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POSTDOCTORAL STUDIES

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Molecular Investigation into Brachydactyly Type A1

By

Mary Elizabeth McCready

THESIS

Submitted to the School of Graduate Studies in partial fulfillment of the
requirements for the degree of

Doctorate of Philosophy

Department of Biochemistry, Microbiology and Immunology,
Program in Human and Molecular Genetics,
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Abstract

Brachydactyly type A1 (BDA1) is a rare, autosomal-dominant genetic trait that affects normal limb and bone development, resulting in short or absent middle phalanges. Despite being the first human trait described in terms of autosomal dominant Mendelian inheritance, the underlying molecular cause had not been examined until relatively recently. In 2001, mutations of the *Indian Hedgehog (IHH)* gene were identified in three Chinese families with BDA1. Preliminary studies in our laboratory further demonstrated linkage of BDA1 to chromosome 5p in a Canadian kindred with a mild variant of the trait. The goal of this thesis was to characterize the BDA1 locus on chromosome 5 by reducing the critical region and examining the transcribed portions of candidate genes for mutations. To reduce the chromosome 5p critical region in the Canadian kindred, 23 microsatellite markers were examined and the number of family members analyzed was increased to include 22 affected and 16 unaffected individuals. By doing so, we defined a 7 cM (3.03 Mb) critical region flanked by the recombinant markers D5S1986 and D5S426. Fifteen candidate genes within the region were examined for mutations, however none were identified. Further characterization of the chromosome 5p critical region was made by recruiting twelve additional families into the study. Of the twelve families, five had a G→A transition at codon 100 of the *IHH* gene. The mutation causes an aspartate to asparagine amino acid substitution and supports a role for IHH in normal bone development. Linkage analysis to chromosome 5 in two other pedigrees indicated that the trait was not linked to this locus in these families, and provided further support for genetic heterogeneity underlying the aetiology of BDA1. IHH mutations were not found in the remaining five families, thus increasing the likelihood that the chromosome 5 locus is involved in these pedigrees.

Linkage could not be established in these families, however, since the trait was sporadic, and not familial. Identification of additional BDA1 families in which the trait is linked to chromosome 5, and further mutational analysis will be required to identify the gene from chromosome 5p that causes BDA1.

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They say that it takes a village to raise a child. I believe that the same holds true for completing a doctoral thesis. I would like to thank my “village” since without them I could not have accomplished this body of work. First and foremost, I would like to thank Dr. Dennis Bulman, for his mentorship, his patience and his enthusiasm. While I truly appreciate all that he has taught me about molecular genetics, the lessons I have valued most are those that are hardest to teach - how to become a researcher. While in this laboratory I have found a new confidence in my abilities and have renewed my love for science. Thank you for providing that opportunity. To those from “the Bulman lab” past and present, in particular Heather MacDonald, thank you for all of your help with this research. The technical assistance, the discussion, and the coffee breaks were invaluable. I would also like to thank Dr. Christine Armour and Dr. William Farabee. If it were not for their discoveries, I would not have had the pleasure of working on this specific project. To all of our collaborators, thank you for your clinical expertise and for providing us with DNA samples. Thank you to individuals from all thirteen pedigrees for participating in the study. To Dr. Valerie Wallace, and to members of my advisory committee, Drs. Rashmi Kothary, John Bell and Sylvia Vidal, thank you for the discussions about my research and about IHH. I would particularly like to thank OGSST and CIHR for funding. Finally, and most importantly, thank you to all of my family for your support through out my graduate career. Thank you John, for standing by me through all of the late nights, through a move to a new city, and through many stressful days. I couldn’t have done it without you. And to my son, thank you just for being you.

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List of Abbreviations

2q	long arm of chromosome 2
3PAP	phosphatidylinositol-3 phosphate 3 phosphatase adaptor subunit
5p	short arm of chromosome 5
ACFD	acrocapitofemoral dysplasia
ADAMTS	a disintegrin-like and metalloprotease with thrombospondin repeats
ADAMTS12	a disintegrin-like and metalloprotease with thrombospondin type 1 motif, 12
AER	apical ectodermal ridge
AMACR	alpha-methylacyl-CoA-racemase
ASOH	allele specific oligomer hybridization
Asp	aspartic acid
Asn	asparagine
AURK	aurora kinase B
BAC	bacterial artificial chromosome
BDA1	brachydactyly type A1
BDA2	brachydactyly type A2
BDB	brachydactyly type B
BDC	brachydactyly type C
BLAST	basic local alignment search tool
BMP	bone morphogenetic protein
BMPR1B	bone morphogenetic protein receptor 1B
bp	basepair
BSA	bovine serum albumin
C1QTNF3	c1q and tumour necrosis factor related protein 3
cDNA	complementary deoxyribonucleic acid
CDMP1	cartilage-derived morphogenetic protein-1
chr	chromosome
Ci	cubitus interruptus
CIHR	Canadian Institutes for Health Research
CiR	cubitus interruptus repressor fragment
cM	centimorgans
Cos2	costal 2
Cys	cysteine
dATP	deoxyadenosine triphosphate
DHH	desert hedgehog
dH₂O	distilled water
DLX5	distal-less homeobox-5
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
En	engrailed
EST	expressed sequence tag

FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FISH	fluorescence <i>in-situ</i> hybridization
Fu	fused
GDF	growth and differentiation factor
GOLPH3	golgi phosphoprotein 3 (coat-protein)
Glu	glutamic acid
HCl	hydrochloric acid
HGP	Human Genome Project
Hh	hedgehog
Hh-Np	hedgehog N-terminal peptide
HOX	homeobox
IAP	Inhibitor of Apoptosis Protein
IHH	indian hedgehog
Ile	isoleucine
KCl	potassium chloride
Leu	leucine
LMX1	LIM homeobox transcription factor 1
LOD	logarithm of the odds ratio
LOH	loss of heterozygosity
Lys	lysine
MATP	melanoma antigen AIM-1
Mb	megabase
mg	milligram
Mg	magnesium
MgCl₂	magnesium chloride
ml, µl	millilitre, microlitre
mM, µM	millimolar, micromolar
mRNA	messenger RNA
MSX1	MSH homeobox homolog 1 (<i>Drosophila</i>)
MSX2	MSH homeobox homolog 2 (<i>Drosophila</i>)
MT	microtubule
N/A	not applicable
NCBI	National Centre for Biotechnology Information
ng	nanogram
NHGRI	National Human Genome Research Institute
NPR3	natriuretic peptide receptor C/ guanylate cyclase C
nt	nucleotide

OGSST	Ontario Graduate Scholarship in Science and Technology
PC4	activated RNA polymerase II transcription cofactor 4
PCR	polymerase chain reaction
PDZK3	PDZ domain containing 3
PKA	protein kinase A
PPR	parathyroid hormone/ parathyroid hormone related protein receptor
Ptc	patched receptor
PTHrP	parathyroid hormone related protein
PZ	progress zone
RAI14	retinoic acid induced 14
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
ROR2	receptor tyrosine kinase-like orphan receptor 2
RUNX2	runt-related transcription factor 2
SALPR	somatostatin and angiotensin-like peptide receptor
SD	standard deviation
Ser	serine
SHH	sonic hedgehog
SMA	spinal muscular atrophy
SMO	smoothen receptor
SNP	single nucleotide polymorphism
SOX	SR Y (sex-determining region Y)-box
STS	sequence tagged site
SuFu	suppressor of fused
θ	(theta) recombination fraction
θ_{max}	(theta maximum) maximum recombination fraction
TARS	threonyl-tRNA synthetase
TGFβ	transforming growth factor β
U	Units
UCSC	University of California Santa Cruz
Vs	Versus
ZFR	zinc finger RNA binding protein
Z$_{max}$	maximum LOD score
ZPA	zone of polarizing activity

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Chapter 1 – Brachydactyly:
Background Information and Literature Review

1.1 Brachydactyly Descriptions

The brachydactylies are a group of rare, congenital disorders that affect normal bone development and patterning, resulting in shortened digits. First classified by Bell into types A1, A2, A3, B, C, D and E (1), the brachydactylies were reclassified by Temtamy and McKusick (2) and Fitch (3) based on the bones and digits involved (see Table 1).

Table 1: Description of the Different Classifications of Brachydactyly (2;3)

Brachydactyly Classification	OMIM Accession	Description
Type A1; Fitch Type 9 Farabee Type	112500	Broad hands with concomitant shortening of all digits. All bones of the hands or feet may be reduced in size, but shortness is most apparent in middle phalanges of digits 2 through 5, and the proximal phalanges in digit 1. Middle phalanges may be fused to terminal phalanges.
Type A2; Fitch Type 2; Brachymesophalangy II; Mohr-Wreidt Type	112600	Shortening of the middle phalanx in digit 2, resulting in a triangular shaped bone and clinodactyly.
Type A3; Fitch Type 5; Brachydactyly- clinodactyly; Brachymesodactyly V	112700	Shortening of the middle phalanx in digit 5. Clinodactyly of digit 5 occurs due to sloping of the articular surface on the middle phalanx.
Type A4; Temtamy Type; Brachymesodactyly II & V	112800	Shortening of the middle phalanges in digits 2 and 5. Radial clinodactyly of digit 4. May be a mild expression of Type A1 (3).
Type A5	112900	Absence of the middle phalanges in digits 2 through 5, associated with nail dysplasia and bifid thumbs. Frequently undistinguishable from Type B (3).
Type B; Fitch Type 8	113000	Absence of the terminal portion of digits 2 through 5 including the nail. Proximal phalanges are of normal length. Terminal and middle phalanges are absent or replaced by a small spherical bone.
Type C; Fitch Type 11	113100	<u>Haw's Type</u> : Shortening of the middle and proximal phalanges of digits 2 and 3. Occasional hypersegmentation of proximal phalange. <u>Robin's Type</u> : Shortening of the middle phalange in digits 2, 3 and 5 and the first metacarpal. Distinguished from mild Brachydactyly Type A1 by a longer fourth middle phalanx than the third middle phalanx.
Type D; Fitch Type 1	113200	Short and broad terminal phalanx of the thumb and big toe.
Type E; Fitch Type 4	113300	Short metacarpal in digit 2.
Type E; Fitch Type 5	113300	Short metacarpal in digit 4.
Type E; Fitch Type 6	113300	Short metatarsal in digit 4.
Type E; Fitch Type 7	113300	Short metacarpal and metatarsal in digit 4.
Type E; Fitch Type 10	113300	Short metacarpal and distal phalanges. Short stature.

In 1903, as part of his Ph.D. thesis, William C. Farabee described a large family from Pennsylvania with a hand disorder and correctly surmised that the trait was transmitted as a dominant condition, making brachydactyly type A1 (BDA1) the first human trait described in terms of autosomal dominant Mendelian inheritance (4). Individuals affected by the disorder were characterized by short, broad hands and overall short stature. Examination of radiographs showed that the middle phalanges of affected individuals were absent in digits two through five, and that the proximal phalanx in digit one was short. The feet were likewise affected, having only two phalanges in each toe. Although the disorder did not drastically affect daily living, affected individuals described hyperextensible hand joints and weak grips. The fingers had only one volar crease, and the skin was thick and pulpy. Ten years later two English families with similar characteristics were described by Harry Drinkwater, in which the middle phalanges of affected individuals were either absent or severely hypoplastic (5;6). Based on the similar characteristics and shared kin, Drinkwater proposed that the first family he had described was the English branch of that described by Farabee, although a clear relationship between the two families has not yet been established (6;7). Drinkwater also showed that in affected individuals apparently lacking the middle phalanges, the bone was not always absent; instead the middle and terminal phalanges were fused. This fusion, known as terminal symphalangism, results in a chess pawn-shaped bone (5;6). Fusion between the middle and terminal phalanges is further supported by evidence of children with separate middle and terminal phalanges despite the presence of a single bone in the parent (5-7).

Since the original reports of brachydactyly by Farabee and Drinkwater, a number of families have been described worldwide (for example, 8-15). Phenotypes may range from a severe variant in which the middle phalanges of affected individuals are absent or fused to

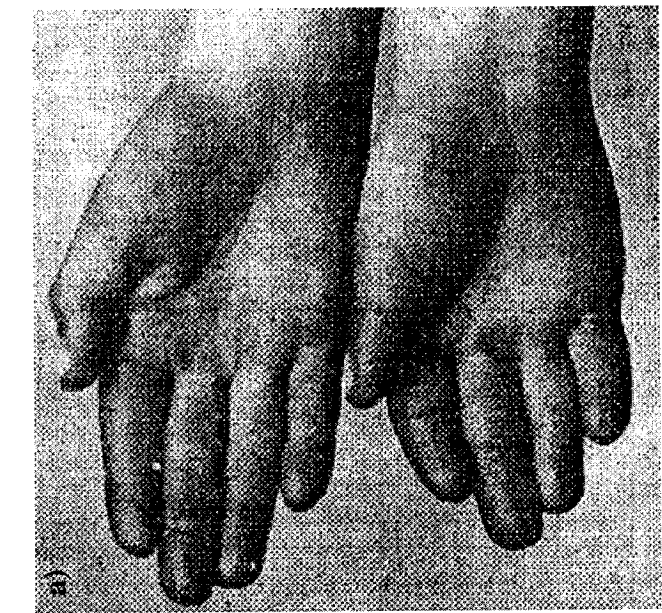
the terminal phalanges, to a mild variant in which the middle phalanges are intermediate in length between unaffected kin and those with severe shortening (see Figure 1) (14). In both mild and severe forms of BDA1, the shortening is most prominent in digits two and five, followed by digits four, then three. Similarly, if terminal symphalangism is present, it is more common in digits two and five than four and three (3).

Although shortening of the middle phalanges is the hallmark feature of BDA1 a number of other features have been reported including short stature (6;14;16;17), prominent sweat glands on the dorsal side of the hands (6), radial/ulnar clinodactyly (5;12;13;15;18;19), shortening of the first, third (15;20), or fifth metacarpal (13), and premature epiphyseal closure (3;7;13;14). In a comprehensive analysis of surviving members from Farabee's kindred, Haws and McKusick further described sloping of the distal end of the radius, an absence of the styloid process of the ulna, abnormally shallow acetabulum and glenoid fossa, and an elliptical, rather than round, humeral head (7).

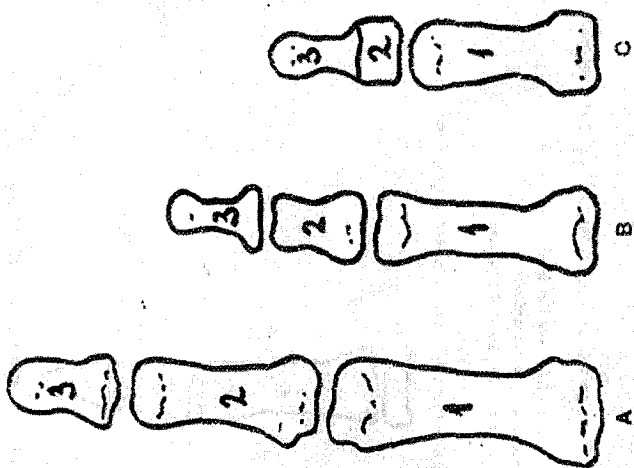
1.2 BDA1 Syndromes

Although BDA1 can occur as an isolated malformation (6;7;12-15;21), it has also been described as part of complex syndromes. Some of the most commonly reported associated disorders include nystagmus (16;17), developmental delay, mental retardation (16-18), and scoliosis (16;19;22). The observation of these characteristics in multiple families suggests that the gene(s) responsible for BDA1 may also be expressed in tissues other than bone (16;19). There have also been reports of a family with BDA1 and degenerative arthritis of the knee due to an abnormal meniscus (22). Although it is conceivable that the abnormal meniscus and BDA1 may have resulted from the same

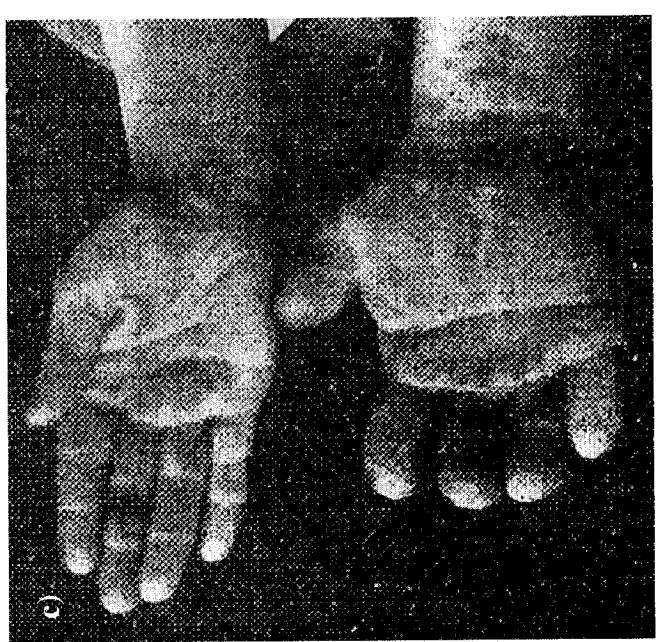
Figure 1: Comparison of unaffected hands to brachydactylous hands. a) & c). The bottom hand in each figure is that of an individual affected with BDA1, while the top hand is that of an unaffected kin. The bottom hand from a) is that of an individual with minor BDA1 and the one from c) is of an individual severe BDA1. b) Drawings comparing proximal, middle and distal phalanx size between A- a normal individual, B- an individual with mild BDA1, and C- an individual with severe BDA1 (14).



a)



b)



c)

Reprinted with permission from Drinkwater H. 1912. Account of a Family Showing Minor Brachydactyly, *J. Genet.* 2, 21-40. Journal of Genetics copyright Indian Academy of Sciences, Bangalore

developmental defect, several of the BDA1 affected individuals had normal menisci, suggesting that the two characteristics may be coincidental or result from mutations in two different genes (22). Of particular interest to this project, was a report in 1995, of a girl presenting with BDA1 and Klippel-Feil anomaly (23). Cytogenetic analysis of this individual revealed a balanced reciprocal translocation between 5q11.2 and 17q23, suggesting that the gene responsible for BDA1 may be located on either chromosome 5 or 17.

In addition to the interfamilial heterogeneity described above, there have also been reports of significant intrafamilial heterogeneity (16;20;24). In some instances this heterogeneity is limited to the severity of phenotype observed between family members, but may extend to include additional developmental defects. In an extreme case Slavotinek and Donnai described a descendant of Drinkwater's third brachydactylous family who had severe manifestations of BDA1 (16). In addition to absent middle phalanges in his hands and feet, Slavotinek and Donnai described an abducted forefoot that was dislocated from the talus and hand deformities including ulnar subluxation of the wrist and fixed flexion deformities of the first metacarpal. The boy also had a variety of other disorders including short stature, scoliosis, nystagmus, and developmental delay. His brother, who was also diagnosed with BDA1, exhibited severely shortened middle phalanges, single volar creases and velvety skin, but the remainder of his examination was normal, with normal development. Slavotinek and Donnai (16) suggested that the phenotype of the first boy was a representation of the more severe manifestations of the disease. The phenotypic differences between the sibs could be explained by the presence of modifier genes or a variation in expressivity (16).

