutations in this gene can cause malformations of both the upper and lower limbs. Moreover, a restricted time frame does not seem feasible, since the *CDMP1* gene is involved in growth [Nakamura et al., 1964; Chang et al., 1994; Storm et al., 1994], and in patterning of the bones of the limbs [Storm and Kingsley, 1996], and because it is expressed in precartilage condensations, in cartilaginous tissues pre-and postnatally, and in hypertrophic chondrocytes [Chang et al., 1994]. We show that patients whose upper and lower limbs are affected by complex brachydactyly have a disease-causing mutation in the *CDMP1* gene. Therefore, we conclude that the degree of complexity of brachydactyly type C cannot be explained by locus heterogeneity alone.

We attribute differences in complexity of the brachydactyly type C phenotype to variation in expression. The phenotypic variation between the families in our study versus those in Robin's families is most likely not caused by locus heterogeneity, but by genetic modifiers and/or environmental factors.

ACKNOWLEDGMENTS

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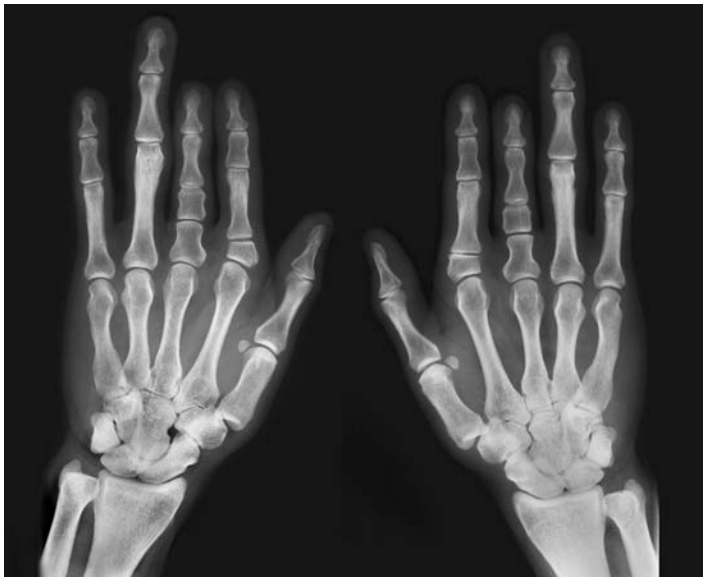

Fig. 5. Radiograph of the hands of the index patient (VI:74) of family 2. Short MC I–IV, MPh II,III, PPh III, and hypersegmentation of PPh II and III.

TABLE I. Measurements of the 19 Tabular Bones of the Left Hand of 13 Patients of Family 2, Compared With Normative Data*

			Sign																	
Patnr.	Sex	Age	MC I	MC II	MC III	MC IV	MC V	PPh I	PPh II	PPh III	PPh IV	PPh V	MPh II	MPh III	MPh IV	MPh V	DPh I	DPh II	DPh III	DPh IV	DPh V
V: 40	F	52,1 yr	−2.9	−1.3	−0.9	−0.3	0.1	0.3	0.3	−6.9	1.9	2.4	−4.9	−11.2	−0.4	−1.0	−0.3	1.4	2.2	1.5	1.3
V: 42	M	72,4 yr	−2.9	−2.3	−2.1	−2.5	−3.0	−2.0	−0.9	0.2	0.1	0.1	−11.3	−12.5	−3.4	−7.5	−3.0	−1.3	−1.3	*	−1.8
VI: 74	F	47,2 yr	−5.0	−2.7	−3.1	−3.3	−1.9	−0.7	−0.7	−6.1	2.4	2.0	−6.2	−5.5	−0.8	−1.5	−1.3	−0.6	−1.2	−1.6	−0.8
VI: 75	M	46,3 yr	−5.9	−2.2	−2.1	−0.8	−1.2	−0.8	−1.7	−8.3	0.9	0.6	−11.1	−11.9	−2.9	−7.6	−0.5	1.2	1.4	0.9	−0.8
VI: 78	F	43,0 yr	−2.0	−1.6	−1.5	−0.4	0.3	−0.2	−2.7	3.6	2.5	1.0	−0.3	−9.7	−8.5	−5.8	−0.6	0.4	0.7	0.4	0.5
VII: 139	F	7,7 yr	−4.1	−1.3	−1.6	−3.4	−2.5	0.7	−1.0	0.4	0.9	0.4	−6.4	−3.1	−1.2	−1.2	0.1	−0.3	−0.4	−0.3	0.2
VII: 141	F	12,8 yr	−2.3	−1.4	−1.8	−1.1	−2.3	−1.0	−0.5	−0.1	−0.8	−1.8	−2.2	−1.6	−1.3	−4.0	−0.9	−0.1	−0.4	−0.3	−1.5
V:89	F	65,1 yr	−4.9	−1.4	−2.4	−0.8	−1.7	−2.3	−2.7	−9.3	0.0	−0.2	−11.4	−11.4	−10.4	−6.4	−1.1	−0.3	−0.1	0.2	0.0
VI:254	M	41,7 yr	−4.1	−1.3	−1.8	−1.7	−2.1	−2.4	−1.6	−3.0	−2.5	−2.0	−13.3	−12.4	−13.5	−10.1	−1.3	0.1	−0.9	−1.2	−1.3
VI:257	M	36,0 yr	−3.8	−1.8	−2.7	−2.7	−2.4	−1.7	−12.0	−5.0	−9.7	−2.3	−4.4	−9.8	−12.7	−9.0	−1.9	0.0	−1.2	−1.3	−0.7
VI:260	M	31,5 yr	−1.4	0.1	0.6	1.3	0.0	−0.8	−0.2	−1.2	0.7	−0.4	−10.7	−7.8	−4.6	−8.5	−1.1	0.5	0.0	0.2	0.0
V:143	F	74,3 yr	−1.1	−0.2	−0.8	0.1	0.1	0.8	0.9	1.0	1.8	2.0	−2.6	−4.3	−1.4	−2.6	1.4	1.5	0.9	1.1	2.0
V:148	M	66,5 yr	−3.2	−2.2	−1.7	−1.8	−2.1	1.7	0.6	0.3	0.7	1.1	*	−13.2	−4.4	−6.9	1.9	3.1	0.9	−2.6	1.3

*When shortening exceeds 2 standard deviations, the result of the measurement is depicted in gray.

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Chapter 6

X-linked recessive inheritance of radial ray deficiencies in a family with four affected males

Galjaard RJH, Kostakoglu N, Hoogeboom AJM, Breedveld GJ, van der Linde HC, Hovius SER, Oostra BA, Sandkuijl LA, Akarsu AN, Heutink P. Eur J Hum Genet (2001) 9, 653-658

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ARTICLE

X-linked recessive inheritance of radial ray deficiencies in a family with four affected males

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Radial ray deficiencies are frequently associated with additional clinical anomalies and have a heterogeneous aetiology. X-linked forms are extremely rare. We report a family in which four male relatives show bilateral absence of the radius with presence of the thumbs and associated anomalies. The segregation of the phenotype is suggestive for X-linked recessive inheritance. This is confirmed by performing linkage analysis using 24 markers spanning the X chromosome in which a maximum lod score of 1.93 for DXS8067 and DXS1001 is obtained. We defined a critical region of maximal 16.2 cM on the X chromosome with haplotype analysis. *European Journal of Human Genetics* (2001) **9,** 653–658.

Keywords: radial ray deficiency; X-linked recessive; linkage mapping

Introduction

Radial ray deficiencies have an incidence of about 1 : 10 000 live births.[1] They occur either isolated or associated with additional clinical anomalies.[1,2] Radial ray deficiencies are clinically and genetically very heterogeneous. Many cases are sporadic and considered to be multifactorial in origin. Although radial ray deficiencies are more commonly present in males than in females,[1] X-linked inheritance has only been described for a few cases. Four families with possibly X-linked VACTERL-H (OMIM 314390) have been published.[3,4] Furthermore, a phenotype of radial aplasia and associated anomalies in a male and his maternal uncle (OMIM 312190), suggestive of X-linked recessive inheritance has been described.[5]

We present a family in which four male relatives have total absence of the radii, presence of the thumbs, and several variable associated anomalies. Since all affected individuals are male, and no male to male transmission was observed, an X-linked recessive mode of inheritance was assumed. We tested 24 microsatellite markers on the X chromosome, performed linkage analysis, and constructed haplotypes. The results of these studies indicate that the phenotype of our patients is linked to Xq24-25.

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Subjects and methods

Patients

There are no consanguineous marriages in this family of Turkish origin. Physical examination of the index patient (III-5, Figure 1) and his parents was done several times in the Netherlands. Patient KM (III-1, Figure 1) and his parents, patient AS and one of his unaffected brothers (II-6 and II-5, Figure 1), were examined in Turkey. Information of the remaining family members was obtained by family history. Photographs were examined of the unaffected sib(s) of patients OS and KM (III-1,2,4, Figure 1) and the other unaffected brother of patient AS (II-7, Figure 1).

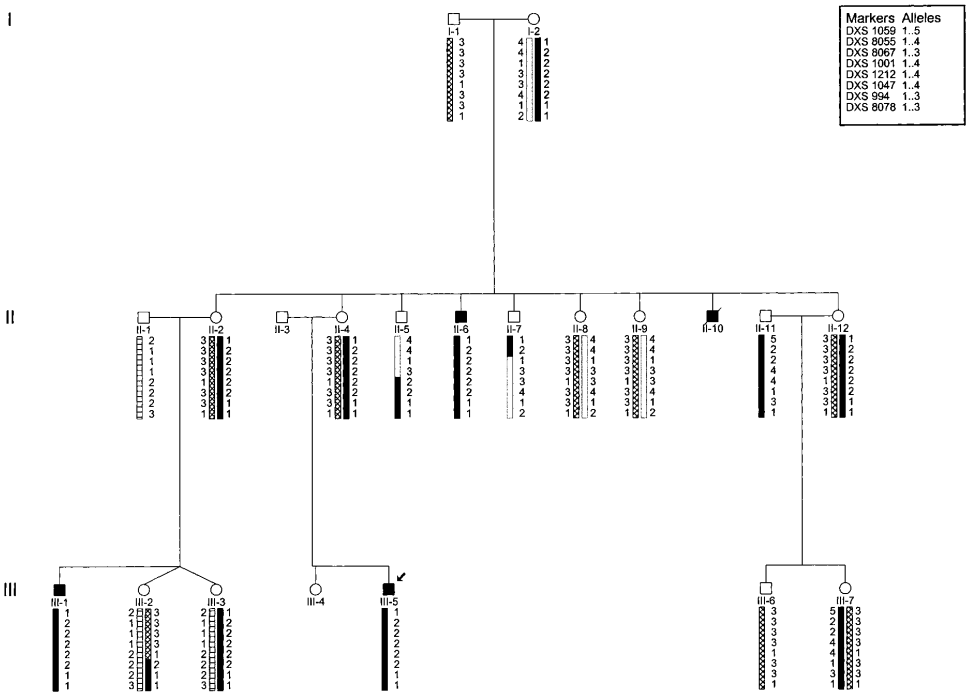

Figure 1 Pedigree diagram of the family. The markers shown are restricted to those between DXS1059 and DXS8078. Distances between markers in cM: DXS1059-2.71-DXS8055-4.9-DXS8067-0-DXS1001-0-DXS1212-6.26-DXS1047-0.77-DXS994-0-DXS8078. The three patients (II-6, III-1, III-5 pedigree diagram) share an identical haplotype telomeric from DXS990. Two unaffected males (II-5, II-7 pedigree diagram) have also part of this haplotype except for the region between DXS8055 and DXS1212.

Cytogenetic studies

GTG -banded metaphases from blood lymphocytes were used for karyotyping the index patient. Skin fibroblasts were tested for chromosome breakage after exposure to diepoxybutane (DEB).

Molecular studies

Blood was obtained from 18 family members after informed consent and genomic DNA was isolated as described before.[6] We tested 24 microsatellite markers; 20 from Généthon,[7] DXS451 and DXS538 from Reed *et al*,[8] and DXS7132 and DXS6800 from the integrated genetic map provided by the Center of Medical Genetics, Marshfield, USA (htpp://www.marshmed.org). We choose this map because the distances between most markers, except DXS451, are well defined. For DXS451 the relative position was estimated according to published data.[8] Analysis of polymorphic markers was performed essentially as described before.[9] PCR conditions were: 10 min denaturation at 94°C followed by 25 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 90 s, with a final extension at 72°C for 5 min. PCR products were separated on denaturing polyacrylamide gels. Alleles were visualised by autoradiograpy.

Mutation analysis

Three sets of primers (available upon request) were designed from genomic sequence obtained from Genbank (Accession no X98253) to amplify the coding region and intron–exon boundaries of the ZNF 183 gene. PCR products from genomic DNA of all the patients and normal (family) members were sequenced on an automated sequencer (Perkin Elmer's ABI Prism 377 DNA sequencer, Foster City, USA) using big dye chemistry. For the IAP 3 gene mRNA sequence was obtained from Genbank (U45880) to design eight sets of primers (available upon request) for amplification and sequencing of cDNA according to standard procedures.