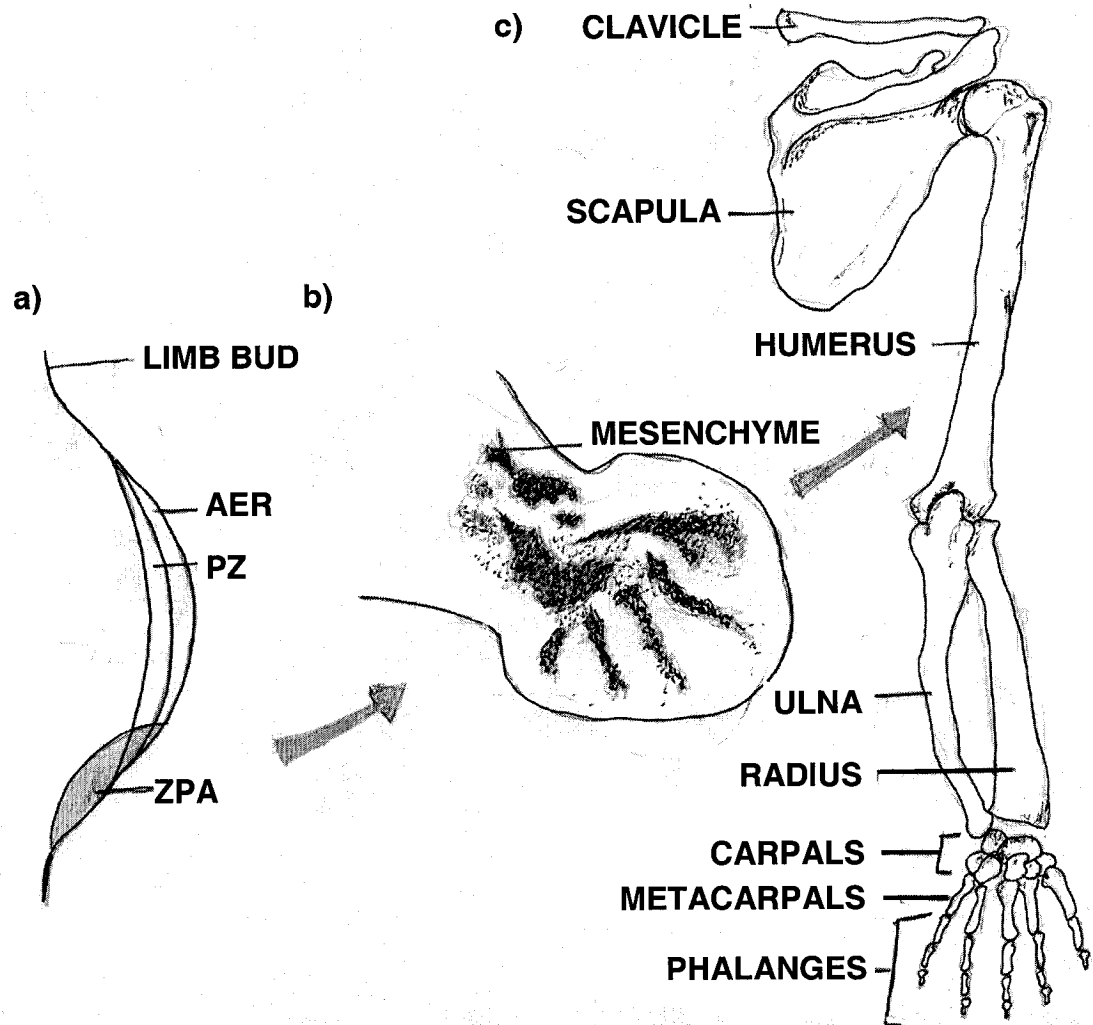
1.3 Limb and Bone Development

Congenital limb anomalies can arise from the disruption of normal limb development at a number of stages including limb outgrowth, mesenchymal condensation prior to chondrogenesis, proliferation of cartilaginous structures, or replacement of cartilage tissue with bone by endochondrial ossification (3). Although the mechanisms regulating appendicular skeletal development have not been fully elucidated, the characterization of genes that cause skeletal abnormalities in humans and mice have provided scientists with unique insights into the molecular pathways that regulate bone patterning and development (reviewed in 25-27). Many of these genes are also involved in the development of other vertebrate systems and include members of the Wnt, fibroblast growth factor (FGF), bone morphogenetic protein (BMP) and hedgehog (Hh) signaling pathways. Expression of transcription factors, such as Sox, Lmx1, Runx2 and members of the Hox family, are also critical for normal limb development, as are numerous matrix components and paracrine hormones (reviewed in 26;28-30). The identification of these genes has been combined with cellular and morphological studies to provide a framework for understanding how the embryonic limb bud grows to form functional arms and legs (reviewed in 31;32).

1.4 Limb Patterning

The pattern of skeletal development within the limb bud of vertebrates is determined by three sets of cellular interactions that inform the cells of its position along the proximodistal, anteroposterior and dorsolateral axes of the limb. These interactions include the apical ectodermal ridge (AER) and the associated progress zone (PZ), the ectodermal cells surrounding the limb and the zone of polarizing activity (ZPA) (refer to figure 2).

Figure 2: Anatomy of the developing forelimb. a) Four cellular regions of the limb bud including the apical ectodermal ridge (AER), the progress zone (PZ), the zone of proliferating activity (ZPA), and the mesoderm. b) condensation of mesenchymal cells to form outlines of future bones. c) Final structure of skeletal arm.



The AER is a structure formed at the tip of the limb bud by thickening of the ectodermal tissue. Its critical role in mediating limb outgrowth was first demonstrated by experiments in which the AER was removed from chick limb buds, resulting in truncation- like defects (33). Removal of the AER at earlier stages of limb development resulted in more extensive truncation defects than in experiments in which the AER was removed later during development (33). Although a number of proteins are expressed in the AER, the key functions of this structure are mediated by members of the FGF family. It has been shown that FGF-soaked beads can substitute for the AER, thus rescuing normal limb development after removal of the AER in chicks (34;35). While mutations of FGF genes have not yet been identified in human diseases, mice lacking *fgf-10* exhibit an amputation-like phenotype in which both hind- and forepaws are truncated (31). Mutations of FGF receptors also result in limb anomalies and have been identified in a number of human development disorders including Apert, Pfeiffer and Crouzon syndromes (reviewed in 36).

In addition to mediating limb outgrowth, the AER also regulates patterning along the proximodistal axis by secreting *Fgf8*, which induces proliferation and prevents differentiation of mesodermal cells in the underlying PZ (reviewed in 36). Summerbell *et al.* proposed the “progress zone” model, which postulated that cells acquire positional information along the proximal-distal axis by measuring the length of time spent in the progress zone (37). As cells in the PZ are always proliferating, and the size of the zone remains constant, cells must also constantly leave the PZ. Those cells that leave the PZ early acquire a proximal specification, where as cells that leave the PZ later acquire a more distal specification (37). Alternatively, Dudley, Ros and Tabin proposed that the various limb segments are specified early in limb development as distinct domains (200). Cell fates within these domains subsequently become progressively determined during limb outgrowth

(200). Interactions between the AER and PZ compartments are necessary for maintenance of these structures during outgrowth of the limb, and likely involve a *Fgf/Wnt3/β-catenin* regulatory loop between the two compartments (38).

Another family of genes that are critical for determining proximal-distal, as well as anterior-posterior asymmetry is the *Hox* family of transcription factors. HOX proteins are evolutionarily conserved throughout the animal kingdom and are characterized by a 60 amino acid DNA-binding motif called the “homeodomain” (39). Many vertebrates have 39 *Hox* genes, which are arranged in four clusters, *HOXA* to *HOXD* (40). Expression of the genes within each cluster is spatially and temporally regulated during development such that the 3’ genes tend to be expressed early in development and in the anterior/proximal body segments, while 5’ genes are expressed later in development and in the posterior/distal segments (41). It has been shown that *Hoxa9–Hoxa13* and *Hoxd9–Hoxd13* are required for normal limb development in vertebrates, while *Hoxa13* and *Hoxd10–Hoxd13* are expressed during proper hand/foot (autopod) development in chicks and mice (42;43).

The primary posteriorizing signal in the limb is produced by the ZPA, a group of mesodermal cells located at the posterior margin of the limb bud. Transplantation of the ZPA to the anterior portion of the limb causes mirror-image duplication of structures along the anteroposterior axis (44). In 1975, Tickle *et. al.* proposed a gradient model for ZPA activity and predicted that the ZPA produced diffusible molecules which could establish a chemical gradient across the limb bud. Positional information of cells along the anteroposterior axis could thus be determined by the concentration of the factors in the gradient (44). The first molecule identified that could substitute for ZPA activity was a vitamin A derivative known as retinoic acid (45). Retinoic acid is secreted by cells in the ZPA and induces expression of sonic hedgehog (SHH) and bone morphogenetic proteins (BMPs) (46;47). It has since been suggested that SHH is the key posteriorizing signal produced by the ZPA, and that it acts initially as a long-range signaling molecule to prime

cells for digit formation (48). Later it acts as a short-range signal to induce Bmp2 expression, which in turn specifies digit identity (48).

Specification of the dorsoventral axis has been less well characterized in the limb than specification of the other axis, although three genes have been identified that effect dorso-ventral asymmetry. These include *Wnt7a*, *Lmx1* and *Engrailed (En)* (49-52). *Wnt7a* is expressed in dorsal ectoderm and induces expression of the homeodomain transcription factor, Lmx-1, in the dorsal mesoderm (49;50). Ectopic expression of *Lmx1* in the ventral ectoderm results in a double-dorsal limb phenotype (46;52), while loss of *Wnt7a* activity in mice results in dorsal to ventral transformations of limb mesoderm (49). These experiments suggest that *Wnt7a* and *Lmx1* proteins are dorsalizing signals. *En*, by contrast, is expressed by cells in the ventral ectoderm (52) and is necessary to repress *Wnt7a* expression (52). Mice lacking a functional copy of *En* display a double dorsal phenotype, further supporting a role for this gene in maintenance of dorsoventral asymmetry (52).

While the definition and maintenance of the proximodistal, anteroposterior, and dorsoventral axis are important for normal limb development, patterning can also be influenced by improper mesenchymal condensation. Some have even argued that proper condensation of mesenchyme tissue is the most important factor affecting limb patterning since abnormally shaped condensations, or condensations with too few mesenchymal cells, can cause misshapen, short or absent skeletal elements (53). Although the factors necessary for proper mesenchymal condensation have not all been characterized, the transcription factor, Sox9, appears to be necessary for both mesenchymal condensation, and establishment of osteochondroprogenitor cells (28;54). Inactivation of *Sox9* from mice limb buds before mesenchymal condensation, results in the complete absence of bone and cartilage, whereas inactivation of *Sox9* after mesenchymal condensation results in generalized chondrodysplasia

with most cells arrested at the mesenchymal stage (54). The importance of Sox genes in skeletal development is further emphasized by the identification of *SOX9* mutations in individuals with the hereditary skeletal disorder, campomelic dysplasia (55), which is characterized by bone hypoplasia, particularly of bones formed by endochondrial ossification.

1.5 Endochondrial Ossification

The development of bone from mesenchymal tissue can occur directly by intramembranous ossification, or indirectly by endochondrial ossification. The first process occurs primarily in the flat bones of the skull. Endochondrial ossification, by contrast, requires a cartilage intermediate and is the primary process by which bone development occurs in the axial and appendicular skeletons. In the limb, bone development occurs exclusively by endochondrial ossification.

As the limb grows along the proximodistal axis, the mesenchymal cells behind the PZ begin to condense to form the basic shapes of the future bone. At the core of these condensations, called blastula, cells differentiate into chondrocytes and start to secrete a cartilaginous matrix rich in type II collagen and proteoglycan aggrecan (25;31). Periphery cells also differentiate and secrete a perichondrial sheath that surrounds the cartilage anlagen. Since bone does not have the capacity to grow, formation of a cartilaginous structure is essential for skeletal growth prior to bone deposition. Proliferation of the chondrocytes and perichondrial cells allows the anlagen to grow lengthwise and radially. Defects in proper cartilage anlagen formation, or in mesenchymal condensation, can result in shortened, abnormally shaped or absent bones (25). Shortly after formation of the cartilage structure, cells in the center exit the cell cycle, and mature into hypertrophic chondrocytes. They

enlarge, begin to secrete type X collagen, and promote mineralization of the matrix. This in turn allows for vascularization and invasion by osteoblasts from the perichondrium. The terminally differentiated chondrocytes finally die through apoptosis and the calcified matrix is replaced with trabecular bone. As the process of hypertrophy extends outwards from the center of the bone the zone of proliferating chondrocytes is restricted to the apical ends. Secondary ossification centers may form at the distal ends of the bone, leaving a small region between the primary and secondary ossification center that continues to proliferate. This region is called the epiphyseal growth plate and is the only portion of the skeletal element that retains growth potential. The cells within this region arrange themselves in columns including a zone of resting chondrocytes followed by zones of proliferating, pre-hypertrophic, hypertrophic and terminally differentiated chondrocytes. The conversion of calcified cartilage to trabecular bone matrix continues until adolescence when the epiphyses close and the growth plate is finally replaced by bone.

1.6 Growth Plate Genetics

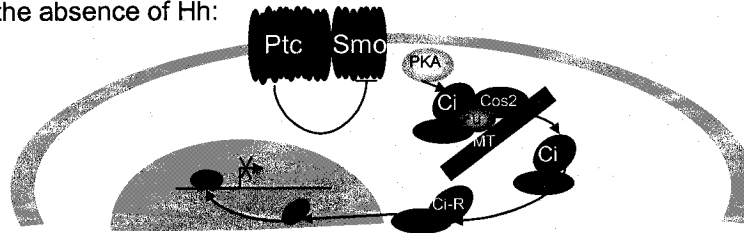
Within the growth plate, the transition of chondrocytes from the proliferative to terminally differentiated states is a highly regulated process. Maintenance of appropriate numbers of cells within each fraction is necessary to ensure normal growth and development. A key modulator of proliferation and differentiation within the growth plate is the Indian Hedgehog (IHH) protein. Mice homozygous for an *Ihh* null allele (*Ihh*^{-/-} mice) show a reduced capacity for chondrocyte proliferation, a shift in the fraction of hypertrophic chondrocytes towards the axial ends, and a lack of osteoblast development within the perichondrium (56).

IHH is part of a conserved family of secreted proteins including IHH, SHH, and desert hedgehog (DHH). These three proteins are highly homologous and are processed through an autocatalytic cleavage event to generate an N-terminal polypeptide containing all of the signaling function, and a C-terminal polypeptide necessary for autoproteolysis (57;58). The N-terminal polypeptide (Hh-Np) is further modified through the covalent attachment of a cholesterol moiety to its C-terminus and palmitoylation of the N-terminus (59;60). Addition of these molecules is necessary for activity and long-range signaling of Hh-Np (61;62). Although the three identified mammalian hedgehog proteins have different functions during fetal development, it has been shown that they each transduce signals through a common pathway including the Patched receptor (Ptc), a twelve-transmembrane-domain protein, and the Smoothened receptor (Smo), a seven-transmembrane-domain protein with homology to the family of G-protein coupled receptors (63;64). In the first model of this pathway, it was believed that Ptc inhibited Smo through a direct interaction at the plasma membrane. Upon binding of the Hedgehog (Hh) protein to Ptc this inhibition was relieved through either a conformational change, or through steric interference (reviewed in 65;66) (see Figure 3a). Recent evidence has challenged this model, however, and suggests that Ptc may indirectly regulate Smo activity by affecting the levels of Smo protein expressed at the plasma membrane (see Figure 3b) (reviewed in 67). Deneff *et al.* demonstrated that, in the absence of Hh, Ptc can reduce the levels of Smo protein, while treatment of cells with Hh, or reduction of Ptc by RNA interference, results in accumulation of phosphorylated Smo at the plasma membrane (68). Moreover, it has been shown that soluble and membrane-tethered forms of Shh are internalized by Ptc through an endocytic process

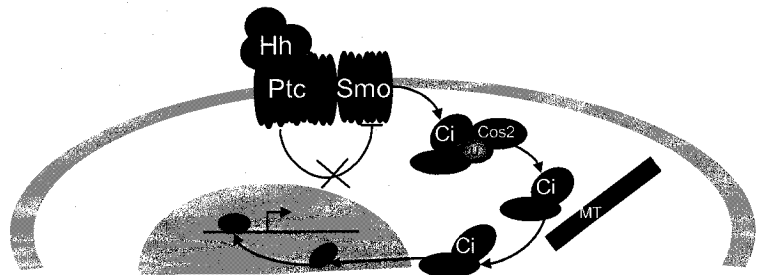
Figure 3: Models of Hh signalling. a) This diagram depicts Hh signalling through the “direct interaction” model in which the Ptc receptor inhibits Smo activity through a direct interaction between the two molecules. The Cubitus interruptus (Ci) transcription factor exists in a multi-protein complex that includes Costal 2 (Cos2), Fused (Fu), Suppressor of Fused (SuFu), and microtubules (MT). In the absence of Smo signalling, the Ci protein is cleaved in a Protein Kinase A (PKA)-dependent reaction. The cleaved fragment (Ci-R) then translocates into the nucleus and inhibits Hh-responsive genes. In the presence of Hh, the interaction between Ptc and Smo is relieved, thus allowing Smo signalling, hyperphosphorylation of Fu and Cos2, release of the multi-protein complex from MTs, stabilization of the full-length Ci protein and transcription of the Hh-target genes (reviewed in 67). b) In the most recent models of Hh signalling Ptc and Smo expression at the membrane are differentially regulated by Hh (68;70). In the absence of Hh, Ptc inhibits Smo activity, perhaps by promoting Smo dephosphorylation (68). Smo protein levels are further regulated postranslationally by PKA, which either stimulates Smo degradation or reduces its rate of synthesis (70). Hh-binding induces opposite changes in the subcellular localization of Ptc and Smo, resulting in a decrease of Ptc at the cell surface and an accumulation of hyperphosphorylated Smo (68). After Hh-binding to the Ptc receptor, the protein complex is internalized by endocytosis (69) and may be trafficked to the lysosomal compartment for degradation (68).

a)

In the absence of Hh:

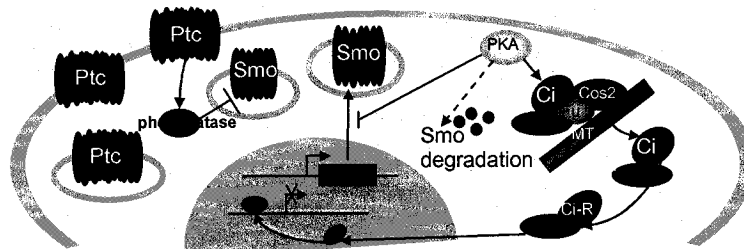


In the presence of Hh:

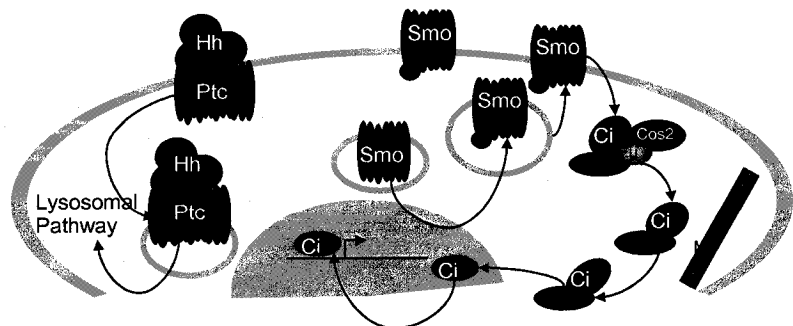


b)

In the absence of Hh:



In the presence of Hh:



Modified from Nybakken, K. and Perrimon, N. 2002. *Curr.Opin.Genet.Dev.* **12**, 503-511; Alcedo, J. Zou, Y. and Noll, M. 2000. *Mol. Cell* **6**, 457-465; and Deneff, B., Neubaser, D., Perez, L., and Cohen, S.M. 2000. *Cell* **102**, 521-531.

and that treatment of cells with Hh results in localization of Ptc and Smo to different compartments within the cell (68-70).

Despite activation of a common signal transduction pathway, and an ability to substitute for each other in a number of functional assays (71), the hedgehog proteins elicit distinct biological responses *in vivo* and *in vitro* (72). It has been hypothesized that the differences in function are partially determined by distinct spatial and temporal expression patterns during development, as well as slight sequence differences between the proteins (72).

During embryogenesis Ihh is expressed throughout the primordial cartilage, but eventually becomes restricted to the pre-hypertrophic chondrocytes in the growth plate (71). Evidence from the misexpression of Ihh in limb development, and from *PTHrP*^{-/-} mutant mice indicates that Ihh and parathyroid hormone-related protein (PTHrP) regulate hypertrophic differentiation through a negative feedback loop (71;73). In this negative feedback model, pre-hypertrophic chondrocytes transiently express Ihh. The secreted Ihh protein induces expression of PTHrP in the adjacent perichondrium and the distal periarticular perichondrium, which in turn blocks differentiation in cells adjacent to the pre-hypertrophic zone expressing the parathyroid hormone/parathyroid hormone - related protein receptor (PPR). As pre-hypertrophic cells become hypertrophic, Ihh levels drop, leading to attenuation of the feedback loop and permitting more cells to proceed to the pre-hypertrophic stage (71;73). The suppression of Ihh expression by PTHrP further prevents excess accumulation of PTHrP in the proliferative fraction of chondrocytes (74). By comparing *PPR*^{-/-}/wild type chimeric mice with *Ihh*^{-/-};*PPR*^{-/-}/wild type and *Ihh*^{-/-}/wildtype chimeric mice, Chung *et al.* (75) demonstrated that interactions between Ihh and PTHrP are also necessary for determining the position of hypertrophic cells in the growth plate with respect to the

periarticular region. In addition to PTHrP-dependent effects on differentiation, *Ihh* can also directly promote chondrocyte proliferation through PTHrP-independent mechanism and may be necessary for driving growth along the longitudinal axis of the bone (76). The absence of a bone collar in *Ihh*^{-/-} mice further demonstrates a role for *Ihh* during osteogenesis, although it is not necessary for initiation of osteoblast differentiation (75).

Many of the pathways important during limb patterning also have multiple roles during endochondrial ossification. Several FGF receptors (FGFR), for example, are expressed in proliferating, hypertrophic and perichondrial chondrocytes (29;77). The best characterized of these is FGFR3, which is upregulated in proliferating chondrocytes and inhibits proliferation by downregulating *Ihh* signaling and *Bmp4* expression (78;79). Activating mutations of FGFR3 have been identified in several chondrodysplasias and chondrodystoses including achondrodysplasia, the most common form of dwarfism (80;81), thanatophoric dysplasia (82), hypochondroplasia (83), Crouzon syndrome (84), Meunke syndrome (85), and the San Diego type of platyspondylic lethal skeletal dysplasia (86). Many of the FGFR ligands that induce signaling during endochondrial ossification remain elusive, but recent evidence from mice suggests that *Fgf18* may be one such relevant ligand. *Fgf18*-knockout mice exhibit a skeletal phenotype similar to mice lacking *Fgfr3*, including expanded zones of proliferative and hypertrophic chondrocytes and increased *Ihh* signaling (87;88).

Another group of signaling molecules important during endochondrial ossification is the BMPs. BMPs, also known as growth and differentiation factors (GDFs), are related to the transforming growth factor β (TGF β) superfamily of signaling factors. They were first identified based on their ability to induce bone formation when expressed ectopically (89;90). The BMP receptors, BMPR1A and BMPR1B, are both expressed during

endochondrial ossification, but appear to have distinct functions (91). *Bmpr1a* regulates chondrocyte differentiation by mediating local and long-range Ihh signaling. It is initially expressed throughout the mesoderm, but becomes restricted to the prehypertrophic chondrocytes later in development (91). *Bmpr1b*, in contrast, is expressed in mesenchymal condensations and is required for chondrogenesis in the mouse and chick limbs (91;92). An important role for BMPs in endochondrial ossification is further supported by skeletal abnormalities in two mouse mutants, *brachypodism* and *short ear*, which carry mutations in the *gdf5* and *bmp5* genes, respectively (93). *Short ear* mice are characterized by small external ears, abnormally shaped sternum, and hypoplastic long bones, while *brachypodism* mice have shortened long bones, and fusion of the middle and distal phalanges (93). Various BMP molecules are expressed in each chondrocyte zone within the growth plate, and have been shown to induce chondrocyte proliferation by antagonizing FGF signaling (reviewed in 30).

1.7 BDA1 Pathophysiology

Although skeletal dysplasias can occur due to defects in any of the steps during endochondrial ossification or bone patterning, it is not clear how BDA1 occurs. Fitch (3) suggested that BDA1 might result from defects in proper mesenchymal condensation resulting in shortened blastema. Since the middle phalanges are the last to ossify they would be the worst affected if the blastema were short. Premature closure of the epiphyses may also contribute to the shortness by abortively stunting growth of the bone along the growth plate (3;6;9). Since an animal model of BDA1 does not exist it is difficult to conduct experiments to identify the precise developmental defect that causes this abnormality. Identification of the gene(s) that cause brachydactyly is therefore necessary to elucidate its

cause. Knowledge of the gene(s) that cause BDA1 may also help us understand the molecular mechanisms that regulate skeletal size and shape in the distal limb.

1.8 Genetics of Brachydactyly

To date genes have been characterized for four types of brachydactyly other than type A1. In the years 1997 and 2000, mutations of *CDMP1* and *ROR2* were identified in families with brachydactyly types C (BDC) and B (BDB), respectively (94-96). More recently, mutations of *BMPR1B* were shown to cause brachydactyly type A2 (BDA2) (97), while mutations of the *HOXD13* gene were identified in individuals presenting with a rare form of brachydactyly in which symptoms overlapped types D and E (98;99).

CDMP1, also known as GDF5, is a member of the TGF- β superfamily and is related to the group of bone morphogenic proteins (BMPs) (100). It is expressed primarily in the primordial cartilage of the appendicular skeleton (100) and is involved in promoting mesenchymal cell recruitment, chondrocyte proliferation, hypertrophic chondrocyte differentiation and joint formation (101;102). In 1997, Polinkovsky *et al.* mapped a variant form of autosomal dominant brachydactyly type C to a region on chromosome 20q11.2 previously shown to include *CDMP1* (94;103). Since mutations of *cdmp1/gdf5* had been described in the autosomal recessive mouse phenotype, *brachypodism* (104), it was considered a strong candidate for brachydactyly type C. When the two *CDMP1* exons were amplified and sequenced, a C→T transition was identified in affected family members, at nucleotide 901. The change converted an arginine to a termination codon, resulting in a truncated protein (105). Additional heterozygous *CDMP1* mutations have since been described in other families with brachydactyly type C, suggesting that haploinsufficiency of

GDF5 is an underlying cause of the trait in these families (106). Mutations in this gene have also been identified in individuals with autosomal recessive Hunter-Thompson acromesomelic chondrodysplasia and Grebe dysplasia (reviewed in 31), further demonstrating a role for CDMP1 in regulating skeletogenesis in the appendicular skeleton.

ROR2, is an orphan receptor tyrosine kinase that belongs to a family of receptors characterized by cytoplasmic tyrosine kinase, proline-rich and serine/threonine-rich domains, a transmembrane domain, and extracellular immunoglobulin-like, frizzled and kringle domains (107). Although its function was unknown until recently, experiments with mutant mice carrying disruptions of the ROR2 tyrosine kinase domain, suggest that the gene promotes growth of the proliferating chondrocytes in the growth plate, by regulating transcriptional activity of the proteins Dlx-5 and Msx2 (108;109). Homozygous mutant mice exhibit a severe skeletal dysplasia phenotype in which all the bones formed by endochondrial ossification were shortened and misshapen (108). Oldridge *et al.* was able to show that the mouse *ROR2* gene mapped to chromosome 13 in a region syntenic to chromosome 9 in humans. Radiation hybrid mapping and fluorescence in-situ hybridization (FISH) analysis were subsequently used to localize the gene to 9q22, in the same region as Brachydactyly Type B (95;110;111). Mutational analysis of the gene in 11 kindreds identified 7 different mutations located in two small regions near the proximal and distal ends of the tyrosine kinase domain, thus supporting a role for *ROR2* in human limb development as well as mice (95;96). Mutations of the *ROR2* gene have also been identified in the autosomal recessive disease, Robinow syndrome (112;113) (reviewed in 114).

Recently two independent groups identified mutations in the *HOXD13* gene associated with limb abnormalities with features of both brachydactyly types D and E (98;99). *HOXD13* is a member of the *HOX* gene family of transcription factors. In humans

involvement of *HOXD13* in autopod development was first demonstrated by identification of mutations in individuals with synpolydactyly (115). Individuals with this disease carried stable expansions of a polyalanine tract within *HOXD13*, and exhibited synpolydactyly between the 3rd and 4th fingers, and the 4th and 5th toes, associated with a partial or complete extra digit in the syndactylous web. Involvement of *HOXD13* in synpolydactyly led Johnson *et. al.* (98) to examine the gene in 128 patients with congenital limb anomalies. A novel heterozygous transversion (940A→C) was identified in two kindreds with brachydactyly. The mutation was predicted to cause an isoleucine to leucine amino acid substitution at codon 314, in an area that encodes the homeodomain. Concurrently, Caronia *et. al.* (99) identified the same missense mutation in a large English kindred. Affected individuals from all three kindreds showed hypoplasia of the metacarpal in digit 3, hypoplasia/aplasia of the distal phalanx in digit 5 and/or bilateral phalangeal duplication of digit 4 accompanied by digits 3/4 syndactyly. A second mutation, at nucleotide position 923, was also identified by Johnson *et. al.* (98), in a family with features of brachydactyly type E. Similar to the Ile314Leu alteration, this second mutation was predicted to cause an amino acid substitution within the homeodomain, resulting in a serine to cystine substitution at position 308 (99). Based on the location of amino acid substitutions in the homeodomain, both groups predicted that the changes may affect DNA-binding and transcriptional regulation of downstream genes. It was shown that the Ile314Leu substitution altered protein affinity for some, but not all DNA recognition sequences, while the Ser308Cys substitution did not affect DNA binding of the *HOXD13* protein *in vitro* (98;99).

The most recent gene associated with brachydactyly is the bone morphogenetic protein receptor 1B (*BMPR1B*) (97). *BMPR-1B* is a transmembrane serine/threonine kinase that is transphosphorylated by type II receptors upon ligand binding, and is important for

transducing signals from the membrane to the nucleus (116). Evidence in both the chick and mouse indicate that it is necessary for proper mesenchymal condensation and chondrogenesis during embryonic limb development (91;92). The gene was assessed for mutations in two German families with BDA2 after identifying linkage to a 21.2 cM region on chromosome 4q21-q25 (97). This led to the identification of a T599A nucleotide change in family one and a C1456T nucleotide change in family two. Both mutations caused amino acid substitutions in domains thought to be involved in phosphorylation, the GS domain (family one) and the NANDOR box (family two) (97). Although *in vitro* kinase assays clearly demonstrated that the mutations affected normal protein function differently, *in vitro* and *in vivo* assays showed that they both inhibited chondrogenic differentiation and thus likely cause the BDA2 phenotype observed in humans (97).

In addition to the genes discussed above, a number of loci have been linked to isolated and syndromic forms of brachydactyly. In 1996, for example, Schuster et al. (117) mapped a syndromic form of brachydactyly type E and hypertension to chromosome 12p12.2-p11.2. More recently, in 2002, a cryptic translocation between chromosomes 6p and 11q was identified in monozygotic twin brothers with craniofacial anomalies, short stature, deafness moderate mental retardation, 5th finger clinodactyly and brachydactyly (118). This suggests that there may be a gene for brachydactyly on either chromosome 6p or 11q. Similarly, the presence of candidate genes for brachydactyly type A2 on either chromosome 20p11.2-pter or 15q25-qter was hypothesized based on the identification of a chromosome 15 deletion and a chromosome 20 duplication in a young girl with congenital skeletal abnormalities, including brachydactyly type A2 (119). The presence of a candidate gene on either chromosome was further supported by similar phenotypes in previous reports of patients with deletions of 15q associated with ring formation, duplications of chromosome

20p and, trisomy 20p (119). Finally, the identification of a duplication on chromosome 17 between q24 and q25.1, and a deletion of 2q37 in two separate individuals with brachydactyly type E suggests that there may be genes at each of these loci which can cause this disorder (54;120).

1.9 Genetics of Brachydactyly Type A1

Despite being the first human trait described in terms of autosomal dominant Mendelian inheritance, when this thesis project was initiated, the genes involved in BDA1 had not been identified. A study by Mastrobattista *et. al.* (21) had excluded many of the known genes involved in limb patterning and development including HOXD, MSX1, MSX2, FGF-1 and FGF-2. In addition, Fukushima *et. al.* had identified a reciprocal, balanced translocation between chromosome 5q11.2 and 17q23, in an individual with Klippel-Feil anomaly and brachydactyly type A1, consistent with a BDA1 locus on either chromosome 5 or 17 (23). This was supported by preliminary evidence from our own lab showing linkage between BDA1 and a locus on chromosome 5p (121). Subsequent to the project's initiation, an independent group showed that BDA1 was linked to chromosome 2q35-36 (15), indicating that the trait is genetically heterogeneous.

Yang *et al.* (15) examined genetic linkage in two large Chinese kindred with BDA1 and mapped the disease locus to an 8.1 cM interval on chromosome 2q35-36 that was flanked by markers D2S2248 and D2S360. Having excluded *PAX3* as the cause of the disease, Gao *et al.* (122) examined *IHH* based on its position within the critical region and its involvement in regulating chondrocyte growth and differentiation during endochondrial ossification (56;71). A G→A transition at position 283 of exon 1 was identified in affected

individuals from the first family, and a G→A transition at position 391 of exon 2 was identified in affected individuals from the second family (122). The missense changes resulted in glutamate to lysine amino acid substitutions at positions 95 and 131 of the amino acid sequence, respectively. Analysis of a third family with similar characteristics, allowed for the identification of a third point mutation in the *IHH* gene (122). This time it was C→A transversion at position 300 of exon 1 and was predicted to cause an Asp100→Glu amino acid substitution (122). Since the crystal structure of the human IHH protein had not yet been determined Gao *et al.* used the crystal structure of a similar gene product, the mouse Shh protein, to determine the approximate location of the three amino acids altered in the affected individuals of these families. Assuming that the mouse Shh and human IHH proteins are folded similarly, the crystal structure of Shh predicts that the three implicated amino acids, Glu95, Asp100, and Glu131 are all positioned close to one another in an external groove of the protein that binds to the Ptc receptor. Based on their positions in this groove Gao *et al.* proposed that these mutations may disrupt proper receptor-ligand binding and that haploinsufficiency of the wildtype IHH protein may contribute to BDA1 pathogenesis in each family (122). Attenuation of IHH signaling may further decrease the growth potential of developing bones by reducing the number of proliferating chondrocytes, and/or increasing the number of cells differentiating from the proliferative to hypertrophic stages. As the middle phalanges are the last phalanges to ossify they may be more affected by fewer chondrocytes than other phalanges (3).

1.10 Recessive IHH Mutation and Acrocapitofemoral Dysplasia

The recent discovery of *IHH* mutations in the autosomal recessive disorder, acrocapitofemoral dysplasia (ACFD), had provided further evidence that the IHH protein is important for normal human skeletal development (123). ACFD was first described in March 2003 and is characterized by short stature, brachydactyly (short middle and distal phalanges), a dysplastic, egg-shaped femoral head with a short femoral neck, and abnormally shaped digital and femoral epiphyses (124). Mortier *et al.* described two families, with a total of five affected individuals (123;124). Examination of the *IHH* gene revealed a homozygous 137C→T transition in affected individuals from family one, and a homozygous 569T→C transition in affected individuals from family two. Both mutations caused amino acid substitutions in the amino terminal portion of the protein responsible for local and long range signaling (59;123). Hellemans *et al.* hypothesized that the mutations cause ACFD by diminishing IHH signaling and thus increasing the rate of chondrocyte differentiation (123).

Chapter 2 – Rationale, Hypothesis and Aims

2.1 Rationale

Genetic manipulation in chicks and mice has provided developmental and molecular biologists with a number of insights into the genetic pathways that regulate normal vertebrate limb and skeletal development. It is unclear, however, how many of these discoveries can be directly translated into an understanding of human developmental processes. By contrast, characterization of genes that cause hereditary and congenital defects in humans can provide a unique opportunity to directly assess pathways which affect normal human growth and development.

The brachydactylies are a group of rare genetic traits characterized by short fingers due to anomalous bone development. In BDA1, affected individuals have short or absent middle phalanges in each digit. Despite being the first human trait described in terms of autosomal dominant Mendelian inheritance, when this thesis project was initiated the genes that caused BDA1 had not yet been identified. As an animal model of this disease does not exist, the reasons to identify the disease-related gene(s) were two-fold: 1) to gain a better understanding of the underlying molecular defect that cause this trait, and thus better understand its pathophysiology, and 2) to provide information about the genetic pathways that contribute to normal digit patterning and development. The recent identification of *IHH* mutations in a number of families suggests that this gene is important for normal growth and patterning of the digits, and supports evidence from mice that *IHH* is a key modulator of both chondrocyte and osteoblast differentiation during limb development (56;122). Despite this discovery, evidence from our laboratory and others suggests that the disease is genetically heterogeneous and can be caused by mutations of genes other than *IHH* (24;121).

In the year 2000, Armour, Hunter and Bulman, described the clinical characteristics of a large Canadian family with mild BDA1 (13). A genetic linkage study was initiated to

identify the gene that caused the trait in this family. Particular emphasis was placed on polymorphic markers from chromosome 5 and 17 based on a report of a girl presenting with BDA1 and Klippel-Feil anomaly (23). Cytogenetic analysis of this individual revealed a balanced translocation between chromosome 5q11.2 and 17q23, suggesting a gene for BDA1 on either chromosome. Preliminary linkage analysis of the Canadian kindred suggested that there was a BDA1 locus on chromosome 5p.

2.2 Hypothesis

Based on previous linkage analysis, we hypothesize that there is a gene on chromosome 5p that is necessary for proper limb patterning and bone development, and that this gene is mutated in a subset of families with brachydactyly type A1.

2.3 Aims

Thesis Objective:

1. To reduce the critical chromosomal region on chromosome 5p that is linked to BDA1 by:
 - a) saturating the critical region with polymorphic markers and examining linkage of each marker to the disease locus;
 - b) examining genetic linkage of polymorphic markers in additional family members and additional families, as they are identified.
2. To characterize the critical region on chromosome 5p by assessing transcribed regions of candidate genes for mutations in individuals with BDA1.

Long-term Laboratory Objectives:

1. To identify the gene on chromosome 5 that is mutated in individuals with Brachydactyly Type A1.

Chapter 3 – Definition of the BDA1 Critical Region on Chromosome 5p

Armour, Hunter, and Bulman recently described a three-generation, Canadian kindred with mild brachydactyly, consistent with type A1 (13). Although clinical and genetic features of this family have been described elsewhere (13;121), a brief description will be provided herein to review progress made as part of the genetic study, prior to initiation of this doctoral thesis.

The Canadian kindred with mild BDA1 was ascertained through an eight year old girl who had been referred to the Genetics Clinic at the Children's Hospital of Eastern Ontario for evaluation of her "abnormal hands". By the age of ten, when a genetic study was initiated, she had relatively short stature, as well as short hands and feet. Her height measured in the 15th centile and her hands and feet measured below the third centile (13). Further radiograph analysis showed evidence of brachydactyly type A1. Armour *et. al.* observed that all of the bones in the hands and feet were short and that shortening was most prominent in the middle phalanges, the proximal and distal phalanges of the first digit and the fifth metacarpal (13). Cone-shaped epiphyses, premature epiphyseal closure, radial clinodactyly in digit four on the left hand, and ulnar clinodactyly in digits two through four on the right hand were also noted (13). Cytogenetic studies did not reveal any chromosomal abnormalities.

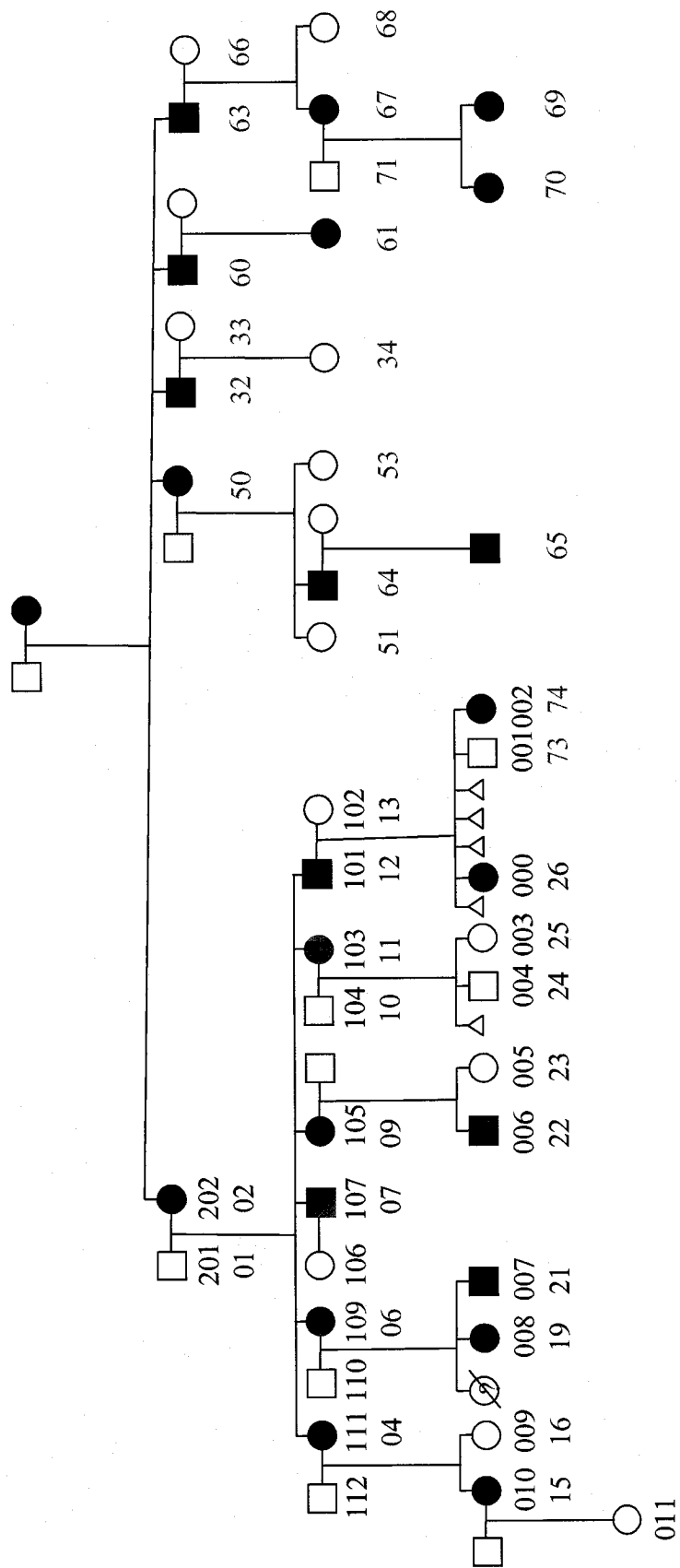
As part of the genetic study, an additional eighteen family members were also assessed including eight affected and six unaffected individuals. Similar skeletal abnormalities were observed in the five affected individuals as observed in the proband, while skeletal examinations of the "unaffected" group were unremarkable. The remaining four family members examined had short hands, but there was no evidence of clinodactyly. Diagnoses for brachydactyly were not possible based exclusively on visual inspection of their hands or radiographs. Consequently, these four individuals were defined clinically as

being “indeterminate” for BDA1. Metacarpophalangeal profiles (plots of each hand bone versus its length, which is expressed as age related standard deviations) were used to provide a more accurate diagnosis of brachydactyly. The profiles of “intermediate” individuals showed a pattern similar to “affected” individuals in that the length of each hand bone was below the mean. For both groups shortening was most prominent in the middle phalanges of digits 2-5 and the distal phalanx of digit 1, with the length of these bones measuring 2 standard deviations below the norm for age-matched controls. As these traits are considered diagnostic of BDA1, the four “intermediate” individuals were subsequently reclassified as being affected (13).