Linkage data

Two point linkage analysis using the LINKAGE program, version 5.01,[10] was performed on all markers tested except for

DXS451, and DXS538, DXS1068, DXS7132, DXS8064 which were not informative in this family. X-linked recessive inheritance of radial anomalies was assumed with full penetrance in males, no anomalies in female carriers, and a gene frequency of 1 : 1000. Multipoint linkage analysis was performed by subsequent four-point analyses on these markers.

Results

Patients

The abnormalities noticed in the four patients are summarised in Table 1. The index patient OS (III-5, Figure 1) was born after an uncomplicated pregnancy and delivery. At birth his weight was 3200 g (10th centile), his length 50 cm (50th centile) and his head circumference (HC) 35 cm (50th centile). The following congenital malformations were noticed: Upper extremities: bilateral short curved forearms, radial deviation with an extension deficit of 90° of the wrists, and short thumbs. Lower extremities: genu varum of the right leg and slight subluxation of the right knee. Radiographs of the upper extremities showed absence of the radii, broad curved ulnae and hypoplasia of the thumbs. Radiographs of the skeleton except for the extremities showed no abnormalities. A Dandy-Walker malformation was seen on a CT-scan. Transposition of the great arteries (TGA), and an atrial septal defect (ASD) type 2 were seen on ultrasound. Ultrasound examination of the abdomen showed no abnormalities. Thrombocytopenia was excluded repeatedly, also during the first year of life. Other hematological disorders were also excluded on routine hematologic laboratory tests. At the age of 2 years and 7 months his mental development was normal. His weight was 11 kg (3rd centile), his length was 86 cm (10th centile), and his HC was 47 cm (3rd centile). His left arm and legs are shown in Figure 2.

Patient KM (III-1, Figure 1) is a 7-year-old male with short stature. He has the same anomalies of the upper extremities as OS and an extension deficit of the 2nd to 4th metacarpophalangeal joints of boths hands (Figure 2). His lower extremities show abducted hip joints, postnatally developed contractures of both knees with popliteal pterygiae (Figure 2), and bilateral absence of the patellae (Table 1). There is no history of a bleeding disorder. Standard laboratory tests showed normal results. Cardiac evaluation did not reveal an abnormality.

Patient AS (II-6, Figure 1) is a 28-year-old male with short stature. He shares the anomalies of the upper extremities with OS. In addition he has hypoplastic middle and distal phalanges of the 2nd to 4th fingers of his left hand. The middle and distal phalanges of the index finger of his right hand are also hypoplastic. All his fingers show an extension deficit. He has genu varum of the right leg, but he has also hypoplasia of the 3rd to 5th toes and the 4th and 5th toes of his left and right foot, respectively. In the past thrombocytopenia was excluded. Cardiac ultrasound revealed a 3rd degree aortic stenosis attributed to rheumatic fever in the

Table 1 Summary of anomalies in patients of present paper

Anomalies	*OS*	*KM*	*AS*	*4th Pat*	*Total*
Short stature	−	+	+	?	2/3
Absent radii	+	+	+	+	4/4
Hypoplastic thumbs	+	+	−	?	2/3
Hypoplastic fingers	−	−	+	?	1/3
Contracture fingers	+	+	+	?	3/3
Hypoplastic toes	−	−	+	?	1/3
Genu varum knee	+	−	+	?	2/3
Contractures knees	−	+	−	?	1/3
Absent patellae	−	+	−	?	1/3
Cardiac anomaly	+	−	+	?	2/3
Dandy-Walker malf.	+	−	−	?	1/3

+ indicates presence; − indicates absence of anomaly; ? indicates not known.

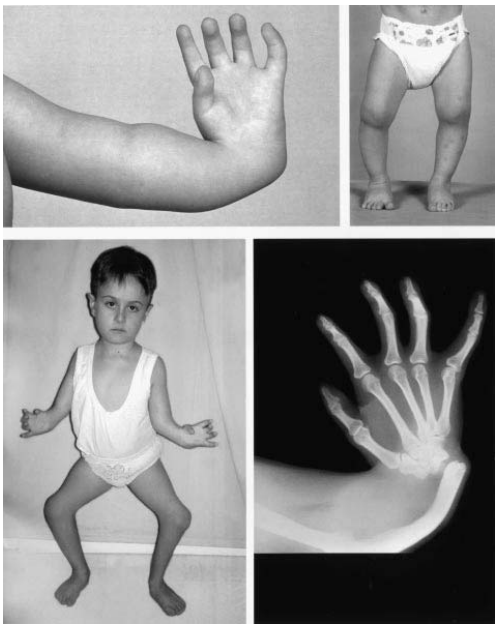

Figure 2 Extremities of the patients OS and KM (III-1, III-5 pedigree diagram, resp.). Left upper photo: Short curved left forearm with radial deviation and an extension deficit of the wrist, and hypoplastic thumb of patient OS. Right upper photo: Genu varum of the right leg of patient OS. Left lower photo: Bilateral short curved forearms, extension deficit of the elbows and 2nd to 4th MCP joints of the hands, hypoplastic thumbs, abducted hip joints, and contractures of the knees with pterigiae poplitiae of patient KM. Right lower photo: Absent radius, short curved ulna and hypoplastic thumb.

past. Ultrasound of the abdomen did not show anomalies. The fourth patient (II-10, Figure 1) had bilateral short curved forearms with an extension deficit of his wrists. He did not have associated anomalies. He died of an infectious disease about 2-3 months after birth. All the other relatives of the male patients are healthy. A radiograph of the forearm of the mother of our index patient did not show an abnormality.

Cytogenetic studies

The index patient has a normal male karyotype. No spontaneous or induced breakage of chromosomes was noticed after DEB exposure.

Molecular studies

Twenty-four markers spanning the X chromosome at regular intervals were tested. With two point linkage analysis a maximum lod score of 1.93 for DXS8067 and DXS1001 at zero recombination frequency was obtained. This result is confirmed by multipoint analysis (Figure 3).

Haplotypes were constructed for the entire chromosome. The patients share an identical haplotype from DXS990 to DXS1193. A double recombinant would be present for individual II-5 in case DXS1212 is positioned centromeric of DXS1001 as indicated by the Marshfield data. Our data indicate that DXS1212 is located telomeric of DXS1001. This is in accordance with data from Généthon and Nagaraja *et al*.[7,11] Therefore, we choose the order DXS8067, DXS1001, and DXS1212 (Figure 1). The unaffected males II-5, and II-7 share part of the haplotype of the patients except for the region between DXS8055 and DXS1212 (Figure 1). The results of the linkage and haplotype analysis show the responsible gene defect must be localised between these two markers. According to the genetic map of the X chromosome of Marshfield, this interval is about 4.9 cM. According the genetic map of Généthon and that of Nagaraja *et al*,[7,11] this interval is about 16.2 cM. We performed mutation analysis for two candidate genes in this interval; the ZNF183 and IAP 3 genes and did not find a disease causing mutation in these genes.

Discussion

We report a family with a radial ray deficiency, presence of thumbs and associated anomalies. In our view, our patients have a previously unreported phenotype. Our differential diagnosis included autosomal recessive TAR syndrome, Fanconi anaemia (FA), X-linked recessive VACTERL-H syndrome, and a phenotype described by Gibson *et al*.[5] TAR syndrome can be rejected when thrombocytopenia is excluded.[12] FA is an unlikely diagnosis in the absence of

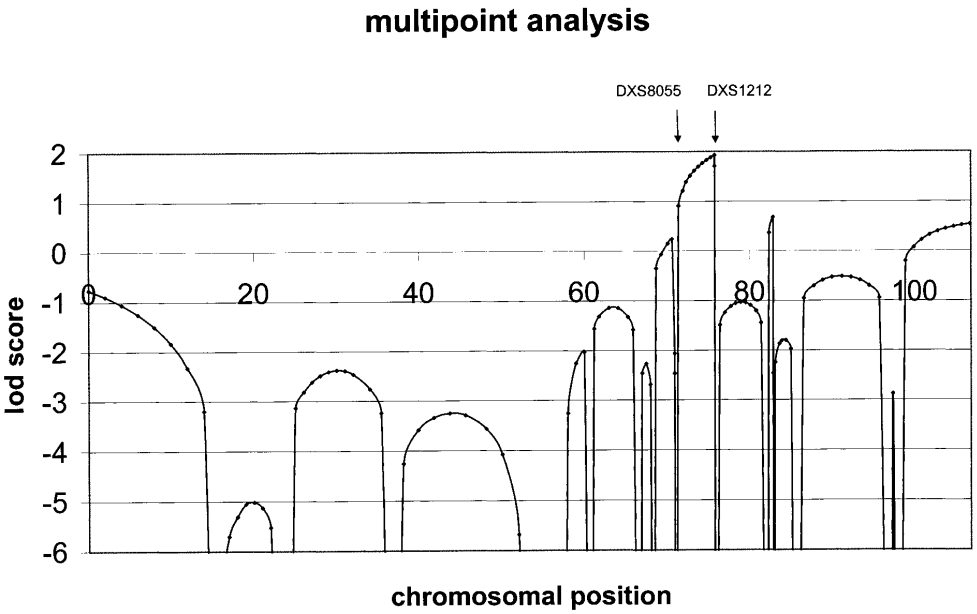

Figure 3 Multipoint lodscores for twenty tested X-chromosomal markers. X-axis: the distance in cM. The most telomeric marker tested DXS996 was used as a point of reference (0), and the one on the right is DXS1193. The highest lodscores are obtained in the region in between DXS8055 and DXS1212.

chromosome breakage after DEB exposure.[13] There are 12 cases published with X-linked VACTERL-H; 10 cases summarised by Lomas *et al*.,[3] and two cases by Froster *et al*.[4] In Table 2 we summarise these cases, the two cases with X-linked radial aplasia,[5] and the cases presented in this study. Our index patient has been evaluated for all defects summarised by Lomas *et al*.[3] He shares only radial aplasia and a cardiac anomaly with the cases described with X-linked VACTERL-H cases. Moreover, his life expectancy is different from that in these patients. The cases described by Gibson *et al*.,[5] only share radial aplasia with our patients. Therefore, we do not think our patients have the same phenotype.

The phenotype of our patients can be caused by a defect of a single gene or several closely linked genes. Alternatively, large variability in the phenotype might be caused by either modifier genes located elsewhere on the genome or environmental factors. A literature search for patients with a radial ray deficiency and a chromosomal anomaly involving our defined critical region revealed no cases, which limits the search for a gene causing the phenotype to a candidate gene approach. Since absence of the radii is the most consistently present anomaly in our patients we hypothesise that the responsible gene must be involved in patterning, differentiation, or apoptosis during human embryonic limb development. In our patients the gene defect could have its major effect on the mesenchymal condensation process of the anterior (radial) part of the upper limb, comparable with the effects of the mutant TBX5 gene.[14,15] Haploinsufficiency of this gene causes mainly anterior limb malformations in patients with Holt-Oram syndrome, whereas haploinsufficiency of the TBX3 gene causes mainly posterior limb malformations in patients with the ulnar-mammary syndrome.[14,16]

Table 2 Summary of anomalies in cases with VACTERL-H and X-linked radial aplasia

Anomalies	*1*[a]	*2*[b]	*3*[c]	*4*[d]	*5*[e]	*6*[f]
Hydrocephalus	4/4	2/2	4/4	2/2	1/1	0/3
Radial anomalies	4/4	2/2	4/4	2/2	2/2	4/4
Anal atresia	2/2	2/2	3/4	1/2	1/2	0/4
Genital anomalies	2/2	1/1	2/3	?	1/2	0/4
Renal anomalies	2/2	2/2	2/2	1/2	?	0/2
T.E.F./Atresia	1/1	1/2	1/3	1/2	?	0/1
Vertebral anomalies	1/1	?	0/1	1/2	?	0/1
Cardiac anomalies	?	?	1/3	1/2	?	2/2
Lung anomalies	?	?	0/3	1/2	?	0/1
Gut anomalies	1/1	1/1	0/3	1/2	?	?
Accessory spleens	1/1	0/1	0/3	?	?	0/2
Microphthalmia	1/1	0/1	0/3	2/2	?	0/3
Cleft palate	1/1	2/2	0/3	1/2	?	0/4
Ear anomalies	2/2	?	0/3	2/2	?	0/4
Died	4/4	2/2	4/4	2/2	?	1/4

Authors: 1[a], Lomas *et al*.[3]: family 1, four cases; 2[b], family 2 cases; 3[c], family 3, four cases; 4[d], Froster *et al*.[4]: two cases; 5[e], Gibson *et al*.[5]: two cases; 6[f], present paper: four cases. Anomalies indicated when known; ? means not known in any case of the family.

The gene defect could also be involved in differentiation or growth of the radius. Disturbance of the chondrofication process could result in a fibrous radius;[17,18] disturbance of the ossification process in a cartilagenous one.[19] Alternatively, the gene defect could also be involved in disturbance of apoptotic cell death eliminating mesenchymal cells which would normally be the precursors of the radius resulting in the absence of the radius. It has been suggested that the radius and the ulna are derived from one mesenchymal condensation separated by apoptotic cell death in the opaque patch.[20,21] Considering the pathogenic mechanisms described above we searched for a likely candidate gene or transcript in our critical region with a possible function in pattern formation, cell differentiation, or apoptosis. Genes coding for zinc finger (ZF) containing proteins are known to be often involved in pattern formation. We searched the OMIM database and found the RING finger containing IAP (Inhibitor of apoptosis protein) 3 gene to be a likely candidate. IAP 3 is mapped to Xq25 by *in situ* hybridisation.[22] It is expressed in all fetal and adult tissues except peripheral blood leucocytes.[23]

We also searched the Human Gene Map (http://www.ncbi.nlm.nih.gov/genemap) and found the ZNF183 gene of the RING finger gene family. This gene is ubiquitously expressed and its function is unknown.[24] We did not find a mutation in these genes in our patients. We are currently searching for additional families as an approach to identify the responsible gene, which could be a starting point for functional studies in order to obtain better insight in the pathogenesis of the disorder.

Acknowledgments

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Chapter 7

A new locus for postaxial polydactyly type A/B on chromosome 7q21-q34

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ARTICLE

A new locus for postaxial polydactyly type A/B on chromosome 7q21–q34

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Postaxial polydactyly (PAP) is the occurrence of one or more extra ulnar or fibular digits or parts of it. In PAP-A, the extra digit is fully developed and articulates with the fifth or an additional metacarpal/metatarsal, while it is rudimentary in PAP-B. Isolated PAP usually segregates as an autosomal dominant trait, with variable expression. Three loci are known for PAP in humans. PAPA1 (including PAP-A/B in one patient) on 7p13 caused by mutations in the GLI3 gene, PAPA2 on 13q21–q32 in a Turkish kindred with PAP-A only, and a third one (PAPA3) in a Chinese family with PAP-A/B on 19p13.1–13.2. We identified a fourth locus in a large Dutch six-generation family with 31 individuals including 11 affecteds. Their phenotype varied from either PAP-A, or PAP-B to PAP-A/B with or without the co-occurence of partial cutaneous syndactyly. We performed a whole-genome search and found linkage between PAP and markers on chromosome 7q. The highest LOD score was 3.34 obtained at D7S1799 and D7S500 with multipoint analysis.
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Keywords: postaxial polydactyly; linkage; fourth locus; chromosome 7q21–q34

Introduction

Postaxial polydactyly (PAP) is the occurrence of one or more extra ulnar or fibular digits or parts of it.[1] The incidence varies from 1/3300 to 1/630 and from 1/300 to 1/100 livebirths in Caucasian- and African-Americans, respectively.[2] The phenotype of PAP is usually subdivided into types A and B. In type A, the extra digit is fully developed and articulates with the fifth or an additional metacarpal/metatarsal. In type B, it is rudimentary and mostly presents as a skin tag (pedunculated postminimus).[1]

PAP is either seen as an isolated malformation or associated with other defects. Associated defects can be restricted to the limbs. If not, they can be part of a syndrome, or of a multiple congenital anomaly case.[3] The syndromic cases have a heterogeneous aetiology, for instance trisomy 13. Partial cutaneous syndactyly between toes 2–3, 4–5, and other, is a frequent finding in individuals with PAP.[3]

Isolated PAP usually segregates as an autosomal dominant trait, with variable penetrance and expression. Penetrance rates of 0.68 and 0.43 have been estimated for types A and B, respectively,[4] although higher estimates have been published.[5]

Currently, there are three loci for isolated PAP in humans. PAPA1 (MIM 174200) has been described in three families with PAP-A/B with a disease-causing mutation in the human transcription regulator GLI3 gene on 7p13.6.[6–8] PAPA2 (MIM 602085) on 13q21–q32 has been reported in a Turkish kindred with PAP-A only.[9] PAPA3 has recently been published in a Chinese family with PAP-A/B on 19p13.1–13.2.[10]

We present a fourth locus in a family with PAP-A/B and partial cutaneous syndactyly on 7q21–q34. In our family

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there are patients with three types of PAP (type A, B or A/B) with or without syndactyly.

Materials and methods

A large six-generation family with PAP and/or syndactyly was ascertained (Figure 1). After informed consent was given, 31 individuals were clinically examined and blood samples were taken from them. Genomic DNA was isolated from peripheral blood lymphocytes as described before.[11] From four affected individuals, deceased at the time of examination, anamnestic data were available (Table 1).

Initially, a genome search was started with 24 individuals of our family including all 11 affecteds with PAP (Figure 1). LOD scores were calculated using the MLINK and LINKMAP programs of the LINKAGE package version 5.1.[12] The following model was used: PAP as an autosomal dominant condition with a penetrance of 0.9, a gene frequency of 0.0003, a phenocopy rate of 0.05, and equal recombination rates between males and females. In this model only individuals with PAP, including V-6, were considered to be affected (Table 1). Allele frequencies were calculated from independent individuals of this family. In total, 26 additional polymorphic markers of chromosome 7 were selected from the Center of Medical Genetics, Marshfield Medical Research Foundation (htpp://research. Marshfield-clinic.org/genetics/). The most recently published high-resolution recombination map of the human genome of Kong *et al* (htpp://genetics.nature.com) was also used. For marker GATA63F08, locus number D7S2202 has recently been assigned. The results of haplotype analysis in this family prompted us to determine if another locus could be associated with the PAP- and the syndactyly phenotype. A whole-genome screen was performed on all affecteds except for V-6, five nonaffected and five control individuals. Microsatellite markers from the Weber Human Screening Set V5 ($n=363$) were tested, DNA pooling and shared segment analysis were performed essentially as described before.[13] DNA quantity and quality were carefully matched to ensure equal amplification.

Results

Clinical findings

The clinical findings of 17 affected individuals with PAP and/or syndactyly of our family are presented in Table 1. They do not have additional anomalies. PAP-A is the predominant polydactyly phenotype. The expression of both the PAP and syndactyly phenotypes is highly variable in our family, especially for descendants of III-7 and IV-5, as examplified in Figure 2. This variability concerns differences in involvement of upper/lower limbs, left/right side, type A and/or B regarding PAP, and the interdigital space(s) (IDS) and extent of syndactyly. However, the PAP phenotype is strikingly consistent in four preceding generations of VI-1 (Figure 3A).

Linkage analysis for PAP

A genome search was carried out with 24 individuals of our family including 11 affecteds with PAP. Evidence of linkage was found with a marker on chromosome 7q35 (data not shown). Additional markers around D7S1799 were tested to identify the smallest region in which the gene involved has to reside. With two-point linkage analysis the highest LOD score obtained was 3.18 for marker D7S1799 at $\theta=0$ (Table 2). Using multipoint analysis, LOD scores above 3.20 were found for markers D7S1799–D7S495, so evidence for linkage is spread over a large genomic region (Figure 4).

Haplotype analysis

Only affecteds with PAP and individual VI-6, with bilateral partial cutaneous syndactyly of IDS 2, share the same haplotype for markers between D7S1799 and D7S495 (Figure 1). In addition, they share their haplotype for markers GATA63F08 and D7S2513 with the clinically normal individuals IV-19 and V-18, and individual VI-9 having only syndactyly (Figure 1). The maximum size of the genomic regions located on chromosome 7q21–q34, between D7S1799 and D7S495, and GATA63F08 and D7S2513 is about 50 and 3.7 cM, respectively.

No other locus associated with the PAP phenotype was identified in this family using the DNA pooling and shared segment analysis (data not shown).

Discussion

We describe a large family with PAP-A/B and/or syndactyly. This family is interesting because of the presence of three types of PAP with or without syndactyly. In addition, a single individual with PAP-B (V-6) does not share the haplotype shared by the other affecteds with PAP-A/B.

There is a longstanding debate in the literature if the PAP-A and PAP-B phenotypes are genetically heterogeneous. Differences between PAP-A and PAP-B concerning incidence, penetrance and side preference have been used to argue that the phenotypic distinction between them is also genetically determined.[1,2,4,14] Until now, only families with individuals with the PAP-A or PAP-A/B phenotype have been used for genotyping; none of them includes the PAP-B only phenotype. In our family individual V-6 with PAP-B only does not share the haplotype with other affecteds with PAP-A or PAP-A/B (Figure 1). In addition, both of his alleles are transmitted to his offspring, who are clinically normal. This indicates his phenotype is probably caused by a genetic defect elsewhere on the genome, or less likely, by a nongenetic factor. This is corroborated by the finding that no other locus, associated with PAP-A/B, was found in this family after we performed a whole-genome search. In order to strengthen our findings we also tested

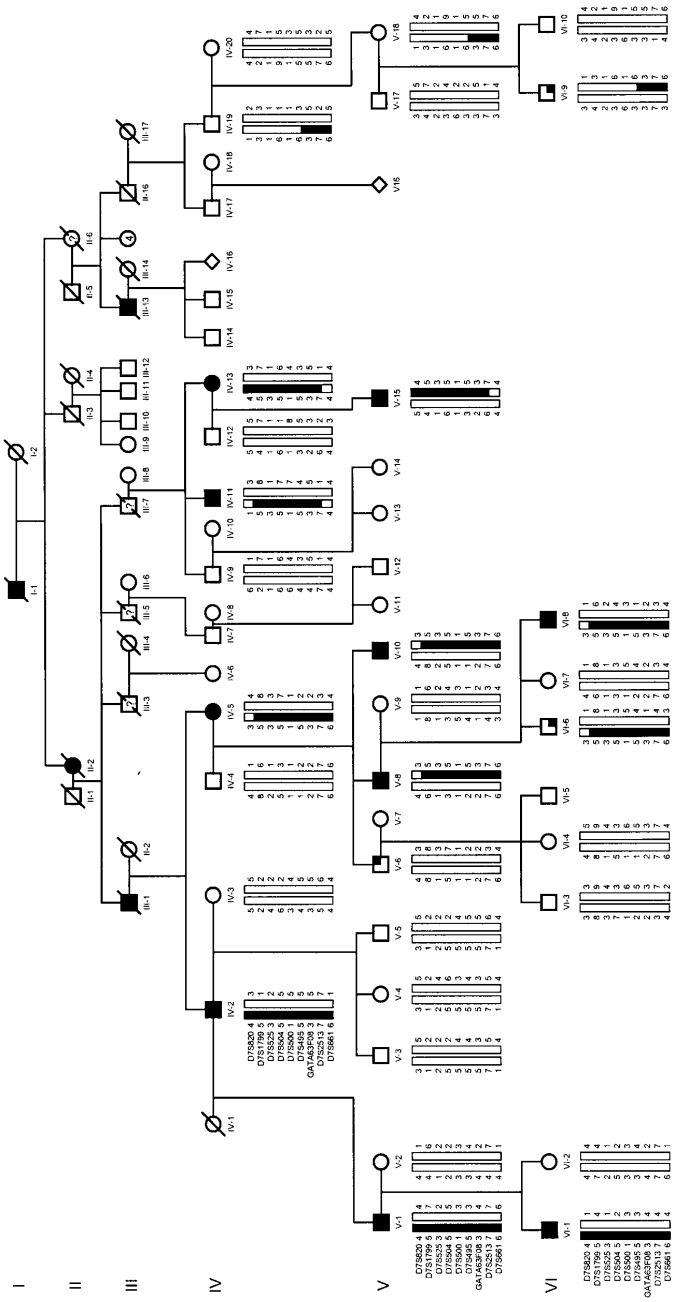

Figure 1 Pedigree diagram of a subset of a family with PAP. Pedigree symbols: black = PAP-A/B with or without cutaneous syndactyly. Black right upper quadrant = PAP-B only. Black right lower quadrant = cutaneous syndactyly digit 2–3 feet only. Haplotypes for eight chromosome 7 markers for 31 individuals. Shared haplotype for markers D7S1799–D7S495 between affecteds with PAP/syndactyly, except for V-6 (phenocopy) and VI-6 (nonpenetrance).

Table 1 Clinical findings of affecteds with PAP/syndactyly

Patient No.	*PAP type left hand*	*PAP type right hand*	*Syndactyly left hand*	*Syndactyly right hand*	*PAP type left foot*	*PAP type right foot*	*Syndactyly left foot*	*Syndactyly right foot*
I-1*	P	P	?	?	P	P	?	?
II-2*	A	A	?	?	A	A	?	?
III-1*	N	A	IDS 4–5	N	A	A	N	N
III-13*	N	N	?	?	P	P	?	?
IV-2	A	A	N	N	A	A	N	N
IV-5	A	A	N	N	N	N	N	IDS 4–5
IV-11	N	N	N	N	A	A	N	IDS 5–6
IV-13	N	A	N	IDS 4–5	A	N	IDS 4–6	N
V-1	A	A	N	N	A	A	N	N
V-6	B	B	N	N	N	N	N	N
V-8	A	A	N	N	A	A	N	N
V-10	A	A	N	N	A	A	N	IDS 5–6
V-15	A	B	N	IDS 2–3	A	A	N	N
VI-1	A	A	N	N	A	A	N	N
VI-6	N	N	N	N	N	N	IDS 2–3	IDS 2–3
VI-8	B	B	N	N	A	A	IDS 2–3	IDS 2–3
VI-9	N	N	N	N	N	N	IDS 2–3	IDS 2–3

*=anamnestic data, P=present, N=normal, ?=unknown, A=PAP-A, B=PAP-B, IDS=interdigital space.