To identify the gene causing BDA1 in this family, DNA was collected from sixteen of the individuals characterized radiographically, and from three unaffected individuals who had married in to the family (refer to Figure 4). A genome-wide screen for polymorphic markers was initiated by Christine Armour in our laboratory, to assess for genetic linkage. Particular emphasis had been placed on markers from chromosome 5 and 17 based on a report of a young girl with Klippel-Feil anomaly and BDA1 (23). Cytogenetic analysis of this individual had revealed a balanced translocation between chromosome 5q11.2 and 17q23, suggesting that there may be a gene for BDA1 on either chromosome (23). The young girl had fusion of the C5 to C7 vertebrae, consistent with Klippel-Feil anomaly, and shortening of the middle phalanges in digits two to five and the proximal phalanges in digit one (23). Interestingly, she also exhibited shortening of the fifth metacarpal, a characteristic observed in members of the Canadian kindred, but not frequently reported in other individuals with BDA1 (121).

As part of the genome-wide screen for genetic linkage, eighteen microsatellite markers from fourteen different chromosomes were assessed. DNA from each

Figure 4: A large, Canadian kindred with mild Brachydactyly. This pedigree shows the kindred described by Armour, Bulman and Hunter (13) as well as family members recruited into the study after the initial genetic study began. Three digits in black represent the pedigree numbers assigned to each individual by the original authors. Numbers in red represent DNA numbers assigned to DNA samples collected in the initial linkage study and used in the genome-wide screen for markers link to BDA1. Numbers in green represent DNA numbers assigned to samples examined after the initial genome-wide screen, but before the doctoral research project. Blue and purple numbers represent DNA samples analyzed as part of the doctoral research project. The two purple samples were collected in February 2001, and were only genotyped at markers within the BDA1 critical region. Squares represent males; circles represent females; triangles represent spontaneous abortions; black symbols represent BDA1-affected individuals; grey symbols represent “indeterminate” individuals; white symbols represent BDA1-unaffected individuals.



family member was genotyped by amplifying micro-satellite markers using commercially available primer sets, and alleles were separated by size on 6% polyacrylamide gels. In doing so, linkage was formerly excluded for twelve markers, while lod scores suggestive for linkage were identified on chromosome 1 and 5, at markers D1S2134 and D5S2500, respectively (maximum lod scores = 0.94 and 0.75 at $\theta = 0.00$). To assess whether these loci were linked to BDA1 in the family, additional microsatellite markers flanking each locus were examined, but further evidence supporting linkage was limited to chromosome 5. To narrow the critical region further, a total of 10 markers were genotyped between D5S817 and D5S1725 (positions 22.88 cM and 97.82 cM on Marshfield's Sex-Averaged linkage map, respectively), and the pedigree was expanded to include an additional 5 people. A maximum lod score of 4.27 ($\theta = 0.00$) was identified at D5S819 (Akeel Baig, unpublished results), providing strong evidence for linkage to chromosome 5p. Using haplotype analysis inferred recombination events were identified in individuals 02 and 32. These events defined a 23 cM critical region between markers D5S2845 and D5S1457 (data not shown).

3.1 Materials and Methods

3.1.1 Family Studies

As indicated above, genetic linkage studies were based on a three-generation kindred previously reported (13). Since this original report, an additional 17 family members have been recruited in to the study. Ten of these individuals had been recruited into the study at the initiation of the doctoral thesis research, and two additional individuals were recruited subsequently (refer to Figure 4). In total, the study was comprised of 38 members including 22 affected individuals. The study was conducted after approval by the Children's Hospital of Eastern Ontario Ethics Review Committee. Informed consent was obtained from each participating family member or their legal guardian (refer to Appendix 1 – copy of consent form). Investigations included a medical history, physical examination and radiograph of hands and some members' feet. Measurements included height, arm span, lower segment hand length, middle finger length, and foot length. From the radiographs each hand bone was measured and a metacarpophalangeal profile was generated for each individual. Drs. Christine Armour and Alisdair Hunter ascertained phenotypes by clinical judgment and by use of the metacarpophalangeal profiles. A pediatric radiologist independently interpreted the radiological findings. All phenotype assessments were made prior to initiation of linkage studies.

3.1.2 Genotype Determination

Peripheral blood samples were taken from all participating family members, and a standard protocol was used to isolate DNA. Genotyping of microsatellite markers was performed using primer sets from the MAPPAIRS™ microsatellite markers (Research Genetics, Huntsville, Alabama) (refer to Table 2). Additional markers from Marshfield's

Table 2: Microsatelite markers used to define the BDA1 chromosome 5p BDA1 locus

Marker Name	Position on Marshfield Sex-Averaged Genetic Map	Heterozygosity	Marker Type	Minimum Size	Primers (5'to3')
D5S817*	22.88 cM	N/A	Tetranucleotide	266 bp	-TGTAACCTTCATGAAGGCAGG -GGGGGAAAAAGAACACTGTA
D5S2846*	33.04 cM	N/A	Tetranucleotide	164 bp	-CTCCTATTCTCCCATGGGT -CATCATTTTAACTTGGGTTTCC
D5S2845*	36.25 cM	N/A	Tetranucleotide	150 bp	-CAAATTCCAAAAGCCTTGAT -GCTGCTTCCCTAAACCTAGA
D5S2848*	39.99 cM	N/A	Tetranucleotide	226 bp	-GAAAAGCAGATTGTATTAGTTAGGG -CAGTTTTGGGAATTGGATTG
D5S819*	41.06 cM	N/A	Tetranucleotide	259 bp	-GTCACCCAAAAGTCATGAGG -TGTACCCGCATGCTATACAA
D5S2854	41.06 cM	N/A	Tetranucleotide	157 bp	-CTTTTGGGAAACAGAAGCAA -CTACAGATGGTACAGTGTAGGACG
D5S1986	44.73 cM	0.73	Dinucleotide	268 bp	-AGCTATTCACCTAGTAGAGATTCT -ACCTAACTGTATTGTCTATGAGAGG
D5S477	45.34 cM	0.76	Dinucleotide	180 bp	-AGCTGGGTAAAGTCCAAGGT -TGCCTCATCAGATTCCCTATT
D5S1350	45.34 cM	N/A	Tetranucleotide	246 bp	-TGACACAGCGAGACTCAGT -TGTCAGAAAAGTAAAAGGTGC
D5S111	47.09 cM	0.58	Dinucleotide	167 bp	-GGCATCATTTTAGAAGGAAAT -ACATTTGTTTCAGGACCAAAG
D5S583	47.09 cM	0.86	Tetranucleotide	250 bp	-GGCTGAGGCAGAAAGTCT -TCATGGGAGGTCTTCACATG
D5S1506*	49.54 cM	N/A	Tetranucleotide	387 bp	-TTGTGGTAGCTTATTATGCAGC -GGAATATTTGTAGCAGTTAGAAGG
D5S631	51.99 cM	0.71	Dinucleotide	193 bp	-GCTTGAACCCGAGAGG -TTGCAGATTTGAAAAGCAG
D5S426	51.99 cM	0.82	Dinucleotide	183 bp	-AAATTCCTTGCTTTCATAGCCA -AGACTAAATAAAATCACTGCCG
D5S663	51.99 cM	0.83	Dinucleotide	205 bp	-TTACTGAGATGCAGTCACCTT -CCTGAAGACATTAATCATGGA
D5S493	51.99 cM	N/A	Dinucleotide	302 bp	-AAGGACACAATGGCATAAAAGG -AGCTCCTCTGTTGCTTCTCT
D5S1994	51.99 cM	0.80	Dinucleotide	109 bp	-CTCAAAGAGTGCATAACCCA -TTAAAGATGAAACTGAAACCCA
D5S395	52.55 cM	0.83	Dinucleotide	189 bp	-GCAGATGGAAAACACCACTT -ACCTGCTGCTGGAAGATTAC
D5S1490	57.3 cM	N/A	Tetranucleotide	146 bp	-AACAATTTCTTCCTTTCAGATAGA -GGTAACAGCATTAGGAAAGACA
D5S1457*	59.30 cM	N/A	Tetranucleotide	123 bp	-TAGGTTCTGGGCATGTCTGT -TGCTTGGCACACTTCAGG
D5S2500*	69.23 cM	N/A	Tetranucleotide	155 bp	-TTAAAGGAGTGATCTCCCCC -GTTACAGTACCTATGGTCATGCC
D5S1501*	82.25 cM	N/A	Dinucleotide	99 bp	-TTATAGAGCAGCCTTCCCTG -AAAAGCCTCCAGTGGGATAT
D5S1725*	97.82 cM	N/A	Tetranucleotide	189 bp	-TGTACTTCAGGCTACCCTGC -CCAGAGAAAGAAAACCAATAGG

* indicates markers examined in initial genetic linkage studies

sex-averaged genetic map (D5S2846, D5S819, D5S1986, D5S477, D5S1350, D5S111, D5S583, D5S631, D5S426, D5S663, D5S395, D5S1490, and D5S1994) were also examined based on their location around markers suggesting linkage. Each of the loci examined were individually amplified in 10 µl polymerase chain reactions (PCRs) containing 10 mM Tris HCl (pH 8.3), 1.5 mM MgCl₂, 100-200 ng genomic DNA, 0.2 mM dNTP, 0.12 µM M13-tailed forward primer, 0.12 µM reverse primer, 0.12 µM IRD-700 labeled M13 forward(-29) primer (LI-COR, Lincoln, NE), and 1 Unit Taq DNA polymerase enzyme. In most instances DNA was amplified over 16 cycles at 94°C for 30 seconds, 66°C - 50°C for 30 seconds (-1°C per cycle) and 72°C for 30 seconds, followed by 23 cycles at 94°C for 45 seconds, 50°C for 30 seconds and 72°C for 30 seconds. PCR products were size separated on 6% acrylamide gels, using the LI-COR DNA sequencer Model 4000 (LI-COR, Lincoln, NE) and analyzed using RFLPscan software (version 3.0). For analysis of genotypes at the NPR3repeat, D5S1994 and D5S111 loci a similar technique was used, except that IRD-700 labeled M13 forward(-29) primers were not included in the PCR. Instead, the forward PCR primer was end-labeled by incubating it for 45 minutes at 37°C with [α -³²P]dATP and 1 Unit of PNK enzyme, followed by a heat inactivation step of 15 minutes at 65°C. We subsequently used 0.06 µM of the ³²P-labelled primer in the PCR. PCR products were size separated on 6% acrylamide gels, and visualized by audioradiography. All haplotypes were generated using Cyrillic software (version 2.1).

3.1.3 Linkage Analysis

Two-point LOD scores were calculated using MLINK, ILINK and LODSCORE, from the FASTLINK version (125;126) of the Linkage software package (105). To permit calculation of multipoint LOD scores, the number of alleles present for each marker was condensed to a total of 3 or 4 alleles without the loss of genetic information. Two-point LOD scores using the condensed alleles were recalculated for each locus to verify that these scores were not affected. Multipoint LOD scores were then calculated using LINKMAP from the FASTLINK version (125;126) of the Linkage software package (105). All LOD scores were calculated using an autosomal dominant model, penetrance of 100%, and a disease frequency of 0.000001. Equal recombination frequencies between males and females were assumed.

3.1.4 Amplification of Single Nucleotide Polymorphisms in the BDA1 Critical Region and Genotype Methodology

Sixteen different single nucleotide polymorphisms (SNPs) were chosen for genotype analysis based on their uniqueness within the genome, and their position on the Human Genome Browser physical map (June and November 2002 version) between D5S1506 and D5S426. Of these SNPs, seven were located within a single 130 bp fragment and were assessed by sequence analysis, four were assessed by restriction fragment length polymorphism (RFLP) analysis, and five were assessed by allele specific oligomer hybridization (ASOH) (refer to Table 3). Each SNP locus was amplified in a 25 µl PCR containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 0.01 mg/ml bovine serum albumin, 1.0-2.5 mM MgCl₂, 50-100 ng genomic DNA, 0.25 mM dNTP, 0.4 µM forward primer, 0.4 µM

reverse primer, and 1 Unit Taq DNA polymerase (refer to Appendix 2 for lists of primers used).

The SNPs within the 130 bp cluster were amplified in a single PCR, treated with shrimp alkaline phosphatase (Amersham Biosciences AB, Uppsala, Sweden) and Exonuclease I (USB Corporation, Cleveland, Ohio, USA) to remove unincorporated primers, and then sequenced in both directions using the Thermosequenase Radiolabelled Terminator Cycle Sequencing Kit (Amersham Biosciences AB, Uppsala, Sweden) according to the manufacturer's suggested protocol. Following amplification the sequence products were size separated on a 6% acrylamide gel and analyzed by autoradiography. PCR products containing the SNPs CTRP3snp, rs1440429, rs2099761, and rs903844, were purified using GFX columns (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's protocol and then digested using specific restriction endonucleases (refer to Table 3. Restriction patterns were then observed on a 1% agarose gel. The remaining loci were genotyped using ASOH as described previously (127).

Table 3: Methodologies used to genotype SNPs between D5S1506 and D5S426

SNP Name	Reference Name	Position (Genome Browser, June'02)	Method of Analysis (Sequence analysis, ASOH probe or Digestion Condition)
rs840388	CTRP3snp	chr5; nt 33965276	<i>Nci</i> I digestion (10 U; 37°C - 2 hours)
rs840345	CTRP3snp1	chr 5; nt 33966745	ASOH (TTTTGACTRCTCTCGTT)
rs840346	CTRP3snp2	chr 5; nt 33966664	ASOH (ACTACATCYGATTCTCT)
rs1366648	snp136	chr 5; nt 34017981	ASOH (TAACCTCAGTYCTTCAT)
rs1440429	snp144	chr 5; nt 34424837	<i>Bsa</i> AI digestion (15 U; 37°C – 2.5 hours)
rs2388676	LOC215i3	chr 5; nt 34433347	Sequence analysis
rs2892568	LOC215i3	chr 5; nt 34433348	Sequence analysis
rs2892569	LOC215i3	chr 5; nt 34433402	Sequence analysis
rs2388677	LOC215i3	chr 5; nt 34433410	Sequence analysis
rs2388678	LOC215i3	chr 5; nt 34433418	Sequence analysis
rs2388679	LOC215i3	chr 5; nt 34433458	Sequence analysis
rs2388680	LOC215i3	chr 5; nt 34433461	Sequence analysis
rs2099761	snp209	chr 5; nt 34518273	<i>Bsa</i> AI digestion (15 U; 37°C – 2.5 hours)
rs903844	snp903	chr 5; nt 34599795	<i>Bst</i> NI digestion (10 U; 2 µg BSA; 60°C- 2 hrs)
rs1992714	snp199	chr 5; nt 34659779	ASOH (ATACAGTRCATAATACCA)
rs466348	RAIsnp2B	chr 5; nt 34695440	ASOH (AGGAGGAMCTTCTGAGC)

Chr = chromosome

Nt = nucleotide

U = units

BSA = bovine serum albumin

3.2 Results

3.2.1 Phenotype

The family from Figure 4 is one with a mild BDA1 phenotype. The principal characteristics of the affected members in this kindred were shortened middle phalanges and short first proximal and distal phalanges, fifth metacarpals and short stature. Consistently the middle phalanges of affected members were below 2 standard deviations (SD) of age-matched norms. Most of the proximal first phalanges and fifth metacarpals of affected individuals were likewise 2 SD below the norms. Affected members also tend to be of short stature. Other findings included coned and prematurely fused epiphyses in the proband and her sibling. Several members, such as the proband, had clinodactyly of one or more digits. A number of individuals also had a broad distal phallux and/or broad slightly adducted forefeet. There were no other abnormalities as reported in a number of other families with BDA1.

3.2.2 Defining a 7 cM BDA1 Critical Region on Chromosome 5p13.3-13.2

To reduce the BDA1 critical region on chromosome 5p from 23 cM, microsatellite markers from chromosome 5 were assessed in an additional twelve family members, not included in the initial genetic studies. Novel chromosomal recombination events in these individuals could potentially provide critical information about which markers co-segregate with the disease phenotype and which ones do not. All ten markers from the initial study were assessed, as well as seven additional microsatellite markers between D5S819 and D5S1457 (D5S1986, D5S1350, D5S111, D5S583, D5S663, D5S1994 and D5S1490). In doing so, a novel, inferred recombination was observed in sample 63, between markers D5S819 and D5S1506, which reduced the size of the critical region to 11 cM.

To further refine the location of the recombinations in samples 63 and 32, which defined the telomeric and centromeric ends of the critical region respectively, the Marshfield Sex-Averaged and GDB genetics maps, as well as the Whitehead and Draft Human Genome physical maps, were examined to identify additional microsatellite markers located between the flanking recombinant and non-recombinant markers. Two microsatellite markers were chosen for analysis based on their position between D5S819 and D5S1986. Likewise, three microsatellite markers were chosen based on their position between D5S1506 and D5S663 (refer to Figure 5). Primers for a highly polymorphic repeat in the *NPR3* gene were also designed and used for genotyping. Genotypes of 36 family members were determined at each locus, while genotypes of two additional samples, 73 and 74, were only assessed for D5S1986, D5S477, NPR3repeat, D5S583, D5S1506 and D5S663. Using two-point lod score calculations a maximum lod score of 7.81 ($\theta=0.00$) was identified at D5S477 (refer to Table 4). Using haplotype analysis, the recombination event in patient 32 was refined to a position between D5S1506 and D5S426. In addition, a double recombination was identified in sample 64, between D5S2848 and D5S477 (refer to Figure 6). These recombination events reduced the size of the critical region from 11 cM to 7 cM. The location of the disease locus within the region between D5S1986 and D5S426 was further supported by multi-point linkage analysis (refer to Figure 7).

Figure 5: Genetic and physical maps of the Chromosome 5p BDA1 locus (November 2000). The five maps shown from left to right are from Marshfield's Sex-Averaged Genetic Map, Marshfield's Sex-Averaged Genetic Map (Reduced), the Genome Database Comprehensive Map, the Whitehead Institute Radiation Hybrid Physical Map, and the Draft Human Genome Physical Map. The position of each microsatellite marker on the map is indicated by a line. Markers examined prior to November 2000 are indicated in red. Markers positioned between the flanking recombinant and non-recombinant markers, D5S819/ D5S1986 and D5S1506/D5S663, that were subsequently used to refine the BDA1 critical region, are underlined and green.