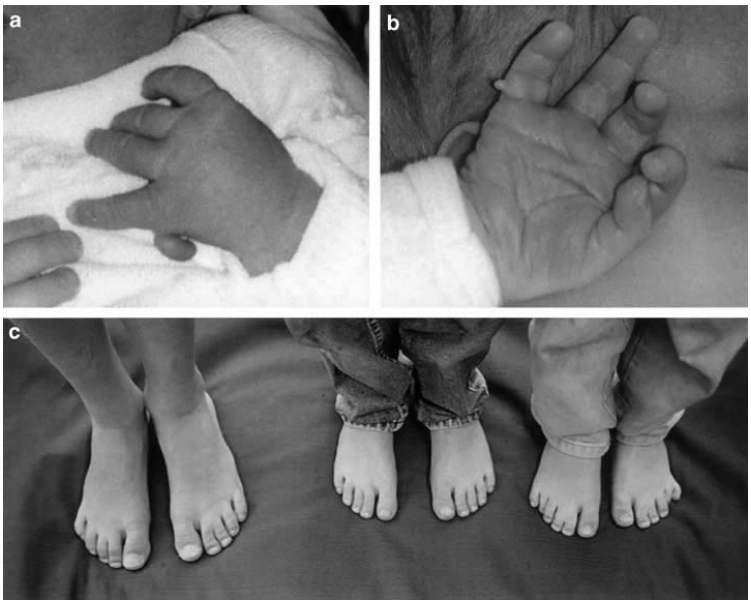

Figure 2 (**a**, **b**) Left and right hand, respectively, of VI-8 with PAP-B. (**c**) From left to right: feet of VI-6 with cutaneous syndactyly digits 2–3 only, normal feet of VI-7, feet with PAP-A/syndactyly digits 2–3 of VI-8.

10 other small families with PAP, but none of them was linked to this 7q region. These findings are indicative of further genetic heterogeneity of PAP, except for the locus harbouring the GLI3 gene. In our family, the PAP-A/B phenotype is genetically homogeneous as described before, but the PAP-A/B and PAP-B only phenotypes are genetically heterogeneous.

Syndactyly is the most frequent associated anomaly in individuals with PAP.[3] In syndactyly type II or synpolydactyly (SPD, MIM186000), the characteristic phenotype is syndactyly between fingers 3–4, and toes 4–5 with or without an extra finger/toe in the syndactylous web.[15] Polydactyly does not occur without syndactyly. SPD is caused by a polyalanine tract expansion in the HOXD13

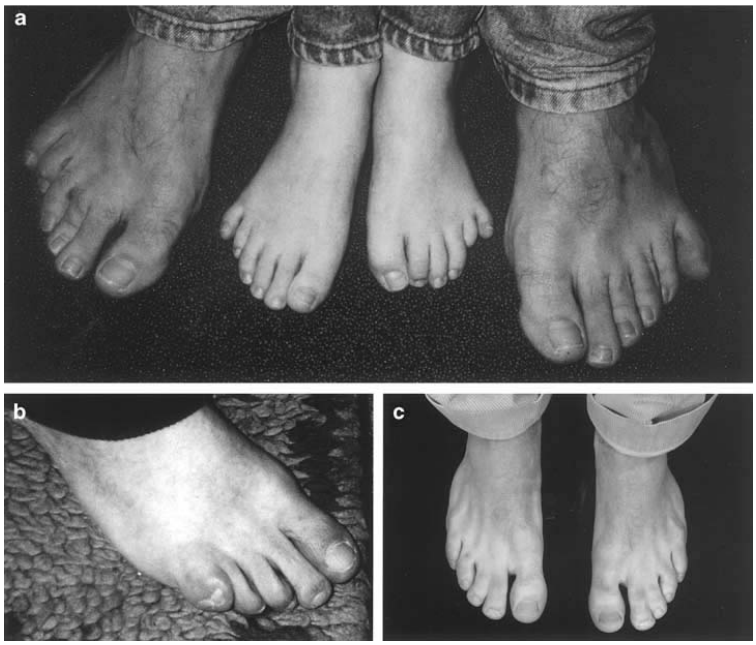

Figure 3 (**a**) Feet of V-1 and VI-1 with PAP-A. (**b**) Right foot of IV-11 with PAP-A and complete cutaneous syndactyly digits 5–6. (**c**) Feet of VI-9 with cutaneous syndactyly digits 2–3 only.

Table 2 Two-point linkage analysis between PAP/B and chromosome 7 markers at different recombination fractions

	Recombination fraction θ						
Markers	*0*	*0.01*	*0.05*	*0.1*	*0.2*	*0.3*	*0.4*
D7S820	−1.97	−1.69	−1.02	−0.57	−0.13	0.03	0.04
D7S1799	3.18	3.15	3.00	2.76	2.15	1.43	0.65
D7S525	2.44	2.43	2.33	2.15	1.66	1.06	0.44
D7S504	1.26	1.24	1.17	1.05	0.75	0.41	0.14
D7S500	2.64	2.59	2.38	2.11	1.54	0.94	0.39
D7S495	1.08	1.07	1.02	0.94	0.72	0.48	0.24
GATA63F08	2.00	1.96	1.81	1.60	1.13	0.65	0.24
D7S513	0.45	0.44	0.39	0.34	0.23	0.14	0.07
D7S661	−0.84	−0.51	−0.09	0.06	0.11	0.05	−0.01

gene on chromosome 2q31. This phenotype can be differentiated from that in patients of our family. Their phenotype include an extra digit 5 without syndactyly, or with syndactyly between fingers/toes not typically present in patients with SPD (Table 1). Isolated syndactyly IDS 2–3 of the feet has an incidence of 1.17/10 000 individuals.[3] We looked if PAP and syndactyly could be genetically homo or heterogeneous in our family. PAP and syndactyly are present in seven individuals (Table 1). Four individuals have PAP type A or A/B. There are two individuals (VI-6, VI-9) with only cutaneous syndactyly IDS 2–3 of both feet (Figures 2c and 3c). All patients with PAP-A/B, share the same haplotype between markers GATA63F08 and D7S2513 (Figure 1). This includes the clinically normal mother (V-18) and maternal grandfather (IV-19) of the individual with bilateral syndactyly IDS 2–3 of his feet (VI-9). There are two possible explanations for these observed phenotypes/genotypes.

First, the region shared by all of them harbours the gene(s) causing the PAP and the syndactyly phenotype, so

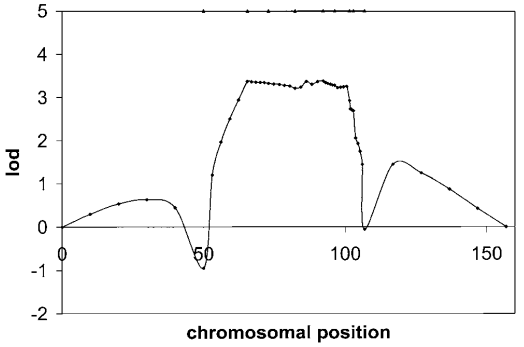

Figure 4 Results of multipoint linkage analysis between PAP-A/B and chromosome 7 markers. Triangles indicate markers used also in the two-point linkage analysis. From left to right: D7S820, D7S1799, D7S525, D7S504, D7S500, D7S495, GATA63F08, D7S2513, D7S661.

the PAP and syndactyly phenotypes are genetically homogeneous and the region would be about 3.7 cM. If so, four individuals with PAP-A/B only are nonpenetrant for syndactyly. In addition, VI-6 and VI-9 with syndactyly only do not show penetrance for the polydactyly phenotype. Moreover, the mother, the maternal grandfather and the great-grandfather of VI-9 are nonpenetrant for both PAP and syndactyly. The PAP phenotype is highly penetrant in offspring of II-1 and II-2. Nonpenetrance of this phenotype in three successive generations in offspring of II-5 and II-6 is an unlikely occurrence.

Besides, the syndactyly phenotype of VI-6 could be a variable expression of the syndactyly phenotype in this branch of the family, with a different locus than that harbouring the gene mutation causing syndactyly of VI-9, taking the relatively high incidence of syndactyly IDS 2–3 of feet in the normal population into account.

Alternatively and more likely, the PAP and syndactyly phenotypes are genetically heterogeneous in our family. In that case, the critical region is defined by the haplotype shared by patients with PAP-A/B only (Figure 1). If so, VI-6 is the only case of nonpenetrance for the PAP phenotype in this family. This is in accordance with rates of penetrance for PAP from other families reported in the literature.[4,8,14,16] Penetrance is probably determined by modifier genes, analogous to the observation of Fawcett *et al*.[17] They described CRABPII knockout mice with an extra bone of digit V and found that the penetrance of this phenotype varies according to their genetic background.[17]

We hypothesize that genetic background also affects the phenotypic expression of PAP-A and PAP-A/B. There are two affected in our family with PAP-A/B (V-15, VI-8 (Figure 2)). PAP types A and B in one individual has been described before.[8,14,18–20] Radhakrishna *et al*.[8] suggested that GLI3 mutations cause PAP-A. In individuals with PAP-A/B, these mutations and environmental or stochastic factors could cause the PAP-B phenotype. In our view, it is difficult to envisage a major impact of the same environmental factor on patterning causing PAPA/B in V-15 and VI-8 in different generations excluding a genetic susceptibility for such a factor, although a stochastic factor could cause the phenotypic expression of PAP-A/B in V-15 and VI-8.

We identified the fourth locus containing one or more genes involved in the PAP and syndactyly phenotype. This is based on our results of the genome scan, the linkage and haplotype analyses. The maximum size of the critical region between D7S820 and GATA63F08 is about 50 cM. This chromosome 7 region excludes genes previously known to be involved in the polydactyly phenotype like the sonic hedgehog- and GLI3 genes. We looked for candidate genes and we found many genes with unknown functions which could be potential candidates, genes coding for zinc-finger containing proteins from which members are known to be involved in patterning in embryonic development, and two members of the Wnt gene family. Wnt7a, located elsewhere, is shown to be involved in dorsal–ventral patterning in the developing limb.[21] Wnt2 (MIM147870) and two variants of Wnt16 (MIM606267) are located on 7q31. There are no data available on expression in limb since this is relatively rarely looked for. Besides, we found a Wnt receptor frizzled homolog1 Drosophila (FZD1), mapped on 7q21. Frizzled1 (fz1) has distinct D–V domains of expression in mesenchyme of developing footpads.[22] Given the large number of candidate genes and the fact that none of these is an obvious candidate, we did not perform mutation analysis in our family. At this point we are pursuing other families with PAP linked to this region in order to be able to narrow down the region containing the gene associated with PAP and syndactyly in this family.

Acknowledgements

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Chapter 8

Isolated postaxial polydactyly type B with mosaicism of a submicroscopic unbalanced translocation leading to an extended phenotype in offspring

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Clinical Report

Isolated Postaxial Polydactyly Type B With Mosaicism of a Submicroscopic Unbalanced Translocation Leading to an Extended Phenotype in Offspring

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Postaxial polydactyly (PAP) is characterized by the presence of one or more extra ulnar or fibular digits or parts of it. PAP type B presents frequently as a skin tag on the hand(s). It is usually an isolated malformation, but in 6.6% it is associated with other congenital abnormalities, mostly well recognizable syndromes. We present a male with PAP-B only and his daughter with an extended phenotype including mental retardation and minor dysmorphisms. Both share a cytogenetically balanced t(4;7)(p15.2;q35), present in mosaicism in the father. We found microdeletions associated with the breakpoints. The chromosomal regions described here have not been previously associated with the PAP-B phenotype. We present the first case of an individual with isolated PAP-B and a submicroscopic chromosome abnormality.

KEY WORDS: postaxial polydactyly; mental retardation; translocation (4;7); microdeletion; mosaicism; chromosome 4p15.3; chromosome 7q35-36.1

INTRODUCTION

Postaxial polydactyly (PAP) is characterized by the presence of one or more extra ulnar or fibular digits or parts of it. The most widely accepted phenotypic classification distinguish types A and B [Temtamy and McKusick, 1978]. In PAP-A, the extra digit is fully developed and articulates with the fifth or an additional metacarpal/metatarsal. In PAP-B, it usually presents as a skin tag (pedunculated postminimus).

Incidence figures of PAP vary from 1/3300 to 1/630, and from 1/300 to 1/100 livebirths in Caucasian- and African-Americans, respectively [Temtamy, 1990]. This is mainly due to differences seen in PAP-B [Castilla et al., 1973; Woolf and Myrianthopoulos, 1973].

PAP is usually present as type B of the left hand(s), without associated congenital anomalies [Castilla et al., 1973,1997,1998; Woolf and Myrianthopoulos, 1973; Scott-Emuakpor and Madueke, 1976].

It segregates as an autosomal dominant trait, with variable expression and penetrance [Castilla et al., 1973; Scott-Emuakpor and Madueke, 1976]. It is genetically very heterogeneous, with four genetic loci for isolated PAP in humans [Akarsu et al., 1997; Radhakrishna et al., 1997; Zhao et al., 2002; Galjaard et al. unpublished communication], and several other loci for PAP as part of syndromal cases. Moreover, PAP with multiple congenital abnormalities is associated with numerical chromosome abnormalities, mainly trisomy 13, structural abnormalities including duplications and deletions, and an inversion/microdeletion.

We present a male individual with isolated PAP-B of the hands and his daughter with PAP-B and associated abnormalities. He is mosaic for a cytogenetically balanced translocation that is present in all cells in his daughter. We found microdeletions of chromosome regions near the breakpoints not previously known to be involved in the PAP-B phenotype.

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PATIENTS AND METHODS

The family was ascertained by evaluation of mental retardation and dysmorphisms of a female index patient. She and her family were clinically evaluated and blood samples were taken after informed consent. An EBV-transformed cell line was made from the index patient and her father. GTG-banded metaphases were used for karyotyping.

FISH was done essentially as described before [Pinkel et al., 1986; Eussen et al., 2000] on metaphase spreads of skin fibroblasts. DNA was isolated as described by a simple salting out procedure [Miller et al., 1988]. YAC's and BAC/PAC clones were obtained from the CEPH and RPCI-4,5,11 libraries at BAC/PAC Resources (htpp://www.chori.org/BACPAC). Data on these YAC/BAC/PAC's were obtained via Whitehead Institute (www.genome.wi.mit.edu), and the Chromosome 7 database (www.genet.sickkids.on.ca). Clinical data on PAP and physical maps were obtained from OMIM and NCBI's Human map viewer via (www.ncbi.nlm.nih.gov), respectively. Celera's database was used for physical maps (http://publication.celera.com). Data on chromosome abnormalities in individuals with PAP were obtained from POSSUM version 5.5 (The Murdoch Institute for Research into Birth Defects, Melbourne, Australia).

RESULTS

Clinical Findings in Patients

The index patient was born after a normal pregnancy and delivery. At birth her length was 53 cm (90th centile), she weighted 3750 g (75th centile) and her head circumference (HC) was 32.5 cm (10th centile). She had postaxial polydactyly type B (PAP-B) of the left hand (Fig. 1).

She was clinically evaluated intermittently from the age of 2.5 months because of microcephaly present untill the age of 13 years, developmental retardation, and hypotonia. At the age of 4.3 years she had a verbal IQ of 58 and non-verbal IQ of 62–72. She was dysmorphologically evaluated at the ages of 8 and 13 years. The following anomalies were noted: strabismus, upward slanting of the palpebral fissures, slightly bifid nose with anteverted nares and long columella, prominent upper frontal teeth, a high arched palate, low-set ears with flattened crux superior and a rightsided preauricular pit, widely spaced nipples (Figs. 2 and 3), and cubiti valgi. The liver was 3 cm, the spleen 2 cm palpable. Moreover, a coloboma of the papil was seen on ophthalmological examination. Screening for metabolic disorders gave normal results.

Physical examination of the father was normal, except for bilateral PAP-B of the hands which were removed after birth.

His wife and their other daughter have a normal phenotype, but the latter attends a school for children with learning difficulties. The family history is uneventful.

Cytogenetics and FISH

Cytogenetics. The karyotype of the index patient is 46,XX,t(4;7)(p15.2;q35) in blood lymphocytes and

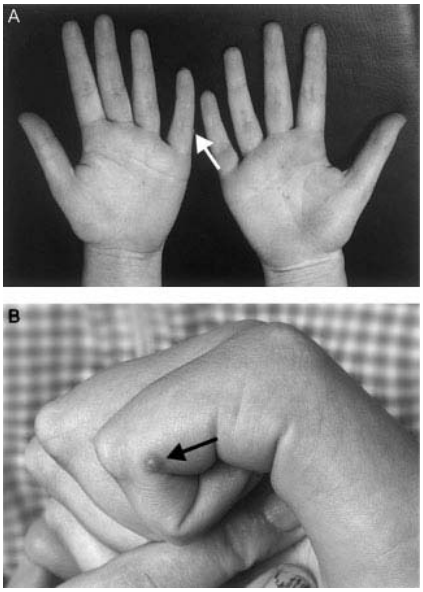

Fig. 1. PAP-B of the left hand of the index patient at the age of 13 years.

Fig. 2. Face of the index patient at 13 years showing upward slanting palpebral fissures, strabismus, slightly bifid nose, and long columella.

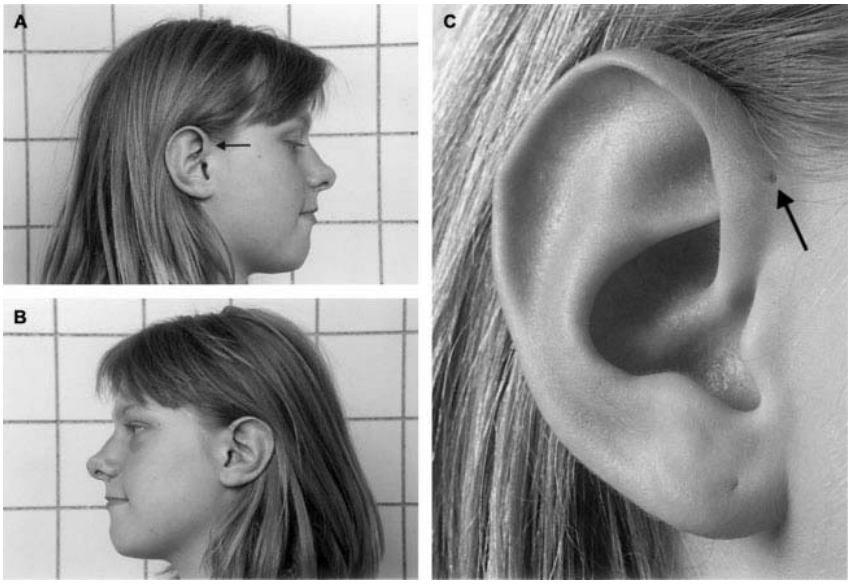

Fig. 3. Flattened crux superior of the ears of the index patient and rightsided preauricular pit (A,C).

skin fibroblasts. The karyotype of her father is 46,XY,t(4;7)(p15.2;q35)[35]/46,XY[15] in blood lymphocytes and 46,XY,t(4;7)(p15.2;q35)[48]/46,XY[2] in skin fibroblasts; so the translocation is present in 35/50 and in 48/50 metaphases in blood lymphocytes and skin fibroblasts, respectively. The karyotypes of his wife and their other daughter are 46,XX.

FISH. FISH with YAC's, BAC's, and PAC's from chromosome 4p14-16 and 7q35-36 was performed for fine mapping of the translocation breakpoints. The results, summarized in Table I, lead to the following conclusions:

Translocation. The translocation in fibroblasts of the index patient is shown using a whole chromosome paint with chromosome 4 in red and chromosome 7 in green (Fig. 4A). Part of the short arm of one of the chromosomes 4 (4pter-4p15.32) is translocated to the long arm of one of the chromosomes 7, broken in 7q35. This chromosome is the der(7) (Fig. 5).

Part of the long arm of one of the chromosomes 7 (7q36.1-qter) is translocated to the short arm of one of the chromosomes 4p15.31, the der(4) (Fig. 5).

Breakpoint 4p15.32. FISH with BAC C0481P14 of chromosome 4p15.32 showed a signal on 4p15.32 of the normal chromosome 4, and on the der(7) (Fig. 5). The overlapping BAC RP11-173B23 showed only a signal on 4p15.32 of the normal chromosome 4. Therefore, all, or most of it is deleted on the der(4). The breakpoint is in or adjacent to the overlap of both BAC's (Fig. 5).

Breakpoint 4p15.31. BAC RP11-10P19 of chromosome 4p15.31 showed a single signal on the normal chromosome 4 (Fig. 5). All or most of it is deleted on the der(4). The BAC adjacent to this is RP11-12E5 which showed a signal on both the normal chromosome 4 and the der(4) (Fig. 5). Therefore, all or most of it is present on the der(4). The breakpoint is either in the chromosome region inbetween these BAC's or in one of the BAC's themselves.

Breakpoint 7q35. PAC RP5-1137M13 of chromosome 7q35 showed a signal on both the normal chromosome 7 and the der(7) (Figs. 4B and 5). The overlapping PAC RP5-958B11 showed a single signal on the normal chromosome 7, so all or most of it is deleted (Fig. 5). The breakpoint is in or adjacent to this overlap.

Breakpoint 7q36.1. PAC RP4-753H13 of chromosome 7q36.1 showed a single signal on the normal chromosome 7, so all or most of it is deleted on the der(7) (Fig. 5). The adjacent BAC RP11-728K20 showed a signal on both the normal chromosome 7 and the der(4) (Fig. 5). The breakpoint is either in the chromosome region in between these BAC and PAC or in the BAC/PAC itself.

Since there are loci known on chromosome 7q36 for hand abnormalities including polydactyly [Heutink et al., 1994; Tsukurov et al., 1994], fine mapping of the breakpoints of chromosome 7 was performed. BAC RP11-163I18 contains the centromeric chromosome 7 breakpoint (7q35) harbouring the *CASPR2* gene. Polymorphic markers were designed and tested to determine the number of alleles present in the index patient. A marker 10 kb upstream of exon 24 of the *CASPR2* gene on RP11-163I18 was heterozygous, indicating the presence of two alleles (data not shown). Therefore,

TABLE I. FISH Analysis With Chromosome 4p15 and 7q35-36 YAC/BAC/PAC's on Metaphase Chromosomes of a t(4;7)(p 15.2;q35) Carrier

Chromosome 4p14-16	Signal on normal 4p	Signal on der(7)	Signal on der(4)	Chromosome 7q35–36	Signal on normal 7q	Signal on der(7)	Signal on der(4)
Cosmid/YAC				YAC			
L228A7	+	+	–	C647f12	+	+	–
657-E4	+	+	–	C846e8	+	+	–
739D7	+	–	+	HSCE7E162	+	+	–
				HSCE7E124	+	–	–
BAC/PAC				HSCE7E487	+	–	–
				C873c3	+	–	+
116D01	+	+	–				
RP11-685B15	+	+	–	BAC/PAC			
RP11-274B16	+	+	–				
C0483I23	+	+	–	RP4-800L12	+	+	–
RP11-576E20	+	+	–	RP5-1137M13	+	+	–
C0481P14	+	+	–	RP11-163I18	+	+	–
RP11-173B23	+	–	-	RP5-958B11	+	–	–
C0315N08	+	–	–	RP5-1151M05	+	–	–
C0162P16	+	–	–	RP4-800G07	+	–	–
RP11-724A24	+	–	–	RP4-751H13	+	–	–
RP11-683L20	+	–	–	RP11-728K20	+	–	+
RP11-84N19	+	–	–	RP11-543G3	+	–	+
RP11-96B10	+	–	–	RP4-584D14	+	–	+
RP11-85L2	+	–	–	RP11-632K21	+	–	+
RP11-339D20	+	–	–	RP5-1051J4	+	–	+
RP11-10P19	+	–	–				
RP11-12E5	+	–	+				
RP11-578A19	+	–	+				
269N16	+	–	+				
RP11-735L15	+	–	+				
276O17	+	–	+				

+, Positive specific signal with FISH.

the breakpoint is most likely downstream of the stop-codon of the *CASPR2* gene.

Microdeletions. Several BAC/PAC's from chromosome 4p15.3 and 7q35-36.1 showed a single signal on the normal chromosome only. This is indicative of microdeletions of the derivative (der) chromosomes. The microdeletion of the der(4) was localized between 4p15.32 (telomeric) and 4p15.31 (centromeric), which is about 2.8 Mb. Likewise, a deletion of about 2.3 Mb was found on the der(7) between 7q35 (centromeric), and 7q36 (telomeric).

DISCUSSION

We present a patient with developmental delay, minor facial dysmorphisms, PAP-B of the left hand, a history of hypotonia and microcephaly. Her father had only bilateral PAP-B of the hands. Both are translocation carriers with microdeletions on the breakpoints of 4p15.3 and 7q35-36.1. It illustrates that in rare cases chromosome studies could be considered in individuals with only PAP. Furthermore, it stresses the importance of additional studies for submicroscopic abnormalities in cases with mild or multiple congenital anomalies and cytogenetically balanced translocations.