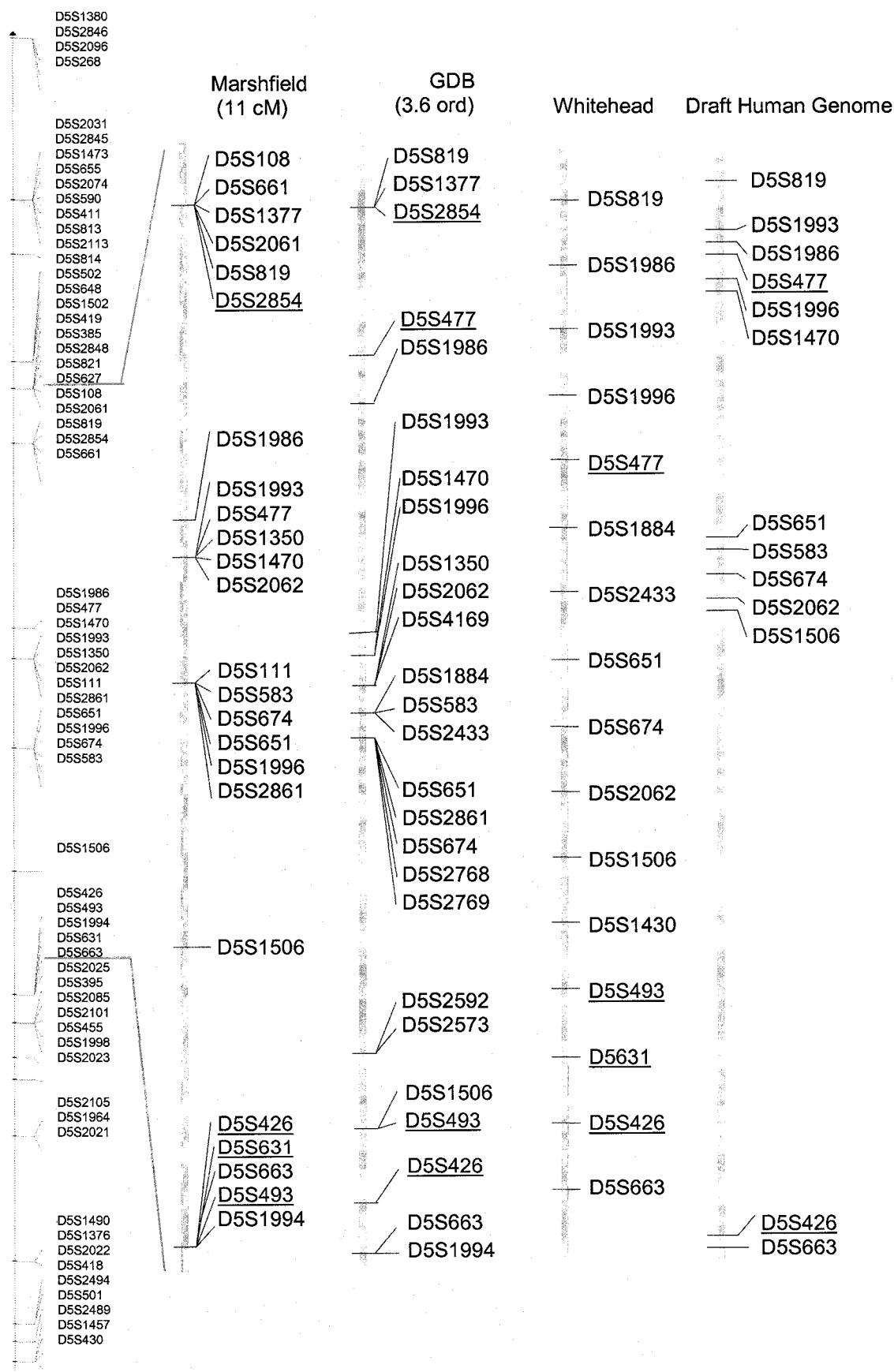
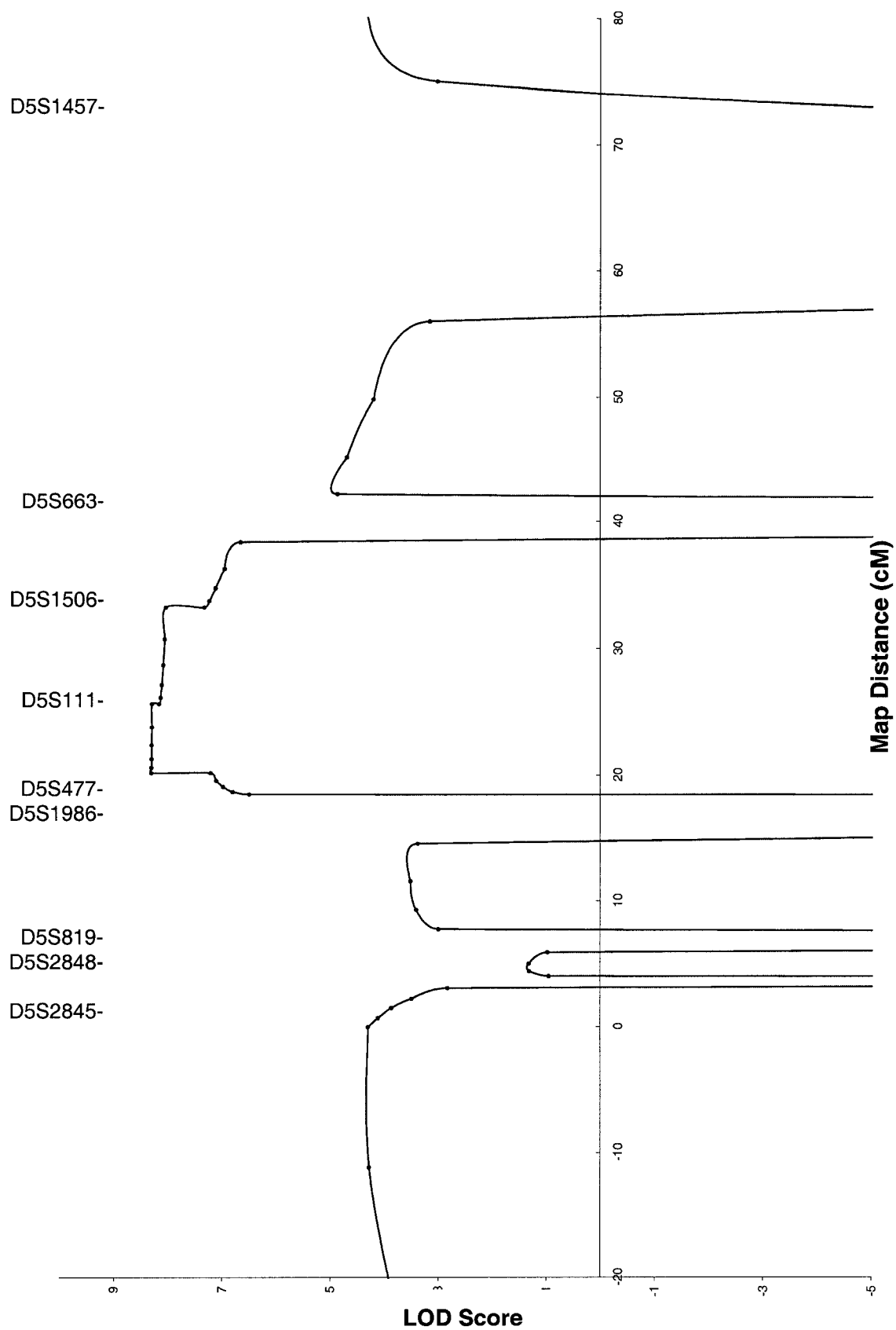


Table 4: LOD Scores of microstatellite markers from Chromosome 5

Marker	Map Position	LOD Score at $\theta =$										Zmax	θ_{max}
		0.00	0.01	0.05	0.10	0.20	0.30	0.40	0.58	0.74	0.98		
D5S817	22.88 cM	-∞	-6.38	-1.85	-0.22	0.85	1.42	0.74	0.58	0.98	0.263		
D5S2846	33.04 cM	-∞	-1.66	0.79	1.54	1.78	1.42	0.74	0.58	0.98	0.176		
D5S2845	36.25 cM	0.90	0.88	0.79	0.69	0.47	0.28	0.11	0.90	0.001			
D5S2848	39.99 cM	-∞	2.71	3.70	3.78	3.25	2.34	1.18	3.80	0.082			
D5S819	41.06 cM	-∞	3.64	4.58	4.57	3.86	2.74	1.35	4.63	0.072			
D5S2854	41.06 cM	-∞	2.96	3.90	3.91	3.25	2.23	1.03	3.96	0.073			
D5S1986*	44.73 cM	-∞	2.78	3.13	2.98	2.34	1.54	0.68	3.13	0.048			
D5S477*	45.34 cM	7.81	7.68	7.17	6.49	5.04	3.45	1.69	7.81	0.001			
D5S1350	45.34 cM	3.78	3.70	3.39	2.98	2.14	1.28	0.48	3.78	0.001			
NPR3 [*] _{repeat}	N/A	7.26	7.14	6.66	6.03	4.68	3.20	1.56	7.26	0.001			
D5S111	47.09 cM	5.81	5.71	5.30	4.78	3.67	2.47	1.16	5.81	0.001			
D5S583*	47.09 cM	4.27	4.21	3.95	3.61	2.86	2.00	1.05	4.27	0.001			
D5S1506*	49.54 cM	3.89	3.82	3.52	3.13	2.33	1.49	0.62	3.89	0.001			
D5S631	51.99 cM	5.82	5.72	5.32	4.79	3.67	2.45	1.14	5.82	0.001			
D5S426	51.99 cM	-∞	0.18	1.94	2.40	2.33	1.77	0.90	2.46	0.137			
D5S663*	51.99 cM	-∞	6.56	6.72	6.32	5.10	3.57	1.78	6.77	0.032			
D5S493	51.99 cM	-∞	1.43	2.48	2.63	2.27	1.58	0.71	2.63	0.095			
D5S1994	51.99 cM	-∞	5.05	5.29	4.99	4.01	2.76	1.33	5.31	0.038			
D5S395	52.55 cM	3.70	3.62	3.31	2.95	2.25	1.51	0.68	3.70	0.001			
D5S1490	57.30 cM	-∞	-1.44	0.96	1.65	1.77	1.31	0.62	1.83	0.159			
D5S1457	59.30 cM	-∞	0.02	2.37	2.98	2.92	2.20	1.11	3.08	0.138			
D5S2500	69.23 cM	-∞	-5.00	-1.75	-0.57	0.23	0.35	0.18	0.03	0.650			
D5S1501	85.25 cM	-∞	-4.17	-0.97	0.15	0.81	0.78	0.46	0.85	0.239			

Figure 6: Definition of a 7 cM critical region: Haplotype analysis. Shown are the haplotypes for each individual at 17 loci between D5S819 and D5S493 . Haplotypes that cosegregate with the disease are delineated by a box. The two digit value immediately below each symbol refers to the DNA sample number. A sample number is not assigned to the two individuals in the first generation as DNA was not available. Squares represent males; circles represent females; black symbols represent BDA1-affected individuals; white symbols represent BDA1-unaffected individuals. Recombination events that define the 7 cM critical interval are indicated by arrows

Figure 7: Multipoint linkage analysis of the BDA1 locus on chromosome 5p.



3.2.3 Characterization of the Chromosomal region between centromeric flanking recombinant and non-recombinant markers, D5S1506 and D5S426

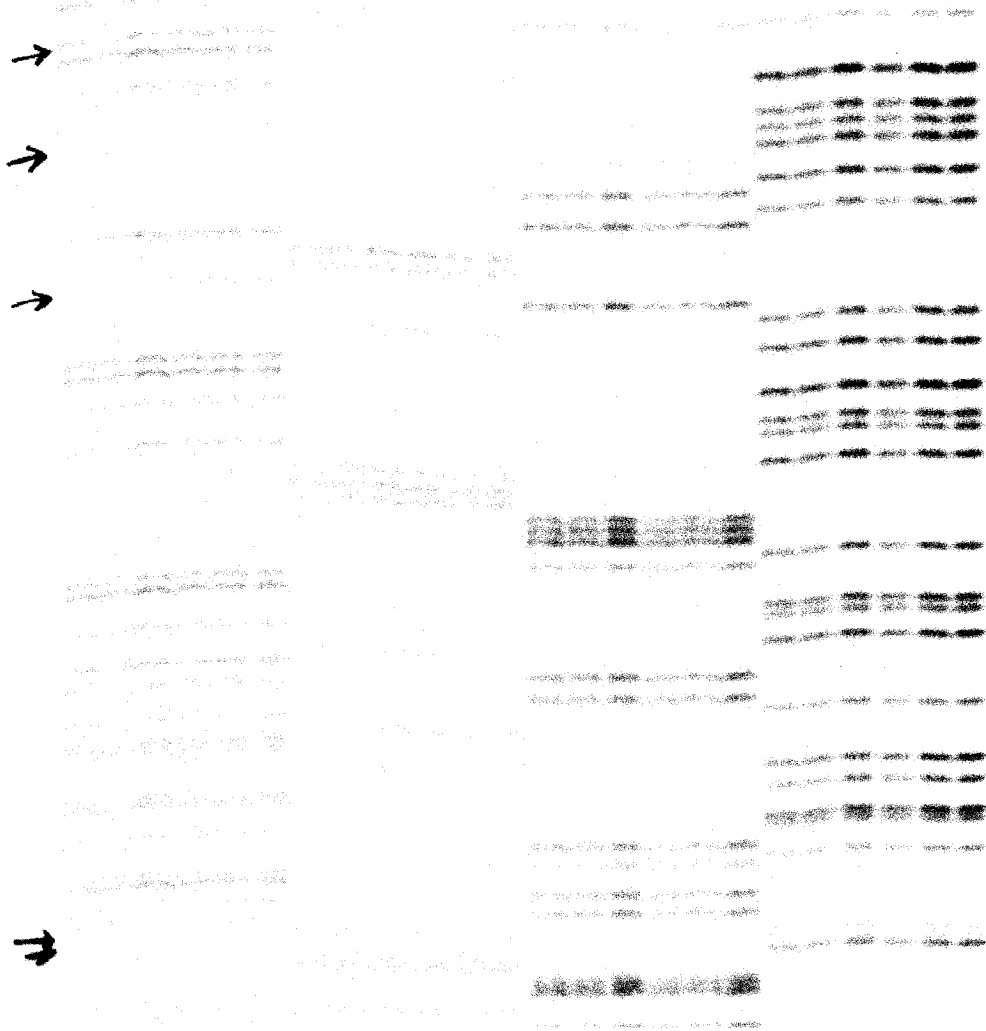
Based on the November 2002 version of the draft human genomic sequence (UCSC Genome Browser - <http://genome.ucsc.edu>), the distance between the flanking non-recombinant marker, D5S477, and the flanking recombinant marker, D5S1986, was 0.17 megabases (Mb). At the centromeric end, however, the gap between flanking non-recombinant and recombinant marker was much larger, corresponding to 1.5 Mb. This region also contained a number of genes that were duplicated on chromosome 5q13, including several spinal muscular atrophy (*SMA*) genes and apoptosis inhibitor (*IAP*) pseudogenes (128). In an effort to reduce the size of the gap between these markers by better defining the position of the recombination in sample 32, and potentially excluding the *SMA* and *IAP* genes from the critical region, a number of SNPs were examined. Many of the SNPs identified through the draft human genome sequence in this region (June 2002 version), were duplicated within chromosome 5p13.2 or between chromosome 5p13.2 and a distant location (frequently chromosome 5q or 6p). In total, 16 SNPs were identified that, according to the genome browser, occurred only once in the genome, and could be assessed easily by sequence analysis, RFLP, or ASOH. Three of these were located in the gene *CTRP3*, seven were located in a 130 bp fragment in *LOC20621*, and one was located in the gene *RAI14*. The rest were positioned throughout the region between D5S1506 and D5S426 (refer to Table 2).

Analysis of the LOC215i3, snp144, and snp903 SNPs suggested that all 38 individuals from the Canadian pedigree were heterozygous for the two alleles (for example, refer to Figure 8). As one would expect to observe approximately 25% of offspring to be homozygous for a given allele when both parents are heterozygous, it seemed unlikely that

Figure 8: Sequence analysis of LOC215i3 from six family members using the reverse primer. Arrows depict the locations of SNPs rs2388676, rs2892568, rs2892569, rs2388677, rs2388678, and rs2388679.

A
C
G
T

15 16 04 19 21 06 15 16 04 19 21 06 15 16 04 19 21 06 15 16 04 19 21 06



every individual assessed was truly heterozygous, suggesting that these results were an artifact. In addition, analysis of snp136 showed that none of the individuals examined carried the “T” allele. It was thus uninformative. All four loci were consequently excluded from future linkage or haplotype analysis.

Five of the 16 SNPs examined showed inheritance patterns that were consistent with Mendelian laws of segregation. They were therefore further examined by haplotype analysis to assess whether they cosegregated with the BDA1 locus in the family (refer to Figure 7). Although each SNP appeared to co-segregate with the trait, none of the loci examined were informative in the individual carrying the defining recombination, sample 32. Consequently, it was not possible to assess whether the markers were recombinant or not.

Despite positioning of the *SMA* and *IAP* genes within the BDA1 critical region as part of the November 2002 human genome sequences, they have since been removed from the region due to standard annotation processes. The physical distance between the flanking recombinant and non-recombinant markers at the centromeric end of the critical region remains large and is measured as 1.1 Mb (UCSC Genome Browser, July 2003 release). The presence of a *SMA*-like gene has not been excluded, however, since on April 2, 2004 the National Center for Biotechnology Information (NCBI) showed a gene, LOC401179 (similar to SMA3 protein), on its physical map between D5S1506 and D5S426.

3.3 Discussion

The family discussed in this chapter represents one with a relatively mild form of BDA1. Their phenotype is characterized by short middle phalanges of digits 2 through 5, a hallmark feature of BDA1. Unlike other families with more severe variants of the trait (4-6;12;15;19), terminal symphalangism and aplastic middle phalanges were not noted. The mild nature of the phenotype is further emphasized by the need for metacarpophalangeal profiles to diagnose several individuals whose clinical status could not be assessed by visual examination alone (13). Other common findings among family members included shortened distal phalanges, a short fifth metacarpal, malformed epiphyses and clinodactyly.

Previous reports of families with brachydactyly have shown that individuals can inherit the disease as an isolated malformation (4-7;12;14;14;15), or in association with other disorders. Slavotinek and Donnai described a boy with severe brachydactyly, valgus deformities, developmental delay, nystagmus, and scoliosis (16). Scoliosis was also described in a family with BDA1 and degenerative arthritis due to discoid meniscus (22) and in a family with brachydactyly, tall stature and foot deformities (19). In examination of affected members described in this report, there were no other features identified other than BDA1.

To define the BDA1 locus in the above family, we examined linkage between the abnormality and polymorphic markers from chromosome 5. Emphasis was placed on markers from chromosome 5p based on previous genetic studies suggesting linkage to this region (121). Consequently, we were able to define a 7 cM critical interval on chromosome 5p13.2-13.3, between D5S1986 and D5S426, that cosegregated with BDA1 in this family. A maximum LOD score of 7.81 ($\theta = 0.00$) was obtained at D5S477, providing strong evidence for a BDA1 locus at this location. The presence of a gene in this region that is necessary for

proper bone development and digit patterning is further supported by observations of long, slender fingers, termed arachnodactyly, in patients with trisomy 5p involving 5p13.3 (123;129-132). Other limb malformations associated with trisomy 5p, and with tetrasomy 5p, include fifth finger clinodactyly, toe abnormalities and club feet (123;129;131-133). Conversely, limb abnormalities are not frequently reported with 5p deletion syndromes, such as cri-du-chat syndrome (reviewed in 134). However, the most common symptoms associated with cri-du-chat syndrome, including a cat-like cry and mental retardation, appear to be linked to terminal regions of the chromosome, within 5p15.2-pter (134). Interstitial deletions which involve chromosome 5p13, but not more terminal regions, have been described in at least two reports. Associated symptoms in these reports included shortness of the upper limbs, oligosyndactyly with absence of the fourth finger and metacarpal, short stature, scoliosis and arachnodactyly (135;136).

There is a discrepancy between the position of our locus and the reported translocation break point found in the girl with BDA1 and Klippel-Feil anomaly. Cytogenetic analysis of this girl suggested that there was a reciprocal translocation between chromosome 5q11.2 and 17q23 (23). If the anomaly described in the previous report is due to a disruption in a gene at 5p13.2-13.3, the translocation may also be associated with a more complex rearrangement. Similar phenotypic characteristics between the girl reported by Fukushima et al (23) and our kindred include brachydactyly type A1, and wide spacing of the first and second toes. Although not reported as such, the radiographs in the publication by Fukushima *et al.* (23) also appear to show shortened fifth metacarpals, a feature found in our kindred. In addition, there was a report of a sib of individual 007 and 008 in Figure 4, who had died as a neonate of a chromosomal abnormality, short fingers and congenital anomalies (Teratology of Fallot and Klippel-Feil anomaly) (13). Despite these similarities, it remains

possible that there is an additional locus on chromosome 5q11.2 or chromosome 17 that is responsible for BDA1 in the girl reported by Fukushima *et al.* (23). Alternatively, she may carry an independent mutation in either the *IHH* gene or the gene at 5p. Mutations in this gene have been identified in at least six different families with BDA1 (20;24;122;137). Attempts have been made to acquire tissue, or DNA from the individual reported by Fukushima *et al.* so that an independent investigation can be conducted assessing involvement of the chromosome 5p or *IHH* loci. These attempts, however, have proven unsuccessful thus far.