In the father the translocation is present with different levels of mosaicism in blood lymphocytes and skin fibroblasts, indicating a postzygotic mitotic event. The lower degree of mosaicism in skin fibroblasts is probably due to the lower mitotic turnover compared to blood lymphocytes. It is tempting to speculate that the absence of normal cells is associated with the extra phenotypic abnormalies in the index patient, compared to her father. The absence of mental retardation in the father is most likely due to absence or a low degree of mosaicism in cells of his brain.

Theoretically, PAP-B could be caused by a spontaneous mutation of a gene in a locus different from those involved in the translocation/microdeletions in the father and his daughter. However, the co-occurrence of two rare events is extremely unlikely. Furthermore, as the PAP-B phenotype and the translocation are shared by the father and his daughter we assume they are associated.

The phenotypes of our patients could be caused by haploinsufficiency of one or more genes located in or adjacent to the microdeletions. To verify this we searched the literature for patients with deletions of 4p15 and 7q35-36. We found a single patient with a deletion 4p15.3 [Davies et al., 1990], and four with a deletion 4p15.2-15.3 [Chitayat et al., 1995]. They share developmental delay (4/5), abnormal helices of the ears (4/5), a high arched palate (3/5), and strabismus (2/5) with our index patient. Two patients with an interstitial deletion of 7q35 have been reported [Frijns et al., 1988; Fagan et al., 1994], who share developmental delay (2/2), strabismus (2/2), a high arched palate (1/2), and microcephaly (1/2) with our index case. From our point of view the number of patients is too small to define a deletion-4p15, or -7q35 phenotype. The developmental delay and

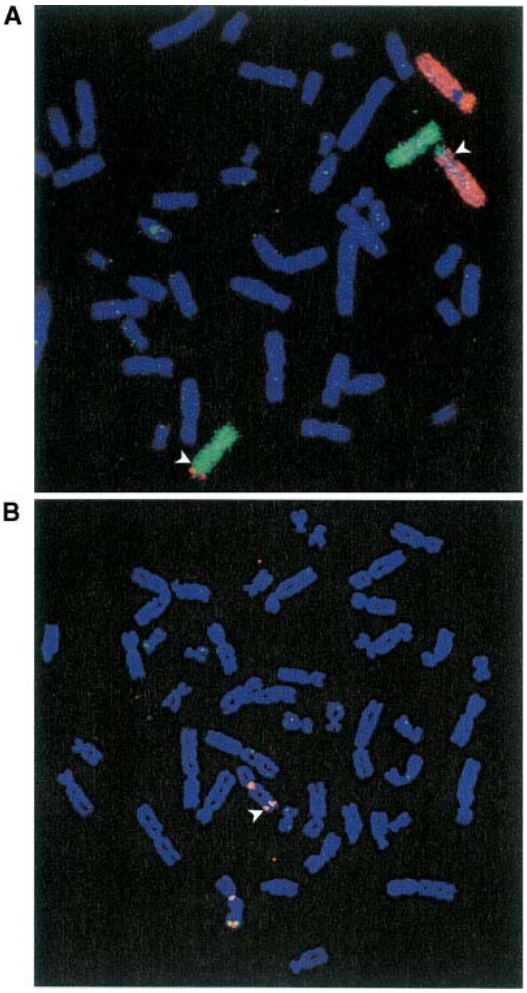

Fig. 4. **A**: Whole chromosome paint of chromosome 4 (red) and 7 (green) showing a cytogenetically balanced t(4;7)(p15;q35) in metaphase chromosomes of skin fibroblasts of the index patient. Arrowheads indicate breakpoints. **B**: FISH with chromosome 7q35 PAC's on metaphase chromosomes of skin fibroblasts of the index patient showing two signals of RP11-1137M13 (red) on the normal chromosome 7 q35 and der(7), and only one signal of RP5-1151M05 (green) on the normal 7q35, indicating a deletion of this PAC on the der(7) (arrowhead).

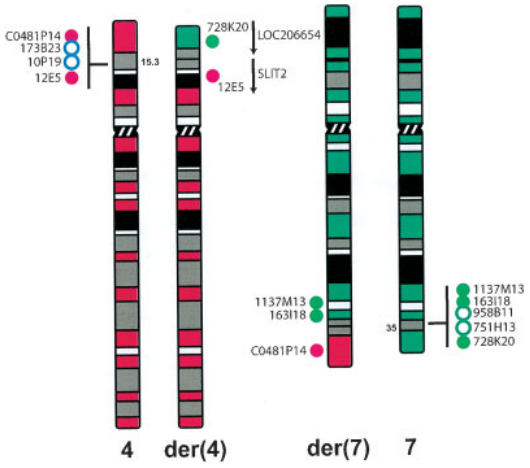

Fig. 5. Schematic illustration of the t(4;7)(p15.2;q35). The BAC/PAC's nearest to the four breakpoints are indicated; in red those of chromosome 4p15, in green those of 7q35-36. The circles in blue of 4p15 and in green of 7q35-36 are only present on the normal chromosomes and deleted in the derivatives. For the der(4) breakpoint the (predicted) genes *LOC206654* and *SLIT2* could be fused to each other.

facial dysmorphisms shared by them and our index patient is likely to be due to haploinsufficiency of one or more genes in the deleted regions 4p15.3 and/or 7q35-36.1.

Patients with larger deletions including 4p15 and 7q35, described in the literature, do not show polydactyly, so the PAP-B phenotype in our patients is most likely not only due to haploinsufficiency of a gene localized in one of the deleted regions. Based on this, we hypothesized that the translocation could lead to fusion of genes, part of genes or regulatory regions. Therefore, we searched for genes in or adjacent to the breakpoints.

The 7q35 breakpoint [on the der(7)] is most likely located downstream of the coding region of the *CASPR2* gene, so could influence mRNA levels by disrupting the 3′ UTR. However, the *CASPR2* gene is not a likely candidate, involved in the pathogenesis of the PAP-B phenotype, because it is only expressed in the central-, and peripheral-nervous system, and shown to be involved in rapid conduction of the action potential in myelinated axons [Poliak et al., 1999]. Since the physical maps of the chromosome region 4p15.32 are still not reliable enough, we can not point out a gene to which it could be fused.

The LOC206654 on chromosome 7q36.1 could be fused to (part of) the *SLIT2* gene on 4p15.31 on the der(4) (Fig. 5). There are no relevant additional data on LOC206654. SLIT2 is known to be involved in migration of neuronal precursors, as well as being expressed in the developing limb [Holmes et al., 1998; Holmes and Niswander, 2001; Vargesson et al., 2001], and could therefore be considered a candidate gene. We are currently investigating if other families with phenotypes including PAP are linked to either 4p15.3 or 7q35-36.1. They could be helpful for further attempts to identify the gene associated with the PAP-B phenotype in this family and possibly reveal if the PAP-B phenotype is caused by a mutation in the same gene as the associated anomalies in our index patient.

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Chapter 9

Discussion

Discussion

About 2000 dysmorphologic entities described in Winter and Baraitser's London Dysmorphology Database (2003) have at least one limb involved. A total of about 120 gene loci have so far been associated with human limb malformations and approximately 80 genes have been identified (Grzeschik 2002). The progress of human gene mapping, homology studies and new possibilities to genetically modify experimental animals especially mice have enabled a start of the unraveling of the molecular genetic basis of normal and abnormal limb development. The results have been used to modify existing classifications based on anatomical features and patterns of inheritance (Swanson 1976; Temtamy and McKusick 1978; Winter and Tickle 1993; Stoll *et al.* 1998; Hennekam 2002). Before a new classification system can be defined we need more understanding of the pathogenesis especially where mutations in the same gene cause different phenotypes and in cases where the same phenotype is caused by mutations in different genes as described in Chapter 3.

There are several combined limb malformations that are inconsistently categorized into the existing classifications. To avoid this an alternative recording of all anomalies has been proposed that allows a full and consistent description (Luijsterburg 2002). However, in view of the very large number of clinical entities, the variability in expression of a phenotype within families, a certain type of classification is required for the recording of diagnostic findings as well as in the search for the molecular causes. Therefore we have used the classification of Temtamy and McKusick.

Ninety percent of the human genes cloned by positional cloning were mapped first in families with traits/diseases following a Mendelian inheritance pattern (Botstein and Risch 2003).

We aimed to map the disease-causing gene in two families with a complex brachydactyly type C (BdC) phenotype (Chapter 5). Two loci were already known, one on chromosome 12q24 associated with complex BdC and another on 20q11.2. The latter locus contains the *CDMP1* gene in which dominant mutations have been described in BdC patients with a non-complex phenotype (Polymeropoulos *et al.* 1996; Polinkovsky *et al.* 1997). From this it was inferred that the clinical heterogeneity of the BdC phenotype is due to locus heterogeneity (Robin *et al.* 1997). We showed that the complex BdC phenotype described in Chapter 5 did not segregate with markers on chromosome 12q24, but with markers on chromosome 20q11.2. We identified two different mutations in the *CDMP1* gene, including one previously reported in patients with a non-complex phenotype in a family from the same geographical region as our family suggestive for a common ancestor. We concluded that an identical mutation in the *CDMP1* gene can cause a complex as well as a non-complex BdC phenotype. Therefore, the clinical heterogeneity can not only be attributed to genetic heterogeneity. This has indeed been confirmed by others (Everman *et al.* 2002; Savarirayan *et al.* 2003). It was even shown that the BdC phenotype originally mapped to chromosome 12q24, is caused by a mutation in the *CDMP1* gene on chromosome 20q11.2 (Everman *et al.* 2002). In retrospect, the linkage found by Polymeropoulos *et al.* (1996) turned out to be a false-positive finding. This could be due to a low density of tested markers and use of markers with high frequency alleles, since other potential hazards of linkage studies including genetic heterogeneity, a high phenocopy rate, or misdiagnosis are not obviously present in this study.

Considerable inter-and intrafamilial clinical variability in phenotypic expression was present

in our BdC patients. Two individuals with a minor expression of BdC were originally misdiagnosed as being unaffected, but were shown to carry the disease allele. The potential discrepancy between phenotype and genotype in individuals with a minor expression of BdC can be solved by more precise clinical diagnostics using metacarpo-phalangeal profiles (MCPP). This turned out to be decisive in the exclusion of non-penetrance of BdC in our family. Non-penetrance of this phenotype has been shown using MCPP plots (Everman *et al.* 2002), so molecular diagnostics of can be a useful addition to the standard clinical assessment.

We have explained the variability in expression of BdC by genetic/environmental modifiers. Others have adopted this and included stochastic factors (Everman *et al.* 2002; Savarirayan *et al.* 2003). In stochastic models of gene expression two alleles function independently (Cook *et al.* 1998). Stochastic activation and inactivation of both genes result in variant/fluctuating product levels. Product levels do not only depend on the kinetics of gene activity but also on product degradation. Some genes are supposed to have unstable expression kinetics, which would render them susceptible to stochastic delays of gene initiation or interruption of gene expression. This is buffered in the presence of two gene copies but a mutation of one allele may lead to a product level below the minimal threshold necessary for normal functioning during a critical developmental period.

In our families with BCD there is no difference in the clinical severity between close and distant relatives, which is in favor of involvement of stochastic factors. Our assumption that genetic modifiers are important in the expression of BdC is based on the fact that litters of the inbred homozygous *Gdf5* (*CDMP1* mouse homologue) mutants show hardly any variation in the severity of their phenotypes (Gruneberg and Lee 1973).

The complexity of the pathogenic mechanisms of *CDMP1* mutations is illustrated by the following. Patients with Grebe syndrome are either homozygotes or compound heterozygotes for mutations in the *CDMP1* gene. Some obligate heterozygous parents have BdC and others are normal (Quelce-Salgado 1968; Kumar *et al.* 1984; Curtis 1986; Costa *et al.* 1998; Savarirayan *et al.* 2003). This might be explained by a dose effect of CDMP1 on the expression of the phenotype. In some families the nature of the mutation of the *CDMP1* gene is such that its protein can not be functional (Faiyaz-Ul-Haque *et al.* 2002a). In other families with an indistinguishable Grebe syndrome phenotype a dominant negative effect of the *CDMP1* gene mutation on other BMP proteins has been postulated (Thomas *et al.* 1997). This dominant negative effect is supposed to prevent secretion of BMP's as a result of their dimerization by the mutant CDMP1. The difference in clinical severity of bone shortening between patients with either Grebe syndrome, Hunter-Thomson syndrome or DuPan syndrome, all caused by recessive *CDMP1* gene mutations, remains unexplained.

It has been postulated that the timing of ossification determines which bones become shortened in patients with mutations in the *CDMP1* gene (Costa *et al.* 1998). According to Costa *et al.* (1998) mutations in the *CDMP1* gene exerts an effect in a narrow time span late in enchondral ossification. The bones shortened in patients with BdC are indeed the last to ossify, but it is unlikely that mutations in the *CDMP1* gene would only affect ossification during this limited time since CDMP1 has multiple functions in chondrogenesis which already starts during mesenchyme condensations. This is illustrated in *Gdf5* null mice displaying mesenchyme condensations which are abnormal in shape and growth 1.5 days after their appearance leading to brachypodism (Gruneberg and Lee 1973; Storm *et al.* 1994).

The early and more extensive effect of a *CDMP1/Gdf5* mutation renders the hypothesis of Costa *et al.* (1998) unlikely.

We have mapped the first X-linked gene in a family with a radial ray deficiency (Chapter 6). Two different genes involved in maintenance of DNA stability have been identified causing syndromal radial ray deficiency in Fanconi anemia (MIM 227600) and Rothmund-Thomsen syndrome (MIM 268400). In addition in patients with Holt-Oram syndrome radial ray deficiency can be caused by mutations in the autosomal *TBX5* gene involved in FGF signaling/AER maintenance (MIM 142900). The pathogenesis of the radial ray deficiency in these three syndromes is still to be determined and can not be derived from the functions of these genes. In other patients with a radial ray deficiency and associated anomalies for instance in trisomy 18, the absence or an abnormal course of the radial artery has been observed (Van Allen *et al.* 1982 and references therein). Since vasculogenesis precedes chondro- and myogenesis the radial ray deficiencies might result from abnormal vascularization. A relative deficiency of oxygen and nutrients is proposed to inhibit chondrogenesis.
In patients with a radial ray deficiency several tissues are abnormal (Heikel 1959). Arterial hypoperfusion might affect the expression of genes involved in patterning. Amniotic sac puncture in mice leading to arterial hypoperfusion of the limbs results in newborns with severe bone shortening and syndactyly of their autopods. Inhibition of interdigital apoptosis and overexpression of the *Msx1* gene in interdigital mesenchyme was observed. It was postulated that arterial hypoperfusion of distal limb alters the expression of genes regulating limb development including those involved in interdigital apoptosis (Kaufman and Chang 2000).

Preaxial ray deficiency has also been described as a feature caused by teratogens like anticonvulsants and thalidomide/softenon (Holmes 2002). Thalidomide has been proposed to inhibit angiogenesis but that does not explain the selected distribution of the anomalies. Interaction of thalidomide with patterning genes has been described and provides a more logical explanation of the limb phenotype. There are studies that relate the use of thalidomide during pregnancy to dysregulation of a transcription factor, which affects the expression of genes involved in patterning and outgrowth of the limb (Parman *et al.* 1999; Hansen *et al.* 2002a; Hansen *et al.* 2002b). In these studies it is postulated that thalidomide causes oxidative stress resulting in a shift in cellular redox environment that leads to diminished binding of a redox sensitive transcription factor NF-κB to its target genes (Hansen *et al.* 2002a;
Hansen *et al.* 2002b). Downregulated expression of *Fgf8* is present in the AER, *Shh* in the ZPA and *Twist* in the PZ (Parman *et al.* 1999; Hansen *et al.* 2002a; Hansen *et al.* 2002b). Up to now this seems the most feasible explanation of the teratogenic effects of thalidomide. It stresses the importance of studies that relate environmental factors to genetic factors.
In our patients, however, no teratogens have been used nor was there an indication for disturbances in vascularization. We have therefore sought to explain the radial ray deficiency in our family by a model for embryonic limb patterning as proposed by Shubin and Alberch (1986). This model has been influential (Cohn 2001) in conceptualization and explains embryonic limb patterning by branching and segmentation of precartilagenous skeletal elements along the P/D axis. This was inferred from comparison of these elements in

different species. They assumed that the humerus branches into a radius and ulna and subsequently the digital arch arises through several branching steps from the ulna in a posterior to anterior sequence. According to this model, the affected elements in our patients with a radial ray defect described in Chapter 6 could theoretically be explained by an impairment of the bifurcation step of the humerus leading loss of the radius. According to this model, an X-linked recessive gene mutation should be involved in the branching of the humerus into an ulna and radius and the determination of the identity of these two elements. However, a family has been described with an X-linked recessive phenotype that includes an ulnar ray defect with loss of digits 2-4 (van den Berghe *et al.* 1978). This is not compatible with the model of Shubin and Alberch in which the ulna is a precursor of all fingers. This implies that we cannot yet explain the molecular pathogenesis of the radial ray deficiency in our patients.

We have studied a family with postaxial polydactyly (PAP) with patients exhibiting all three forms: PAP-A, PAP-B and PAP-A/B (Chapter 7). It had been shown that PAP is genetically a heterogeneous disorder with at least three loci (MIM 174200, MIM 602085, MIM 607324) to which our studies have added two other loci (Chapters 7, 8). We have experienced that heteroanamnesis can be a source of error in a linkage study in cases where individuals cannot be clinically examined. PAP-B and mild cutaneous syndactyly as minor traits are then potentially underreported. This could also result in erroneous estimation of the penetrance rate in a family. The hands are generally more reliably reported to be affected than the feet on hetero-anamnesis. The hazard of non-professional self-judgement on heteroanamnesis is illustrated by a patient with polysyndactyly who denied having polydactyly by counting five toes, which consisted of two fully fused digits five and six. This emphasizes the necessity of clinical examination in a linkage study.
We are the first to report a genetic study of patients with PAP-A, PAP-B and PAP-A/B (Chapter 7). Several authors proposed PAP-A and PAP-B to be the result of mutations in different genes. (Castilla *et al.* 1973; Temtamy and McKusick 1978; Kucheria *et al.* 1981; Temtamy 1990). This was based on differences in incidence, penetrance and involvement of the left or right limb. However, this assumption is disputable.
GLI3 deficient patients have either PAP-A or PAP-A/B but no isolated PAP-B, which shows that a *GLI3* mutation can cause the PAP-A and PAP-A/B phenotypes in one individual (Radhakrishna *et al.* 1999). Our study includes one patient with PAP-B only who does not carry the allele that harbors the gene causing PAP-A or PAP-A/B. The conclusion must be that PAP-A and PAP-A/B are likely to be caused by a mutation in one gene with a different expression and PAP-B by a mutation in a different gene.
Incidence figures for isolated PAP in Caucasians varies between 1/3300 to 1/630 and the incidence figure for syndactyly between the second and third toes is 1.17/10.000 live-births (Temtamy 1990; Castilla *et al.* 1998). In the literature as well as in our family many more patients with PAP have cutaneous syndactyly than would be expected by chance. We showed that syndactyly in consecutive generations of different branches of our family is most likely caused by a mutation in another gene than the gene causing polydactyly, which should be very rare indeed. Therefore, the genetic etiology of PAP and syndactyly in our family and that described in syndromes in Chapter 3 must be different.
Complex chromosomal rearrangements causing congenital anomalies are relatively rare, encompassing only 1.8% of all 27.000 known human mutations (Botstein and Risch 2003).

We have studied a rare nuclear family with an affected father and daughter sharing PAP-B and a complex chromosomal rearrangement (Chapter 8). We hypothesized that the translocation results in a fusion of genes or misplacement of a regulatory region. Not all translocation breakpoints did contain reliable and complete sequence data, supporting the notion that the Human Genome is not yet fully sequenced. Using FISH we identified four breakpoints and two microdeletions in chromosome 4p15.2 and 7q35. Literature search revealed patients with larger (micro) deletions than those in our patients without exhibiting limb malformations. This renders it unlikely that the genes lost are directly related to the pathogenesis of PAP-B. Theoretically the PAP-B phenotype could be non-penetrant or underreported in these relatively limited number of published cases. We favor an alternative explanation in which a regulatory element might be translocated or lost similar as recently described in patients with triphalangeal thumbs (Lettice *et al.* 2003), but this of course requires further genetic analyses.

After discussing our experimental work it seems appropiate to mention that much has been learned about the molecular processes involved in limb development and its malformations in human by homology studies, genetically modified mice and clinical and genetic studies of families with congenital malformations in several generations. In clinical practice the impact of this knowledge is still relatively modest. The most prominent contribution to clinicians is the molecular diagnostics of malformations. There is a measurable impact of the current knowledge about limb malformations on genetic counseling of patients and/or their relatives. In the Netherlands, about 6 % of the genetic counselees seek advice concerning anomalies of the skeleton and connective tissue (Ten Kate, *personal communication* 2003). Skeletal anomalies comprise 2-5 % of the observed anomalies during prenatal ultrasound examination in Rotterdam. Mendelian inherited skeletal abnormalities, which can be prenatally diagnosed by DNA analysis or biochemical studies comprised 4.4 % of all indications in this group (Annual report Dutch working group prenatal diagnosis 2001). Members of the families studied by us have asked for genetic counseling and in the case of familial radial ray deficiency also for prenatal diagnosis. Even in cases of a minor limb anomaly genetic counseling is being appreciated. In our experience couples at risk want to be informed about the cause of a particular malformation/syndrome, its recurrence risk, the expected nature and severity and possibilities of prenatal diagnosis.
It is clear that such information in the future will be more widely available once the molecular basis of the large number of congenital limb malformations is well understood as well as the relationship between the genotype and the phenotype. This forms a strong motivation to perform further clinical and experimental research, including minor anomalies. Of course genetic research of limb anomalies is also fun and is done for the sake of genuine interest in the fascinating biology of limb development.

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Summary

Samenvatting

Summary

Human congenital limb malformations are supposed to be the second most frequent occurring birth defect after those of heart and vessels. Although exact data on the incidence of limb anomalies in liveborns are not yet available it is known that about 1/250 stillborns have a congenital limb malformation. Around 2000 dysmorphologic entities including a limb anomaly have been described. Different classification systems based on anatomic bone structures and genetic factors have been used. The identification of gene defects underlying limb malformations is likely to modify their classification and provides new opportunities for genetic counseling and prenatal diagnosis. Until now about 120 genes have been mapped and 80 have been identified. However, in most instances the molecular pathogenesis is still unknown.

Chapter 1 gives an overview of the timing of the various stages of human limb development during weeks 4-8. Also the differentiation of various tissues is described as well as the positioning of specific cell types and the role of signaling centers in limb outgrowth and patterning along the proximal-distal and anterior-posterior axes. This information has mainly been obtained from studies of experimental animals like the fruit fly, chicken and mouse.

The same is true for the search of responsible gene defects and molecular pathogenic pathways in limb defects reviewed in *Chapter 2*. Molecular studies on experimental animals have shown that a relatively small number of gene families regulate limb morphogenesis. These include growth factors, like Fgf's, Wnt's, Tgfβ's, as well as signaling molecules like Hedgehogs. The encoded proteins may exert different functions by interaction with other proteins at various stages of development even within the same tissue.

Linkage studies in families with limb malformations form the major basis for the identification of responsible gene/protein defects. Homology studies and gene modification in animal models may then reveal pathogenic mechanisms. C*hapter 3* reviews the genes involved in those human limb malformations for which experimental evidence on pathogenic pathways has been obtained.

The aim of this thesis was the identification of gene loci involved in the limb anomalies brachydactyly, radial ray deficiency and polydactyly and to obtain information about the genotype-phenotype correlations (*Chapter 4*).

Brachydactyly type C (BdC) is a highly variable phenotype which minor clinical expression should be diagnosed by metacarpo-phalangeal profiles (*Chapter 5*). Involvement of the upper limbs only (non-complex form) was already shown to be caused by mutations in the *CDMP1* gene on chromosome 20q11.2, whereas the complex form (involvement of upper and lower limbs) mapped to another locus. We showed that clinical heterogeneity cannot only be attributed to genetic heterogeneity but is most likely due to genetic modifiers. In the two families described by us in *Chapter 5* we found patients with BdC of the hands only and patients with all limbs affected. All patients within a family were found to carry the same mutation in the *CDMP1* gene. We therefore reject the hypothesis that different pathogenic mechanisms of the complex and non-complex forms are based on a mutational effect in the narrow time span during which upper limb development precedes that of the lower limbs.

The fact that the mouse gene homologue is expressed during most of the chondrogenesis renders this hypothesis unlikely.

We report in *Chapter 6* a family with four males showing bilateral absence of the radius in presence of the thumbs and associated limb and cardiac anomalies. The segregation of the phenotype suggests X-linked recessive inheritance which was confirmed by linkage analysis. We could define a critical region of maximal 16 cM to the X chromosome and excluded two candidate genes.

Currently most polydactylies result from mutations in genes encoding proteins of the SHH-GLI3 pathway. Three forms of postaxial polydactyly have been described: type A (extra fifth digit), type B (rudimentary form of the fifth digit) and the combination of the two (PAP-A/B). Previously three loci were assigned to various forms of PAP and mutations in the *GLI3* gene were found to be related to PAP-A and PAP-A/B. In *Chapter 7* we describe a large family with all three types of PAP. We performed a whole genome search and found linkage between markers of chromosome 7q and patients with the forms PAP-A and PAP-A/B. This implied the finding of a new locus, which however was not present in the patient with PAP-B. The occurrence of all forms of PAP within one family and the diversity in location of the anomalies in hands and feet might point to variable gene expression or local compensating pathways. It remains, however, difficult to explain the PAP-B phenotype by another mutation since this would imply the presence of two gene mutations for relatively rare malformations within one family.