We have shown evidence of linkage to chromosome 5p13.3-13.2 in a Canadian kindred, while a previous report by Yang *et al.* (15) identified a BDA1 locus on chromosome 2q35-36. In their report, two Chinese families were found to be linked to the same region on chromosome 2, and did not have a common haplotype within the defined interval. Identification of a second locus, on chromosome 5 indicates that BDA1 is genetically heterogeneous.

Our kindred had similar features to the families described by Yang *et al.* (15) although there are some differences in phenotype. Many members in their study were completely lacking middle phalanges, a phenotype that was not observed from our family. Similarly, some individuals had shortened third metacarpals while this was not a marked feature in our affected individuals. As originally described by Fitch (3), all hand bones may be proportionately reduced in size and is the case for our affected family members. For affected members the distal and proximal phalanges, and metacarpals were shortened below 2 standard deviations compared to age related norms. The first proximal and fifth metacarpal were also frequently short (13). Yang *et al.* (15) described a number of features within the family that were apparently beyond Fitch's description such as severely shortened distal

phalanges, shortened 3rd metacarpals and shortened proximal phalanges of the fifth digit. These differences are not surprising given that different loci have been implicated.

Based on Marshfield's Sex-Averaged genetic map, the BDA1 locus on chromosome 5p, corresponds to a 7 cM interval. This translates to a 3-4 Mb region on the current physical maps that contains 14 known genes and 18 predicted genes (NCBI October'03). Characterization of a BDA1 locus on chromosome 2 has led to the subsequent identification of mutations in the *IHH* gene (122), suggesting a role for the Hh signaling pathway in the pathogenesis of BDA1. Despite this, none of the genes from the chromosome 5p BDA1 locus are known to be involved in this pathway. Alternative criteria must therefore be used in selection of candidate genes for analysis (refer to Chapter 5 for analysis of candidate genes). The large size of the chromosome 5p BDA1 region could complicate this process due to the large number of genes that potentially need to be examined. The size of the critical interval, and thus the number of genes which may have to be examined, can be reduced by two primary methods - by analyzing additional family members and/or additional families, and by analyzing more polymorphic markers within the critical region. Both of these techniques can allow identification of novel recombination events, while analysis of additional markers can also be useful for refining the location of recombination events already identified within a family. Unfortunately, within the scope of this doctoral research thesis, it was not possible to recruit additional family members in to the study. Moreover, current physical and genetic maps indicated an absence of microsatellite markers between the flanking recombinant and non-recombinant markers that could be used for further analysis. This second factor was of particular concern since the distance between the centromeric flanking markers, D5S1506 and D5S426, was between 1 to 1.5 Mb in size, depending on which version of the draft human genome sequence was being examined. The

number of genes within this region, and their suitability as candidates, also varied depending on the version of the physical maps being examined. In the November 2002 version, these genes included several *SMA* and *IAP* genes that were duplicated on chromosome 5q13, and would have been difficult to analyze for mutations using the sequencing method described in Chapter 5.

Attempts to refine the critical region were made by examining several SNPs located between D5S1506 and D5S426, but none of these were informative. The microsatellite marker, D5S631, is also located in the region between flanking recombinant and non-recombinant markers (according to UCSC July'03 version), but again was uninformative in the individual with the recombination. An inability to identify informative markers across the 1-1.5 MB region in this study, stresses the need to analyze numerous polymorphic markers to refine the position of recombination events within a critical interval. To reduce the size of the region additional SNPs should be assessed from the region between D5S1506 and D5S426. In the most recent versions of the draft human genome sequence (April and July 2003) the position of D5S1506 was placed further upstream than in prior versions, and the *SMA* and *IAP* genes have been moved from the critical region. As a result, there may be polymorphic markers (SNPs or simple tandem repeats) within the interval between D5S1506 and D5S426 that were not considered previously for analysis, but may provide important information about the boundaries of the non-recombinant region linked to BDA1. Alternatively, it will be necessary to recruit additional family members in to the study, or to identify other families with BDA1, in which the abnormality is linked to the same locus on chromosome 5.

While examining SNPs from the critical interval, a number of loci were identified that were heterozygous for all individuals examined, however, Mendelian laws of

segregation predict that such a result is unlikely for this sample set. It is possible that these observations may have occurred due to contamination by DNA from a heterozygous individual. This seems unlikely, however, since negative controls were run with each PCR, but failed to show amplification products. Contamination problems were also addressed by reordering primers, although no change in the results were observed with new primer sets. Alternatively, the observation of “heterozygous” genotypes may indicate that the loci are duplicated in the genome. In such a case the apparent SNP would represent nucleotide differences between the two sites rather than true SNPs.

In summary, we have shown that BDA1 is linked to a 7 cM critical region on chromosome 5p13.2-13.3 in a four-generation family affected with the disease. As previous studies have shown linkage of BDA1 to chromosome 2q35-36 (15), linkage to a different locus in this study suggests that BDA1 is genetically heterogeneous. Further analysis of the 7 cM region should yield the nature of the gene involved.

Chapter 4 – Assessment of Additional BDA1 Families;
***IHH* Mutational Analysis and Linkage Studies**

The inheritance of BDA1 as an autosomal dominant trait has been well documented over the last century (1;4-6;14), but the gene(s) involved in its pathology had not been examined until recently. In 1995, Mastrobattista *et al.* (21) studied the molecular genetics of two kindreds with BDA1. Although these kindreds were too small to permit genome wide studies, the authors were able to exclude a number of genes known to be involved in limb and bone development including *HOXD*, *MSX1*, *MSX2*, *FGF1* and *FGF2* (21). The first genetic study to identify linkage of BDA1 to a chromosomal location was reported in 2000 by Yang *et al.* (15). They examined two large Chinese kindreds with a severe variant of BDA1 and showed evidence for linkage to a locus on chromosome 2q35-36 (15). The haplotypes that cosegregated with the abnormality covered the same contiguous markers from chromosome 2 in each family, suggesting that BDA1 was caused by mutations of the same gene in both kindreds. The absence of a common haplotype, however, suggested that these mutations likely occurred independently in each family, and were not due to a mutation in a common founder (15). The BDA1 locus was defined by recombination events in two individuals from the first family, resulting in a 8.1 cM critical interval, between D2S2248 and D2S360 (15).

Characterization of a BDA1 locus on chromosome 2q led to the subsequent identification of *IHH* mutations in three families with BDA1 (122). Positional mapping of the *IHH* gene to the BDA1 locus and its requirement for proper hypertrophic differentiation of chondrocytes (71) made it a strong candidate for involvement in BDA1 pathogenesis. To screen for mutations, the *IHH* promoter and coding regions were amplified and sequenced from genomic DNA of affected and unaffected individuals from each family (122). Three mutations were identified, one in each family. The observed point mutations caused substitutions at amino acids Glu95, Glu131 and Asp100, respectively (122). Based on the

crystal structure of mouse Shh (138), a highly similar molecule to human IHH, Gao *et al.* predicted that these three amino acids were closely situated on the surface of the IHH protein, and that the substitutions may disrupt IHH binding to its “natural” receptor (122). Since their report, *IHH* mutations have been described in at least three additional families with BDA1 (20;24;137), thus supporting a role for IHH in normal human digit development and in BDA1 pathophysiology. Homozygous mutations of *IHH* were also identified in individuals with acrocapitofemoral dysplasia, an autosomal recessive disorder characterized by short stature, short limbs, brachydactyly, macrocephaly, a narrow thorax, and cone-shaped epiphyses (123).

Although BDA1 is caused by *IHH* mutations in a subset of individuals with the condition, the identification of a second locus on chromosome 5p13.3-13.2 demonstrates that this condition is genetically heterogeneous (refer to Chapter 3) (121). Acknowledgement of this heterogeneity is important as the presence of multiple loci represents a potentially confounding factor when attempting to recruit families into the current genetic study examining chromosome 5p. Consequently, linkage to chromosomes 5p13.3-13.2 and 2q35-36 and/or evaluation of *IHH* mutational status was assessed in all individuals with BDA1 who were being considered for recruitment into the study. These procedures were necessary to establish which locus was involved in each case.

4.1 Materials and Methods

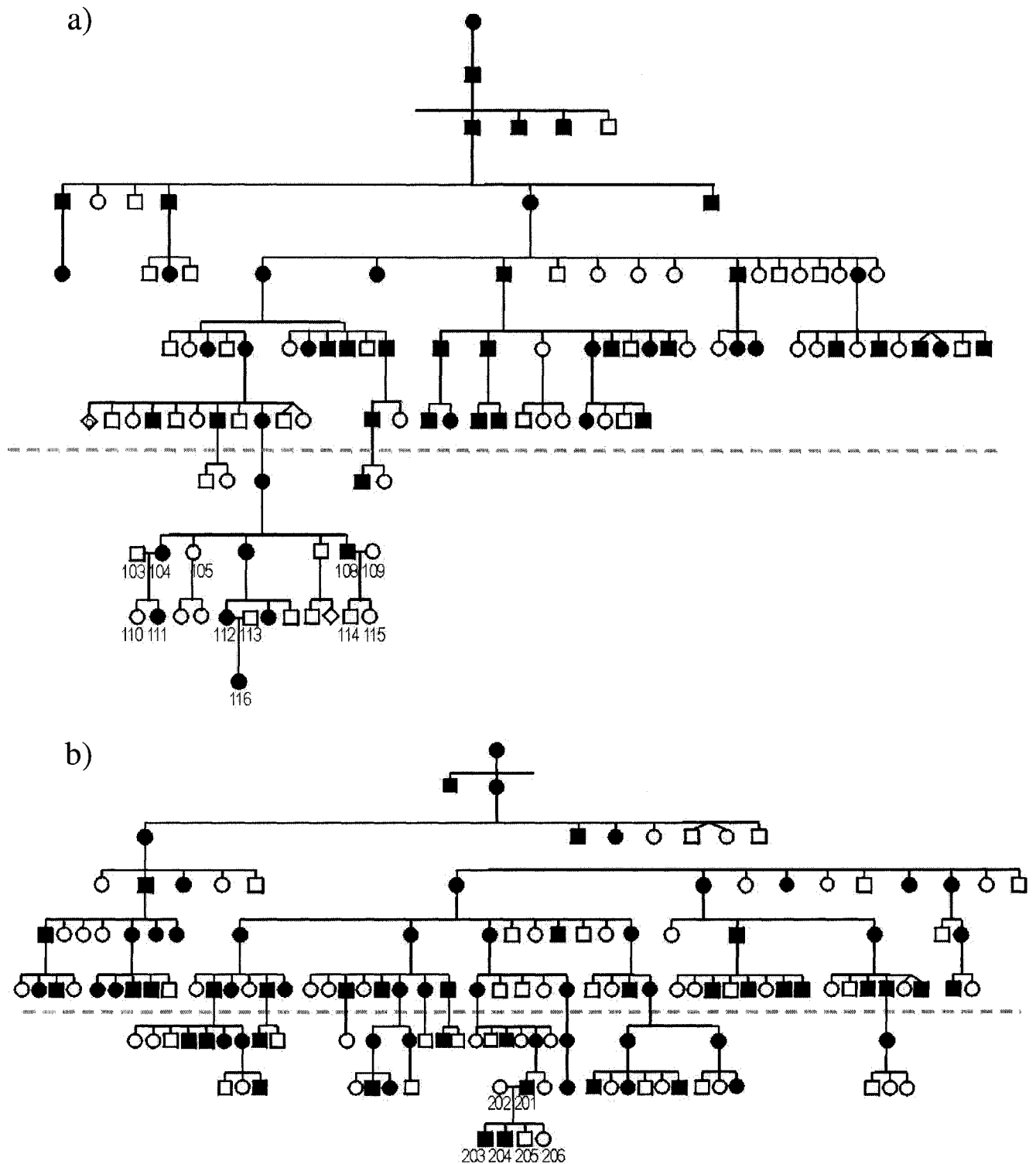
4.1.1 Family Studies

To identify families with mutations at the chromosome 5p BDA1 locus, we established collaborations with physicians in Canada, England, the United States of America, and New Zealand. While contact with many of these physicians was initiated based on published case reports of BDA1, other collaborations were developed as a result of requests for *IHH* mutation screening. In total, family members from twelve kindreds were examined, including 31 affected individuals (refer to Table 5 and Figures 9-12). As with analysis of the pedigree from Chapter 3, the study was conducted after approval by the Children's Hospital of Eastern Ontario Ethics Review Committee. Informed consent was obtained from each participating family member or their legal guardian. Investigations included a medical history, physical examination, and blood collection for DNA extraction. Radiographs of the hands and feet were also provided in some cases. All phenotype assessments were made prior to initiation of linkage and/or mutation analyses studies.

Table 5: Family Studies - Summary of kindred origins and individuals involved in the study.

Pedigree Number	Origin	Total # of Individuals Participating in Study	Total # of BDA1- Affected Individuals
2	Liverpool, England	12	5
3	Manchester, England	6	3
4	Houston, Texas, USA	11	5
5	Houston, Texas, USA	6	4
6	Wellington, New Zealand	3	1
7	Oxford, England	1	1
8	Ottawa, Ontario	1	1
9	Ottawa, Ontario	1	1
10	Washington and Oregon, USA	1	1
11	Edinburgh, Scotland	1	1
12	Los Angeles, California	1	1
14	Pennsylvania, USA	8	7

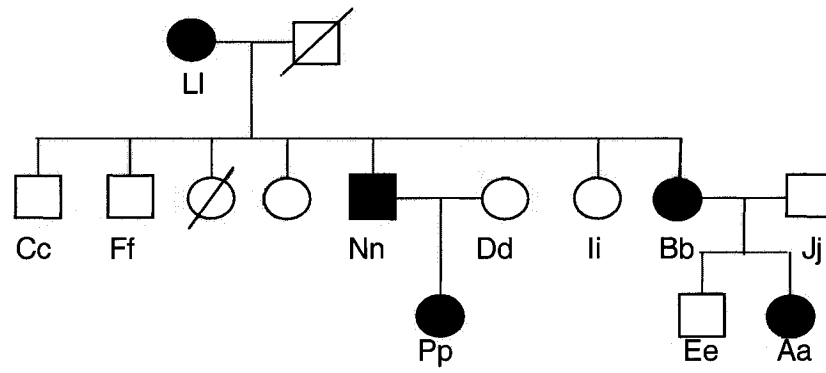
Figure 9: Extended Drinkwater pedigrees - pedigrees 2 and 3. Shown are the a) first brachydactylous family described by H. Drinkwater (1907/8), and b) the third brachydactylous family described by Drinkwater (1915). Each pedigree has been extended to include descendants of the originally described pedigrees. The dashed line demarks the originally published, and the extended pedigrees. Squares represent males, while the circles represent females. BDA1 affected individuals are marked by the filled symbols. Numbers below the symbols indicate the sample number assigned to each individual's DNA. Only those individuals for whom DNA was available were assigned DNA numbers.



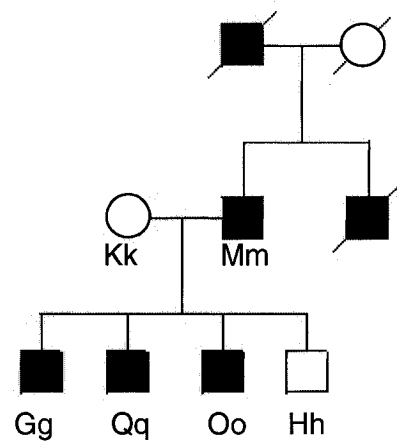
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Figure 10: Familial relationship of individuals from pedigrees 4, 5 and 7. These drawings depict the relationships between individuals in a) pedigree 4, b) pedigree 5, and c) pedigree 7. Squares represent males; circles represent females; black symbols represent affected individuals, diagonal lines indicate that an individual is deceased. Individuals from whom DNA was collected are indicated on each pedigree by an alphabetical or numerical value below their symbol. These values correspond to the identification number assigned to each DNA sample.

a)



b)



c)

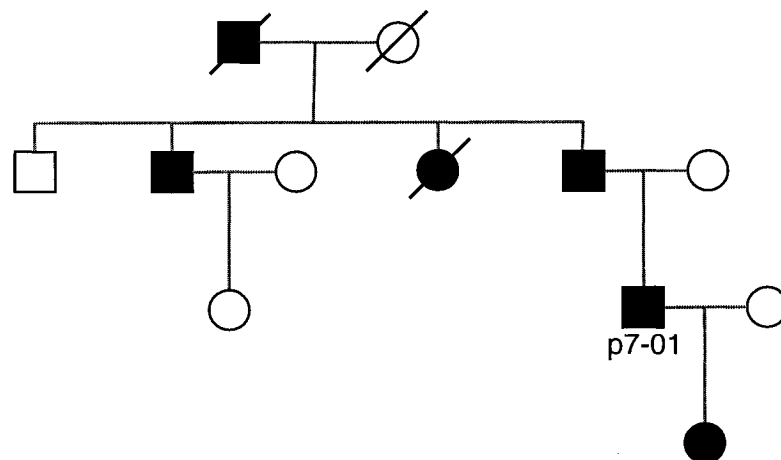
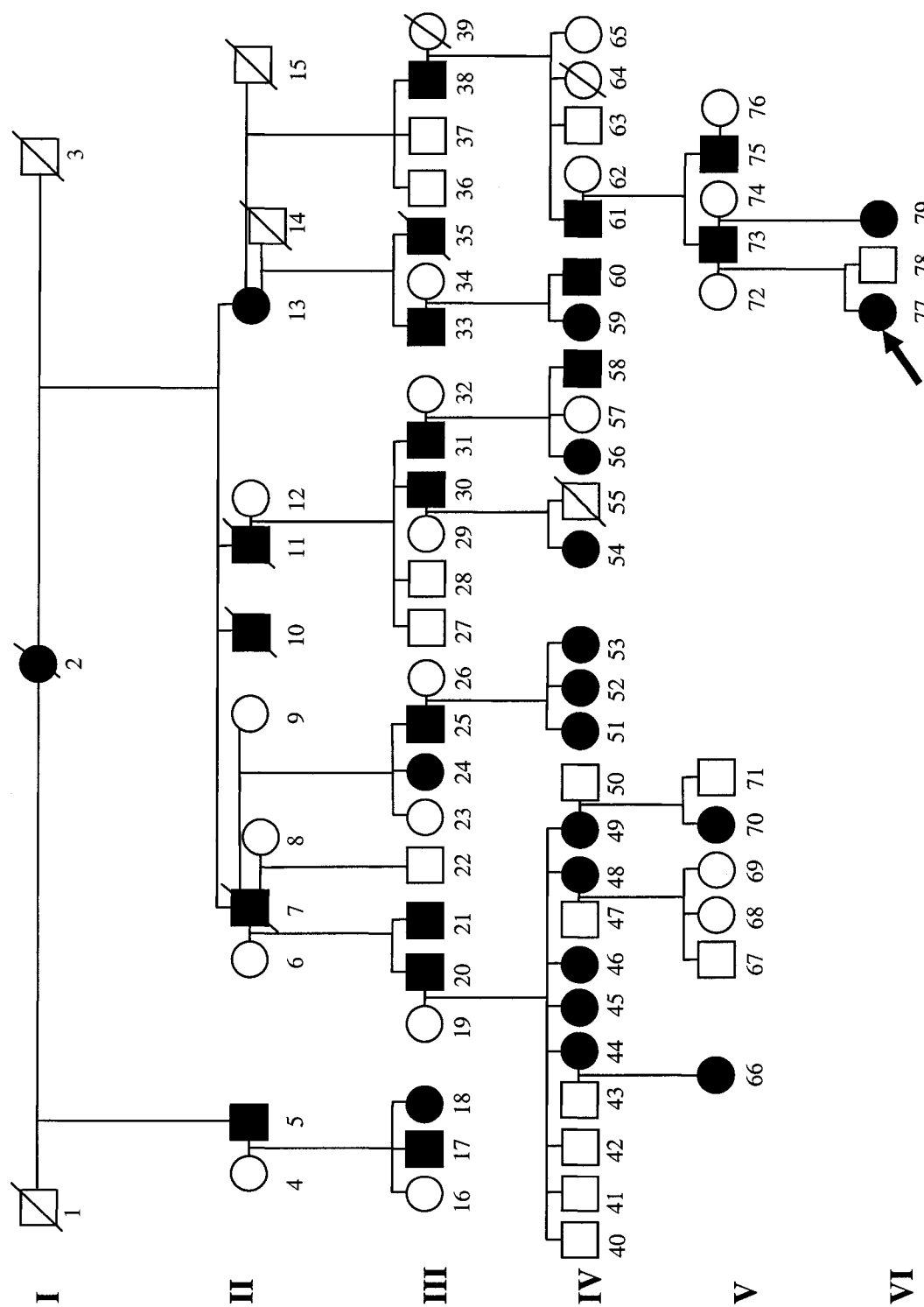


Figure 11: Extended pedigree from kindred 10. Shown is the extended pedigree of the proband from kindred 10, indicating affected and unaffected relatives (11), however affection status was not confirmed by a medical examiner in each case. The proband is indicated by an arrow. Squares represent males; circles represent females; black symbols represent affected individuals, diagonal lines indicate that an individual is deceased.