Until now no gene defect has been mapped for PAP-B. In *Chapter 8* we describe a family with a father and a daughter sharing PAP-B and a cytogenetic balanced translocation for which the father is a mosaic. The daughter has also mental retardation and minor facial dysmorphisms that can be explained by the microdeletions near the breakpoints of chromosomes 4 and 7. These, however, do not explain PAP-B since several patients with a variety of congenital abnormalities but normal limbs have been reported with chromosome deletions larger and encompassing those found by us. Since our patients have a translocation PAP-B might be the result of a fusion gene or a change in gene regulation.

In C*hapter 9* we discuss the main topics of embryonic limb development, genes involved in human congenital limb malformations and the results of our experimental work. We also mention the impact of the current knowledge about limb malformations on genetic counseling of patients and their relatives.
Members of families with limb malformations want to be informed about the cause, recurrence risk and possible interventions also in the case of minor anomalies.
Especially in prenatal diagnosis it is important to be able to predict the severity of a phenotype beyond the mere identification of a causative mutation. This requires a better understanding of the structure and function of the proteins involved in limb development and of the pathogenic mechanisms including environmental factors leading to limb malformations. Apart from its importance for clinical practice genetic research of limb anomalies is also fun and of genuine interest in the fascinating field of developmental biology.

Samenvatting

Aangeboren ledemaatafwijkingen bij de mens worden verondersteld de tweede meest voorkomende groep van misvormingen te zijn na aangeboren hartafwijkingen. Nauwkeurige gegevens over de incidentie van ledemaatafwijkingen bij pasgeborenen ontbreken, maar wel is bekend dat 1/250 doodgeborenen een ledemaatafwijking heeft. Er zijn bijna 2000 verschillende dysmorfologische beschrijvingen inclusief ledemaatafwijkingen beschreven. Verschillende classificatie systemen, gebaseerd op anatomische bot structuren en genetische factoren zijn in gebruik. De moleculaire identificatie van gendefecten die ledemaatafwijkingen veroorzaken zal in de toekomst de classificatie beïnvloeden en schept nu al nieuwe mogelijkheden bij de erfelijkheidsadvisering en prenatale diagnostiek. Op dit ogenblik zijn ongeveer 120 genen in kaart gebracht en 80 geïdentificeerd. In de meeste gevallen zijn echter de gen functies en de moleculaire pathogenese van ledemaatafwijkingen nog onbekend.

Hoofdstuk 1 geeft een overzicht van het tijdsverloop van de verschillende ontwikkelingsstadia van menselijke ledematen gedurende week 4-8. Ook wordt de differentiatie van verschillende weefsels beschreven evenals de positionering van specifieke celtypes, de rol van signaal centra bij de uitgroei van ledematen en de patroonvorming langs de proximale-distale en anteriore-posteriore assen. Deze informatie is voornamelijk verkregen uit studies van experimentele diermodellen zoals de fruitvlieg, kip en muis. Deze diermodellen zijn ook gebruikt bij de zoektocht naar verantwoordelijke gendefecten en pathogenetische mechanismen die ten grondslag liggen aan ledemaatafwijkingen. Een overzicht hiervan wordt gegeven in *Hoofdstuk 2*. Moleculaire studies van experimentele diermodellen laten zien dat slechts een klein aantal gen families de morfogenese van ledematen reguleren. Voorbeelden zijn groeifactoren zoals Fgf's, Wnt's, Tgfβ's en signaal moleculen zoals Hedgehogs. De gecodeerde eiwitten van éénzelfde gen kunnen verschillende functies uitoefenen via interacties met andere eiwitten gedurende verschillende stadia van de ontwikkeling, ook binnen een weefsel.

Koppelingsstudies in families met ledemaatafwijkingen vormen de belangrijkste basis voor de identificatie van verantwoordelijke gen/eiwit defecten. Homologie studies en genetische modificatie in diermodellen kunnen vervolgens inzicht geven in de betrokken pathogenetische mechanismen. *Hoofdstuk 3* geeft een overzicht van die genen die bij menselijke ledemaatafwijkingen betrokken zijn waarvoor experimentele gegevens over de pathogenese beschikbaar zijn.

Het doel van het experimentele werk beschreven in dit proefschrift was de opheldering van gen loci betrokken bij de ledemaatafwijkingen brachydactylie, onderontwikkeling van de radius en de duim en polydactylie; tevens is getracht informatie te verkrijgen over de genotype-fenotype relatie (*Hoofdstuk 4*).

Brachydactylie type C (BdC) is een sterk variabel fenotype waarvan de lichte uitingsvormen klinisch dienen te worden gediagnosticeerd door middel van metacarpo-phalangeale profielen (*Hoofdstuk 5*). Er werd al eerder aangetoond dat de niet-complexe vorm waarbij alleen de bovenste ledematen zijn aangedaan wordt veroorzaakt door mutaties in het *CDMP1* gen op chromosoom 20q11.2, terwijl het gen voor de complexe vorm (bovenste en onderste ledematen aangedaan) elders werd gelokaliseerd. Wij toonden aan dat klinische heterogeniteit niet uitsluitend kan worden toegeschreven aan genetische

heterogeniteit, maar hoogstwaarschijnlijk aan modificatie door andere eiwitten. We verwerpen de hypothese waarin wordt aangenomen dat de complexe en niet-complexe vormen verschillende pathogenetische mechanismen hebben, gebaseerd op een verschil in tijdstip waarop een mutatie tijdens de ontwikkeling een effect heeft. Het feit dat in de muis het gen homoloog gedurende vrijwel de gehele chondrogenese tot expressie komt pleit tegen deze hypothese.

In *Hoofdstuk 6* beschrijven wij een familie met vier mannen met bilaterale afwezigheid van de radius in aanwezigheid van de duim en andere afwijkingen van ledematen en hart. Het segregatiepatroon van het fenotype suggereert een geslachtsgebonden overerving hetgeen werd bevestigd door koppelingsonderzoek. We konden een kritische regio van maximaal 16 cM op het X chromosoom definiëren en twee kandidaat genen uitsluiten.

Tot nu toe worden de meeste polydactyliën verklaard door mutaties in genen die coderen voor eiwitten in de SHH-GLI3 keten. Drie vormen van postaxiale polydactylie zijn beschreven: type A (extra pink), type B (rudimentaire vorm van een extra pink) en de combinatie van beiden (PAP-A/B). Eerder zijn drie gen loci gekoppeld aan verschillende vormen van PAP en bleken mutaties in het *GLI3* gen gerelateerd aan PAP-A en PAP-A/B. In *Hoofdstuk 7* beschrijven we een grote familie waarin alle drie de vormen van PAP voorkomen. Via onderzoek van het hele genoom vonden we koppeling tussen merkers van chromosoom 7q en de vormen PAP-A en PAP-A/B. Dit betekende de vondst van een nieuw locus dat echter niet aanwezig was in de patiënt met PAP-B.

Het voorkomen van alle vormen van PAP in één familie en de verschillen in locatie van de afwijkingen van handen en voeten zouden verklaard kunnen worden door een variabele gen expressie of de aanwezigheid van locale compensatoire mechanismen. Het blijft moeilijk een moleculaire verklaring te geven voor het PAP-B fenotype; de veronderstelling van een andere mutatie zou immers inhouden dat er twee verschillende genen betrokken zouden zijn bij een relatief zeldzame afwijking binnen één familie.

Tot nu toe is er geen gen defect bekend voor PAP-B. In *Hoofdstuk 8* beschrijven wij een familie met een vader en zijn dochter met PAP-B en een cytogenetisch gebalanceerde chromosoom translocatie waarvoor de vader mozaïek is. De dochter heeft evenwel mentale retardatie en lichte faciale dysmorfiën welke kunnen worden toegeschreven aan de door ons aangetoonde microdeleties rond de breukpunten van de chromosomen 4 en 7. Deze bieden echter onvoldoende verklaring voor PAP-B aangezien andere reeds beschreven patiënten met grotere deleties van deze gebieden normale ledematen hebben. Omdat onze patiënten een translocatie hebben zou PAP-B het gevolg kunnen zijn van een fusie gen of een verandering in de gen regulatie.

Hoofdstuk 9 bevat een discussie van de voornaamste gegevens over embryonale ledemaat ontwikkeling, genen betrokken bij aangeboren ledemaat afwijkingen bij de mens en de resultaten van ons experimentele werk. Ook wordt ingegaan op het belang van de huidige kennis over ledemaat afwijkingen voor de erfelijkheidsadvisering van patiënten en hun familieleden.

Leden van families met ledemaat afwijkingen vragen steeds meer om informatie over de oorzaak, het herhalingsrisico en mogelijkheden van medisch ingrijpen, ook in het geval van geringe misvormingen. Voor de prenatale diagnostiek is het belangrijk betrouwbare informatie te kunnen geven over de klinische ernst van een afwijking in relatie tot een bepaalde gen mutatie. Dit vereist beter begrip van de structuur en functie van eiwitten die betrokken

zijn bij de ledemaatontwikkeling en van de pathogenetische mechanismen inclusief omgevingsfactoren die tot misvormingen van de ledematen leiden. Naast het belang voor de klinische praktijk is genetisch wetenschappelijk onderzoek van ledemaatafwijkingen gewoon leuk en van betekenis voor het fascinerende gebied van de ontwikkelingsbiologie.

Abbreviations

A/P	Anterior to Posterior
ACH	Achondroplasia
AER	Apical ectodermal ridge
Alx	Aristaless-like homeobox
ANZ	Anterior necrotic zone
Bag	Bcl-2 associated athanogene
Bd	Brachydactyly
bHLH	basic helix loop helix
Bmpr	Bmp receptor
Cbfa	Core binding factor
CBP	CREB Binding Protein
CCD	Cleidocranial dysplasia
CDMP	Cartilage derived morphogenetic protein
CDPX	Chondrodysplasia punctata
Col	Collagen
CTSK	Cathepsin K
D/V	Dorsal to Ventral
DHC	Dehydrocholesterol
DHCR	Dehydrocholesterolreductase
DNA	Desoxyribonucleic acid
DTDST	Diastrophic dysplasia sulphate transporter
EBP	Emopamil-binding protein
ECM	Extracellular matrix
EEC	Ectrodactyly-Ectodermal dysplasia-Cleft lip/palate
EN	Engrailed
Eph	Ephrin
Er	Ets related
Eya	Eyes absent
FGF	Fibroblast growth factor
Fgfr	Fgf receptor
FGFR	Fibroblast growth factor receptor
FISH	Fluorescence in situ hybridization
Fln	Filamin
Fz	Frizzled
Gdf	Growth and differentiation factor
Gli	Glioblastoma
Hgf	Hepatocyte growth factor
HH	Hamburger and Hamilton
Hox	Homeobox
Hspg	Heparan sulphate proteoglycan
ID	Interdigital

Ig	Immunoglobulin-like
Igf	Insuline-like growth factor
Ihh	Indian hedgehog
IM	Intermediate mesoderm
LBR	Lamin B receptor
Lef	Lymphoid enhancer factor
Lhx	LIM-homeobox
Lmbr	Limb region
LMC	Lateral motor columns
LMS	Limbe-Mammary syndrome
Lmx	LIM-homeobox
LPM	Lateral plate mesoderm
MCP	Metacarpo-phalangeal
MCPP	Metacarpophalangeal profiles
Meis	Myeloid ecotropic insertion site
MIM	Mendelian inheritance in man
Mrf	Muscle regulatory factor
Msx	Muscle segment homeobox
Myf	Myogenic factor
MyoD	Myogenic determination factor
NF-κB	Nuclear factor-κB
NSDHL	NAD(P)H steroid dehydrogenase-like protein
P/D	Proximal to Distal
P63	Protein 63
PAP	Postaxial polydactyly
Pax	Paired box
Pea	Polyomavirus enhancer activator
Pitx	Pituitary homeobox
PNZ	Posterior necrotic zone
PTHrP	Parathyroid hormone related peptide
PZ	Progress zone or
RA	Retinoic acid
RECQ	Recombinant
Rfng	Radical fringe
RTS	Rubinstein-Taybi syndrome
SHFM	Split-hand/split-foot malformation
SHH	Sonic hedgehog
Six	Sine ocules homeobox
SLO	Smith-Lemli-Opitz
Sox	Sry-type high mobility group box
Spdh	Synpolydactyly homologue
Tcf	T-cell factor
TD	Thanatophoric dysplasia

Tgf	Transforming growth factor
UMS	Ulmar-Mammary syndrome
VACTERL-H	Vertebral defects, anal atresia, cardiovascular defects, tracheo-esophageal fistula, renal and limb defects-hydrocephalus
VEGF	Vascular endothelial growth factors
Wnt	Wingless-type MMTV integration site
ZPA	Zone of polarizing activity

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Opleiding

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