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Figure 12: Extended Farabee pedigree - pedigree 14. Depicted is the pedigree first described by William Farabee in 1903, and its descendents (4;7) The limits of Farabee's description are marked by a dashed line, while the limits of Haws and McKusick's description are marked by the dotted line. Individuals from whom DNA was obtained are indicated by a DNA number in brackets under the individual's identification number. Squares represent males; circles represent females; black symbols represent affected individuals; small symbols represent individuals who died in infancy.

4.1.2 Genotyping of Chromosome 2 and Chromosome 5 Microsatellite Loci

Linkage to the BDA1 locus on chromosome 2 was assessed in individuals from pedigrees 2 and 3 by analyzing sixteen microsatellite markers from chromosome 2q35-36 (refer to Table 6). Seven of these markers were also examined in individuals from pedigree 14. In addition, nine microsatellite markers from chromosome 2, and seven microsatellite markers from chromosome 5, were genotyped in individuals from pedigree 6 to ascertain whether there was a contiguous gene deletion on either chromosome, as assessed by homozygosity mapping. The nine markers from chromosome 2 are listed in Table 6 and included microsatellites between D2S2208 and D2S362, but not D2S424. The seven markers from chromosome 5 are listed in Table 2 and included microsatellites between D5S2854 and D5S493, but not D5S631, nor D5S426. Finally, linkage to the BDA1 locus on chromosome 5 was assessed in individuals from pedigrees 4 and 5 by analyzing five microsatellite markers, from chromosome 5p13.3-13.2: D5S819, D5S1986, D5S477, D5S1506 and D5S663 (refer to Table 2). For both linkage analysis and homozygosity mapping, loci were individually amplified as described previously (refer to Chapter 3.1.3), and genotypes were assessed using the RFLPscan software (version 3.0). All two-point LOD scores were calculated using the MLINK and LODSCORE programs as described in Chapter 3.1.4.

4.1.3 *IHH* Mutation Screening

IHH cDNA nucleotide sequences (Accession AB010092 and L38517) were compared to genomic DNA sequences (Accession AC027000) to determine the gene's intron/ exon boundaries. The *IHH* coding region was subsequently amplified by PCR, using intronic primers designed to amplify each exon separately (see Table 7 and Figure 13).

Table 6: Microsatellite markers used to examine linkage and homozygosity on Chromosome 2

Marker Name	Position on Marshfield Sex-Averaged Genetic Map	Heterozygosity	Marker Type (# of bases in microsatellite repeat)	Minimum Size	Primers (5'to3')
D2S1391	186.21 cM	N/A	Tetranucleotide	124 bp	-CTCACTGTCTGGATTCTTGG -TAAGGACAAGTTAAAAAAGCTGG
D2S1384	200.43 cM	N/A	Tetranucleotide	148 bp	-AATAGAGGGCCCTTGCTTAA -TTTGGGATAAAAGGTATTTTGC
D2S2208	205.06 cM	0.89	Dinucleotide	164 bp	-CTATTTGTAAACATGCGGGA -AGCTAAGTACCTGCTCAGGAAA
D2S2944	210.43 cM	N/A	Tetranucleotide	123 bp	-TCTGTCTTAGATGGATGAATGG -GGGATGGATGGAAAAGATTC
D2S164*	214.71 cM	0.84	Dinucleotide	265 bp	-GTCCTAACAGGCCACAGACC - GCTGGCAGTATCACATGACA
D2S1371*	215.25 cM	N/A	Tetranucleotide	108 bp	- GTTGGGGGAAGCATTAAAAT - GATAGCAAGAAGGGCTTTCC
D2S434	215.78 cM	N/A	Tetranucleotide	274 bp	-TAAATCACTAGCCTTTGCCG -GCCATCTGTACTGTTCCCAG
D2S1338*	215.78 cM	N/A	Tetranucleotide	168 bp	- CCAGTGGATTGGAACAGA - ACCTAGCATGGTACCTGCAG
D2S2250	216.31 cM	0.79	Dinucleotide	169 bp	-CTGAAACTCACCGAACACC -CCCAAATAGGCAGGGAAAT
D2S433	216.31 cM	N/A	Tetranucleotide	190 bp	-CCTGGAGTGTCTATTGTTTCC -TGAATCATTGTTTTTACATGG
D2S163	218.45 cM	0.80	Dinucleotide	213 bp	-GTATCCCAGGGCAGGAC -TAGTTTTTGGATGTTTAGCCTC
D2S1242	218.45 cM	0.94	Tetranucleotide	117 bp	-TGACATAGCGAGACCCTGTC -CCATTCTCATCCAGCAGGA
D2S424	218.45 cM	N/A	Tetranucleotide	177 bp	-TGGTGGAGATGTTTAAAGGC -GGGCAGGAAAAGGATTGTAT
D2S1323	221.13 cM	N/A	Tetranucleotide	332 bp	-ACATTGAGCACCAGAATGGT -ATTTGCAGCAGAGACTCTGG
D2S126	221.13 cM	0.83	Dinucleotide	141 bp	-TGCCTAGCACAGAGCCTACT -TCCATGGCATAGAACAAAAGT
D2S2308	227.00 cM	0.84	Dinucleotide	211 bp	- TGCTAATCCCATTTATGAGG - TGGAACCATGCTCTAATC
D2S362	231.27 cM	0.77	Dinucleotide	104 bp	-CTAAAAACAACCTCTGAATGTAGTC -TATTACGGTTCCTAACAGCA
D2S427	236.70 cM	N/A	Tetranucleotide	245 bp	- ATGTAGACTGCTAACAGTGTCTGC - TTGGTCAACATGTCAATTTAGG
D2S2968	251.94 cM	N/A	Tetranucleotide	180 bp	-ATGGGTGGATGTACAAATGG -TCTTAGTTTATGGGCCACCA

* Only analyzed during homozygosity mapping

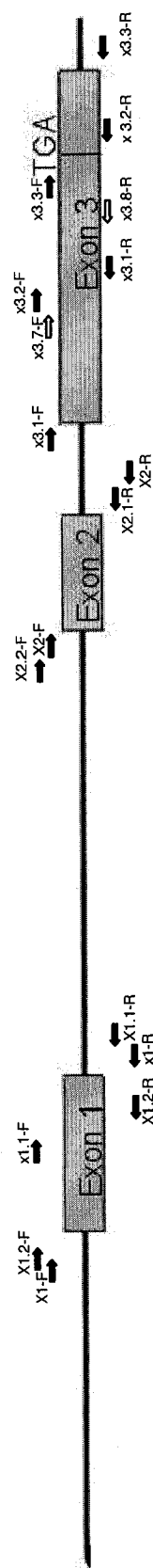
Table 7: Oligonucleotides used for PCR amplification and cycle sequencing of *IHH*

IHH exon	Primer Name	Primer Sequence (5'to 3')	Annealing Temperature (°C)	Final [Mg ⁺⁺] (mM)	PCR Product Size
1	IHHx1-F	TGCCCATCAGCCCACCAG	62*	1.5	470 bp
	IHHx1-R	GAGCGTGCCAGCCAGTCG			
5' end of exon 1	IHHx1.2-F	ATCAGCCCACCAGGAGAC	60*	1.5	324 bp
	IHHx1.2-R	TTGAAGATGATGTCTGGATTGT			
3' end of exon 1	IHHx1.1-F	CCGACGCTATGAAGGCAAGA	60*	1.0	249 bp
	IHHx1.1-R	TTGGCGAGAGGGGCAGGT			
2	IHHx2-F	CGGCTGATTTCTGCTCTG	60	2.5	394 bp
	IHHx2-R	GGCGGGCTCTTCACCTT			
2	IHHx2.2-F	GCGCCTACACCTGCACCT	64	1.5	408 bp
	IHHx2.1-R	CTCGGCACTACTCCTCCTG			
5' end of exon 3	IHHx3.1-F	AAGGGAGGGTCGTTGTG	64	1.5	494 bp
	IHHx3.1-R	TTGTGAGCGGGGCGTAG			
3	IHHx3.7-F**	CCCCACCTTCAGCGATGT	64	1.5	N/A
center of exon 3	IHHx3.2-F	TGCTCTTTACGGCTGACAATC	64	1.5	532 bp
	IHHx3.2b-R	GCAGAGGAGATGGCAGGAG			
3	IHHx3.8-R**	CCCCAGGCGGTAGAGCAG	64	1.5	N/A
3' end of exon 3	IHHx3.3-F	TGGGGCGTCTCCTGCTA	60	2.5	491 bp
	IHHx3.3-R	GCATCGGGTCCAGCCAGA			
Upstream of exon 1	IHHsnp12-F	GGAGACCGCAACCCACG	62	2.5	237 bp
	IHHsnp12-R	TCCCCATCCCCAGTTTG			

*Used 40 cycles during PCR amplification rather than 35.

** Nested primer used for sequencing only, and not PCR amplification

Figure 13: Positional location of amplification primer binding sites within the *IHH* gene. Shown are the approximate location of each primer used to amplify *IHH* sequences. These are represented by arrows that either face the right-hand (forward primers), or the left-hand (reverse primers) side of the page. As all of the primer names begin with “*IHH*”, these prefixes have been omitted from the diagram. The shaded boxes represent the exonic sequences, black arrows represent primers used for amplification and cycle sequencing, and white arrows represent primers used only for cycle sequencing. The position of the stop codon is indicated by “TGA”. The *IHH* gene is not drawn to scale.



Additional exonic primers were also designed to permit amplification of three overlapping PCR products that span exon 3 and to permit sequence analysis of exonic regions greater than 200 bp from the intronic amplification primers (see Table 7). As part of the initial mutation screen in pedigrees 2 and 3, analysis was limited to DNA from one affected and one unaffected individual from each family (individuals 116, 110, 203 and 205). *IHH* mutation screening in pedigrees 6 through 14 was limited to analysis of DNA from the proband of each family. DNA was amplified in 25 µl volumes containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 0.01 mg/ml bovine serum albumin, 1.0-2.5 mM MgCl₂, 50-100 ng genomic DNA, 0.25 mM dNTP, 0.4 µM forward primer, 0.4 µM reverse primer, and 1 unit Taq DNA polymerase. The PCR products were treated with shrimp alkaline phosphatase (Amersham Biosciences AB, Uppsala, Sweden) and Exonuclease I (USB Corporation, Cleveland, Ohio, USA) to remove unincorporated primers. PCR products were then sequenced in both directions using primers (refer to Table 7) and the Thermosequenase Radiolabelled Terminator Cycle Sequencing Kit (Amersham Biosciences AB, Uppsala, Sweden) according to the manufacturer's suggested protocol. Following amplification the sequence products were separated by size on a 6% acrylamide gel and analyzed by autoradiography.

4.1.4 Genotyping at the *IHH* Locus by Restriction Digest and Cycle Sequencing

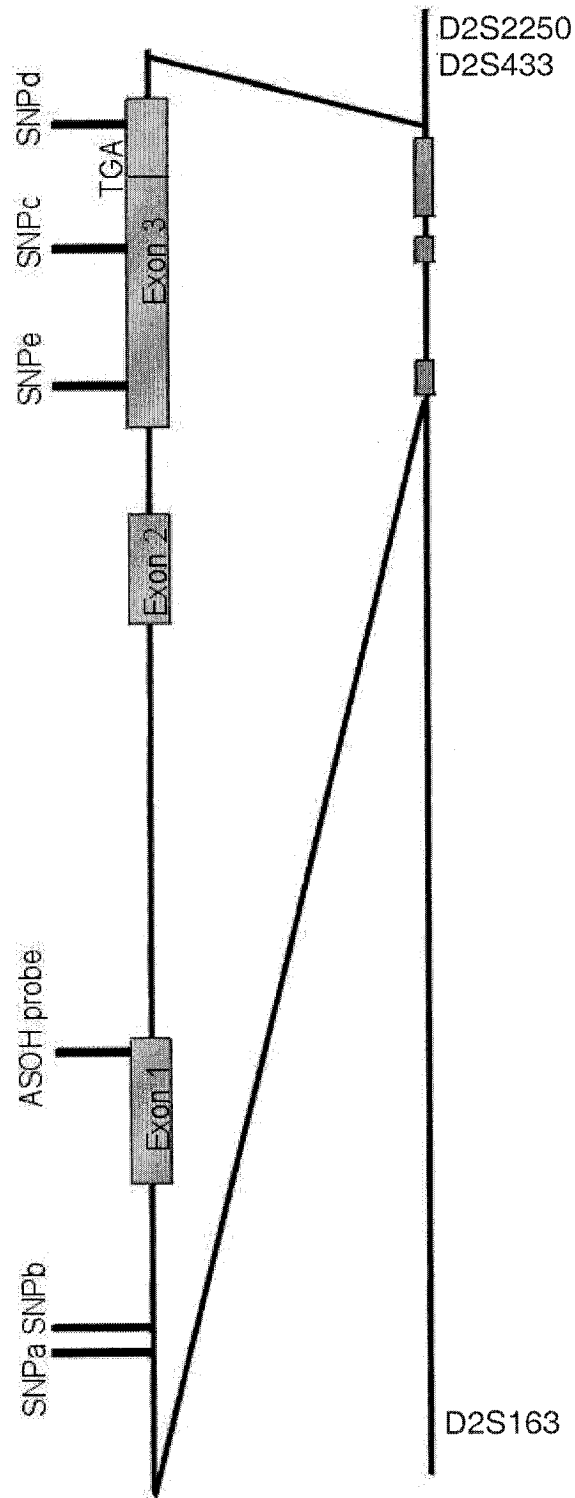
Five SNPs from within the *IHH* gene, SNPa-e, were genotyped in individuals from pedigrees 2, 3 and 14. SNPs a, c and d were also genotyped in individuals from pedigree 6. Four of the 5 SNPs, SNPa-d, have been described previously (Accession # AC027000) and are located immediately upstream of exon 1, or within exon 3 (see Figure 14). SNPs a and b

were amplified in a single PCR using primers specific for the region upstream of *IHH* exon 1 (IHHsnp12-F and IHHsnp12-R in Table 7). The genotypes of individuals at SNP_a and SNP_b were assessed by digesting the PCR products with *XmaIII* (Amersham Pharmacia Biotech, Upsalla, Sweden) and *SexAI* (New England Biolabs Inc., Beverly, MA,USA), respectively, and further verified by sequence analysis. SNP_c and SNP_d were amplified using the IHHx3.2 and IHHx3.3 primers, respectively (see Table 7). Genotypes were determined by digesting the SNP_c PCR product with *SmaI* (Boehringer Mannheim, Indianapolis, IN, USA) and the SNP_d PCR product with *BstUI* (New England Biolabs Inc., Beverly, MA, USA). All of the digests were prepared using 1-2 units of enzyme and 1x the manufacturers suggested buffer in a 20 µl reaction volume. The reactions were then incubated at the manufacturer's suggested incubation temperature for 2 hours (refer to Table 8 for reaction conditions). SNP_e is a novel SNP identified during mutation screening of the 5' end of exon 3 (refer to Figure 14). As neither the C or T allele at this locus generated a restriction endonuclease recognition site, genotypes were assessed by amplifying the 5' end of exon 3 using the IHHx3.1 primers and sequencing the SNP using the IHHx3.1R primer as described earlier (refer to Table 7).

Table 8: Reaction Conditions for Digest with Restriction Endonucleases

Enzyme	Number of Units Used/ Reaction	Incubation Temperature	Bovine Serum Albumin/ Reaction
<i>XmaIII</i>	1	37°C	0.01%
<i>SexAI</i>	1	37°C	2µg
<i>SmaI</i>	2	25°C	N/A
<i>BstUI</i>	2	60°C	N/A

Figure 14: Location of polymorphic loci in and around the *IHH* gene. The bottom diagram within this figure, shows the approximate location of the *IHH* gene on chromosome 2 with respect to nearby microsatellites. The *IHH* locus is expanded in the top diagram to show the intron/exon structure of the *IHH* gene and the position of five SNPs within the gene. Two SNPs, SNP_a and SNP_b, are located in the sequence upstream of exon 1, while the remaining SNPs are located in exon 3. Also shown is the approximate location of an ASOH probe used to examine a sequence variant in exon 1. The *IHH* locus is not drawn to scale.



Reprinted with permission from McCready ME, Sweeney E, Fryer AE, Donnai D, Baig A, Racacho L, Warman ML, Hunter AG, Bulman DE. 2002. A Novel Mutation in the IHH Gene Causes Brachydactyly Type A1: A 95-year-old mystery resolved, Hum. Genet., 111: 368-375. Human Genetics copyright Springer, Germany.

4.1.5 Allele Specific Oligomer Hybridization

Allele specific oligomer hybridization (ASOH) was performed as described previously (127), using γP^{32} -radiolabelled oligonucleotides specific for two different alleles, (5'-TCATGAGGCGGTTGGCG-3') and (5'-TCATGAGGCGGTCGGCG-3'). The oligomers were hybridized at 58°C and 60°C, respectively, and then washed twice using the same temperature. Blots were exposed to X-ray film for between 3 to 12 hours at -80°C. Genotypes were further confirmed by sequence analysis as described in the previous section.

4.2 Results

4.2.1 Phenotype of Familial and Sporadic BDA1 Cases

In addition to the Canadian family described in Chapter 3, individuals from twelve other kindreds with BDA1 were also examined. While several of these families showed inheritance patterns consistent with an autosomal dominant trait, sporadic incidents of BDA1 were noted in some families (refer to Table 9). Cases of sporadic BDA1 were defined by short or absent middle phalanges in digits 2 through 5 of the proband, and normal length digits of both parents. Genetic studies were also extended to include individuals with syndromic forms of BDA1, in addition to those with isolated malformations. Despite slight differences in inheritance (familial versus sporadic) and phenotype (syndromic versus isolated malformations), each kindred shared a diagnosis of BDA1 in the proband. General characteristics of the thirteen kindreds are listed in Table 10, and are expanded on in the proceeding case studies.

Pedigree 2 – Drinkwater’s First Family

Individuals from pedigree 2 are descendents from a family with brachydactyly originally described by Harry Drinkwater in 1907/08 (4). The proband (individual 108 in Figure 9) was the fourth child born to non-consanguineous parents. Radiographic analyses of his hands showed bilateral dysplasia of the carpals, metacarpals and phalanges. The phalanges were short and broad, the middle phalanx in digits 2, 3 and 5 were missing, and spurs were observed on the radial side of the trapezium. At the age of 21 the proband began to experience pain in the right knee, and describes the knee “giving out”, although the patella was never dislocated. At about the age of 40 years he began to experience additional pain in his left knee and both hips. Patellectomies and tendon realignment surgeries were performed

Table 9: Summary of phenotypes observed by physicians in pedigrees 2 through 14

Pedigree	Sporadic Vs Familial BDAI	Metacarpal Shortening	Valgus Deformities	Nystagmus	Developmental Delay	Knee Abnormalities	Short Stature	Musculoskeletal Problems Involving Knee	Vertebral Anomalies ie. Scoliosis	Renal Anomalies	Facial Anomalies	Short Terminal Phalanges	Joint Dysplasia
2	Familial	+				+	+	+					
3	Familial		+	+	+	+	+		+				
4	Familial												
5	Familial												
6	Sporadic				+		+		+	+	+		
7	Familial						+						
8	Sporadic											+	+
9	Sporadic	+					+						
10	Familial	+				+							
11	Sporadic	+							+				
12	Sporadic												
14	Familial	+				+							+

on both knees, as well as two operations to remove scar tissue. During these operations surgeons identified abnormalities of the patella. In addition to problems associated with the knees, the proband described back pain from a ruptured disc and painful feet that required orthotic shoes. A karyotype of the proband was normal and there was no evidence of generalized skeletal dysplasia based on radiographic examination. His height was measured as 6'1½" (97th centile) and was considered within the normal range for males. His mother had been 5'10" (> 97th centile) and his father had been 6'4" (>97th centile).

Similar to the proband from family 1, individuals 104 and 111 were also affected with BDA1 and a variety of musculoskeletal problems, although the extent of these problems varied between individuals. Radiographic analysis of individual 104 showed bilateral shortness of all the hand bones that was most prominent in the middle phalanges. The middle phalanx was absent from digits 2 and 5 in both hands, and spurs were visible on the metaphyses and on the radial side of the trapezium. In addition to shortened digits, individual 104 suffered from a number of musculoskeletal problems. She had never been able to jump or squat and described pain in her back and knees from her teen years. In her fifth decade the pain intensified and walking aids became necessary. Radiographs of her knees and back were not available to assess if there were skeletal abnormalities that contributed to this pain. She was 5'8" tall (~97th centile). Individual 111 also demonstrated brachydactyly and musculoskeletal problems, although not as severe as individuals 104 and 108. Bilateral shortness of the middle phalanges and the third metacarpal ray were observed in radiographic analysis of individual 111's hands, but without further absence of the middle phalanges. Despite normal radiographs of the hip, she also described occasional numbness in her left arm, intermittent pain in her left hip from the age of 18 and an inability to squat. She was 5'4" tall (~60th centile), while both of her parents were 5'8". Similar

musculoskeletal problems to those described above were not identified in the remaining affected individuals from family 2. Hip and leg problems were noted in individual 115, but these appeared to be unrelated to those found in individuals 104, 108 and 111.

Pedigree 3 – Drinkwater’s Third Family

Individuals from pedigree 3 have been described previously, and are descendants of the third brachydactylous family described by Drinkwater in 1915 (16). Individuals from this family share a number of characteristics with pedigree 2, including broad hands and feet with shortened digits due to hypoplastic or absent middle phalanges in digits 2-5. A short proximal phalanx in digit 1, single volar creases, bilateral transverse palmar creases, and velvety skin were also noted among affected individuals. In addition to BDA1 the proband (individual 204 in Figure 9) had bilateral valgus deformities that persisted despite numerous corrective surgeries, a double scoliosis, hand deformities with ulnar subluxation of the wrists and fixed flexion deformities of the first metacarpals, and knee problems due to an irregular femur. Further involvement of the nervous system was also noted, including nystagmus, a squint and developmental delay (16).

Pedigrees 4 and 5

Individuals from pedigrees 4 and 5 were first described by Mastrobattista *et al.* in 1995, as part of a genetic study examining candidate genes for familial brachydactyly (21). The two families contained five and six affected individuals, respectively, and were of Scandanavian and Mexican descent. Besides shortening of the middle phalanges, no other physical or developmental abnormalities were noted (21). In their previous report Mastrobattista *et al.* evaluated five candidate genes for linkage to brachydactyly, HOXD, MSX1, MSX2, FGF1, and FGF2, and were able to exclude each gene in at least one of the two families (21).

Pedigree 6

Individuals from pedigree 6 were first ascertained through the Central Regional Genetic Services at the Wellington Hospital in Wellington South, New Zealand. The kindred is comprised of three individuals including the proband and his parents. Clinical descriptions of the proband indicated marked brachydactyly in association with developmental delay, vertebral and renal anomalies, and short stature. Non-specific facial abnormalities were also described. Clinical examinations of the parents were unremarkable, and did not support a diagnosis of BDA1 in either individual.

Pedigree 7

According to physicians in Oxford, England, there is an extensive family history of BDA1 associated with this kindred; the father, uncle, aunt, grandfather and daughter of the proband are all affected with the trait (refer to Figure 10). Members of this family are residents of Cheshire, North Wales, in the same local region where Drinkwater's families were from. Despite common phenotypes, a clear relationship between the families could not be determined based on genealogical records alone.

The proband, individual p7-01 in Figure 10c, has features compatible with brachydactyly type A1. He has only two phalanges in each finger, and one phalanx in each thumb. His feet are similarly affected and, at the age of 23, he required surgery for a problem with his right cruciate ligament. He has short stature, and measures 5'3" tall.

Pedigree 8

The proband from pedigree 8 was first assessed through the Eastern Ontario Regional Genetics Program at the age of 16 months because of differences in her fingers and toes. She is the first child born to healthy, non-consanguineous parents and was delivered at 41 weeks

after an uncomplicated pregnancy. There are no other family members with hand or foot anomalies.

Upon physical examinations a number of facial anomalies were noted including a bossed and tall forehead, a square face with a small chin, temporal narrowing, and a broad nose with a scooped bridge and an upturned tip. Measurements of the head circumference and body length were normal (50th and 75th centile respectively) but weight was only in the 5th centile. The thorax and abdomen were emancipated in appearance. Thin skin, and abdominal protrusion with an umbilical hernia were also noted. Examination of the limbs did not show differences in the long bones, but abnormalities of the hands and feet were evident. The thumbs were short and spatulate. The index and fifth fingers were likewise short and there was minor clinodactyly of the fifth fingers. Palmar creases were normal, but there was reduced creasing over the distal interdigital space. There was marked shortening of the great toes, and moderate 2-3 syndactyly. The second toes, however, were long and somewhat tree-frogged in appearance. Radiograph analysis of the hands showed that the terminal phalanx of the thumbs and the middle phalanx in digit 5 were short, while the middle phalanx of digit 2 was absent. Each toe had only two phalanges with the terminal phalanges being very short. Karyotype analysis was normal.

Pedigree 9

The proband from this kindred was referred to the Eastern Ontario Regional Genetics Program because of his small size at birth. Short fingers and toes were also noticed at the first examination. Growth retardation at birth was likely related to maternal use of Inderal, a beta-blocker taken for essential tremor, as similar growth retardation was observed with the parent's first pregnancy. Since birth, growth and development has progressed normally. Examination of the proband's hands showed that the second and fifth fingers were short and

had a single interphalangeal crease. The great toe was short, bulbous and upturned. Webbing between the second and third toes was also observed. Radiograph analysis confirmed the presence of two, rather than three phalanges in the second and fifth digits. The toes were likewise affected, while a single phalanx was observed in each great toe. Bilateral shortening of the metacarpals in digits 1, 3 and 4 were also noted. The family history is unremarkable.

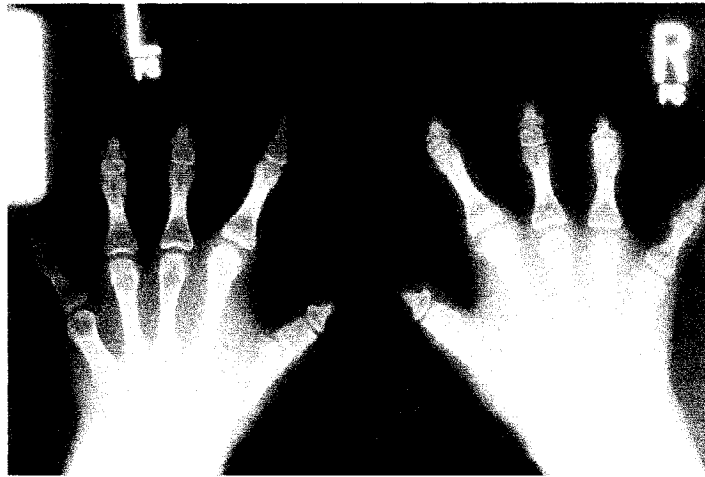
Pedigree 10

Individuals from pedigree 10 were described in 1998, as part of a fourth year-undergraduate thesis project, and reside primarily in Oregon, Washington and Wyoming, USA (11). There is an extensive family history of brachydactyly with reports of individuals with “short fingers” that extends across six generations (refer to Figure 11). Hand measurements and/or audioradiographs were available for five affected individuals (individuals IV-48, IV-49, IV-54, V-70 and V-73 from Figure 11), while physical descriptions of an additional three affected individuals were also provided (refer to Figure 15). The phenotypes described were consistent with BDA1 and included severely hypoplastic or absent middle phalanges in all of the fingers. The toes were similarly affected in some, but not all, of the affected individuals (11). In addition to BDA1, the proband lacked thumbs on both hands. Because this characteristic was not identified in other family members, it is unclear whether it is associated with BDA1 or not.

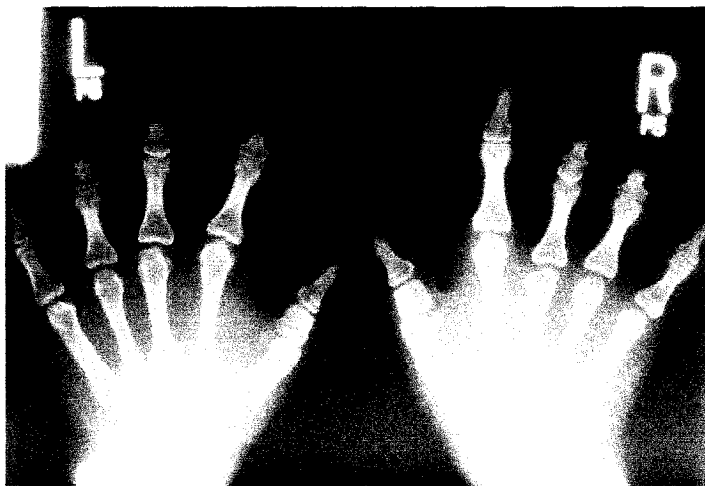
In addition to short fingers, abnormalities of the knees and feet were described in some family members. For example, individual IV-54 had abnormally shaped knee cartilage and pointed patellae, that required surgery at the age of 19 to remove worn cartilage (11). A clinical case study published when the same individual was sixteen years of age indicated that, in addition to absent middle phalanges in all of her digits, the proximal phalanx of each

Figure 15: Hand radiographs of three individuals from pedigree 10. Radiographs are of individuals a)V-70, b)IV-49, and c)IV-48. The middle phalanges in each individual are very short and appear as small rectangular structures on the radiograph. Fusion between the middle and terminal phalanges is also noted in digits 2-5 of individual IV-48, digits 2-5 of individual IV-49 (except perhaps digit 3 on the right hand), and in both digits 5, and the left digit 2 in individual V-70.

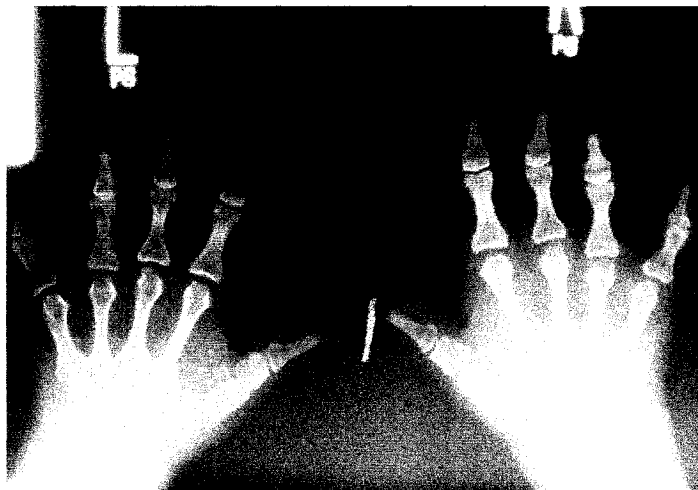
a)



b)



c)



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hallux, and the fourth metatarsal on the right foot were all short (8). Surgery was subsequently required to relieve increasing pain caused by bilateral hallux valgus deformities (inward curved great toe) and bunionettes, and to correct a sublexed and contracted fourth toe (8). Brachymetatarsia was also noted in the individual's father, although surgery was never required. Problematic knees were also described in individual III-38, although the nature of the problem was not expanded upon by the author (11). To our knowledge, there were no other musculoskeletal or developmental abnormalities among family members.

Pedigree 11

The proband from pedigree 11, p11-01, was assessed at the Clinical Genetics Department in the Western General Hospital, Edinburgh, Scotland. A skeletal survey showed short fingers due to a combination of hypoplastic and absent phalanges. Fusion of the proximal and middle phalanges was noted in digit 3 of the left hand, as well as modeling deformities of most of the metacarpals and increased joint space between the carpals. A short angle, concave kyphos (curvature of the spine; frequently referred to as a "hump") at the level of the C5/C6 vertebrae, and a mild lumbar scoliosis convex to the left were also observed. The rest of the skeletal survey was unremarkable and included normal femora, humeri, pelvis, thoracic and lumbar spine. Clinical information regarding the proband's parents were not available, however, reports suggest that both parents and the proband's sister had normal hands.

Pedigree 12

The proband from pedigree 12 is a 32-year old female with BDA1. She has short, square middle phalanges in digits 2 through 5 on her right hand, a short, square proximal phalanx in her left thumb and absent middle phalanges in digits 2 through 5 on her left hand.

It appears as if the trait is sporadic as there were no other affected family members. No further abnormalities, other than BDA1, were described.

Pedigree 14

Individuals from pedigree 14 were first examined in our laboratory after they had contacted us and expressed interest in joining a genetic study investigating the gene(s) that cause BDA1. The proband, individual IX-3/14-01 in Figure 12, described a strong familial history of brachydactyly that was first reported by William Farabee in 1903 (4), and then re-evaluated by Haws and McKusick in 1963 (7). An updated pedigree indicated that he was the grandson of individual VII-68 from Haws and McKusick's publication, and that his mother, sister, brother and nieces were similarly affected with the same condition (refer to Figure 12). An audioradiograph of his hands showed similarities with other family members described by Farabee, Haws and McKusick (4;7), including marked shortening of the middle phalanges on both hands, and shortening of the proximal phalanx in both thumbs, resulting in short, square-shaped bones (refer to Figure 16).

Haws and McKusick had extended Farabee's descriptions of affected individuals from this family, by examining skeletal abnormalities beyond those in the hands and feet. In their examination of two affected individuals, VI-41 and VI-18, they observed shortening of the distal arm, sloping of the distal end of the radius and absence of the styloid process of the ulna. The glenoid fossa was abnormally shallow and the head of the humerus was more elliptical than round. Finally, the acetabulum was shallow, the articular surfaces were irregular and the neck of the femur was short (7). Although, a skeletal survey of VIII-18, was not available to assess if similar traits were present in this individual, hand audioradiographs confirmed bilateral absence of the ulnar styloid process. Examination of

Figure 16: Hand radiographs of the proband from kindred 14 (Individual IX-3).

Radiographs of both hands of individual IX-3 from pedigree 14 show shortening of the middle phalanges resulting in short, square bones. Absence of the styloid process of the ulna (arrow) is also noted.



the proband, and immediate family members, further indicated joint dysplasia, particularly in their knees. The nature of this dysplasia was not further elaborated upon.

4.2.2 Inter- and Intrafamilial Phenotypic Heterogeneity

Despite the common trait of brachydactyly, considerable inter- and intrafamilial phenotypic heterogeneity was observed. This was most apparent in the extent of shortness of the middle phalange of each digit. While some individuals appeared to lack the middle phalanx in some or all of their digits, others showed prominent shortness of the bone without its disappearance. For example, individual 104, from pedigree 2, appeared to lack the middle phalanges in digits 2 and 5, while they were clearly present in her daughter, individual 111. Similar differences in phalanges length were noted by Drinkwater in a mother/daughter pair from his third kindred (pedigree 3) (6). Phenotypic heterogeneity also extended beyond the digits. While symptoms were restricted to BDA1 in many family members, further musculoskeletal, and neurological problems were associated with BDA1 in others. Moreover, short stature among affected individuals, as described by Drinkwater (5;6), was not consistent with height measurements made for three separate affected individuals from pedigree 2, individuals 104, 108 and 111.

4.2.3 Linkage Analysis in Pedigrees 2 and 3

Linkage of BDA1 to chromosome 5p in pedigrees 2 and 3 was assessed prior to initiation of this doctoral research project, by examining six microsatellite markers from the BDA1 locus. Based on the calculated LOD scores and haplotype analysis, linkage to chromosome 5 was not supported in either family (Akeel Baig, unpublished results). To assess if BDA1 was linked to the chromosome 2 locus instead, genotypes at sixteen

microsatellite markers from chromosome 2q were assessed (refer to Table 6). Due to the small size of each kindred examined, formal evidence for linkage (LOD scores > 3.0) was not possible. The theoretical maximum LOD scores for pedigrees 2 and 3 at a linked locus are 2.11 and 0.90 (recombination fraction (θ) = 0.00), respectively. Despite these limitations, LOD scores greater than -2.0 were observed at each locus examined, preventing formal exclusion of BDA1 to these markers (refer to Tables 10 and 11).

Table 10: Chromosome 2q LOD scores – pedigree 2

Marker	Map Position	LOD Score at $\theta =$							Zmax	$\theta_{\max}$
		0.00	0.01	0.05	0.10	0.20	0.30	0.40		
D2S1391	186.21 cM	$-\infty$	-3.21	-1.74	-1.10	-0.50	-0.21	-0.05	-0.00	0.50
D2S1384	200.43 cM	$-\infty$	-4.34	-2.26	-1.40	-0.63	-0.27	-0.09	0.11	0.72
D2S2208	205.06 cM	$-\infty$	-0.85	-0.23	-0.02	0.10	0.10	0.06	0.11	0.25
D2S2944	210.43 cM	$-\infty$	-0.81	-0.17	0.05	0.18	0.16	0.09	0.18	0.23
D2S434	215.78 cM	$-\infty$	0.28	0.82	0.91	0.76	0.46	0.15	0.91	0.10
D2S2250	216.31 cM	$-\infty$	-0.52	0.06	0.22	0.23	0.14	0.04	0.25	0.15
D2S433	216.31 cM	0.78	0.76	0.68	0.57	0.37	0.18	0.05	0.78	0.00
D2S163	218.45 cM	-0.44	-0.42	-0.33	-0.23	-0.10	-0.03	-0.01	-0.00	0.50
D2S1242	218.45 cM	$-\infty$	-1.12	-0.48	-0.25	-0.09	-0.05	-0.02	0.07	0.75
D2S424	218.45 cM	$-\infty$	-1.22	-0.57	-0.32	-0.15	-0.10	-0.06	0.20	0.78
D2S1323	221.13 cM	1.14	1.11	1.01	0.88	0.60	0.32	0.09	1.14	0.00
D2S126	221.13 cM	$-\infty$	0.07	0.62	0.73	0.62	0.38	0.11	0.73	0.11
D2S2308	227.00 cM	0.55	0.54	0.48	0.41	0.25	0.12	0.04	0.55	0.00
D2S362	231.27 cM	$-\infty$	-2.37	-1.06	-0.56	-0.18	-0.05	-0.01	-0.00	0.55
D2S427	236.70 cM	$-\infty$	-2.68	-1.34	-0.80	-0.32	-0.11	-0.01	0.01	0.46
D2S2968	251.94 cM	$-\infty$	-1.40	-0.72	-0.44	-0.19	-0.08	-0.02	-0.00	0.50
Theoretical Maximum Score	N/A	2.11	2.06	1.89	1.66	1.17	0.66	0.20	N/A	N/A

Table 11: Chromosome 2q LOD scores – pedigree 3

Marker	Map Position	LOD Score at $\theta =$							Zmax	$\theta_{\max}$
		0.00	0.01	0.05	0.10	0.20	0.30	0.40		
D2S1391	186.21 cM	0.90	0.89	0.81	0.72	0.52	0.30	0.09	0.90	0.00
D2S1384	200.43 cM	0.90	0.89	0.81	0.72	0.52	0.30	0.09	0.90	0.00
D2S2208	205.06 cM	0.90	0.89	0.81	0.72	0.52	0.30	0.09	0.90	0.00
D2S2944	210.43 cM	0.90	0.89	0.81	0.72	0.52	0.30	0.09	0.90	0.00
D2S434	215.78 cM	0.90	0.89	0.81	0.72	0.52	0.30	0.09	0.90	0.00
D2S2250	216.31 cM	$-\infty$	-1.11	-0.46	-0.23	-0.06	-0.01	-0.00	-0.00	0.55
D2S433	216.31 cM	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10
D2S163	218.45 cM	$-\infty$	-1.11	-0.46	-0.23	-0.06	-0.01	-0.00	0.00	0.10
D2S1242	218.45 cM	0.90	0.89	0.81	0.72	0.52	0.30	0.09	0.90	0.00
D2S424	218.45 cM	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10
D2S1323	221.13 cM	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10
D2S126	221.13 cM	0.90	0.89	0.81	0.72	0.52	0.30	0.09	0.90	0.00
D2S2308	227.00 cM	$-\infty$	-1.11	-0.46	-0.23	-0.06	-0.01	-0.00	-0.00	0.55
D2S362	231.27 cM	$-\infty$	-1.11	-0.46	-0.23	-0.06	-0.01	-0.00	-0.00	0.55
D2S427	236.70 cM	$-\infty$	-1.11	-0.46	-0.23	-0.06	-0.01	-0.00	-0.00	0.55
D2S2968	251.94 cM	$-\infty$	-1.11	-0.46	-0.23	-0.06	-0.01	-0.00	-0.00	0.55
Theoretical Maximum Score	N/A	0.90	0.89	0.81	0.72	0.52	0.30	0.09	N/A	N/A

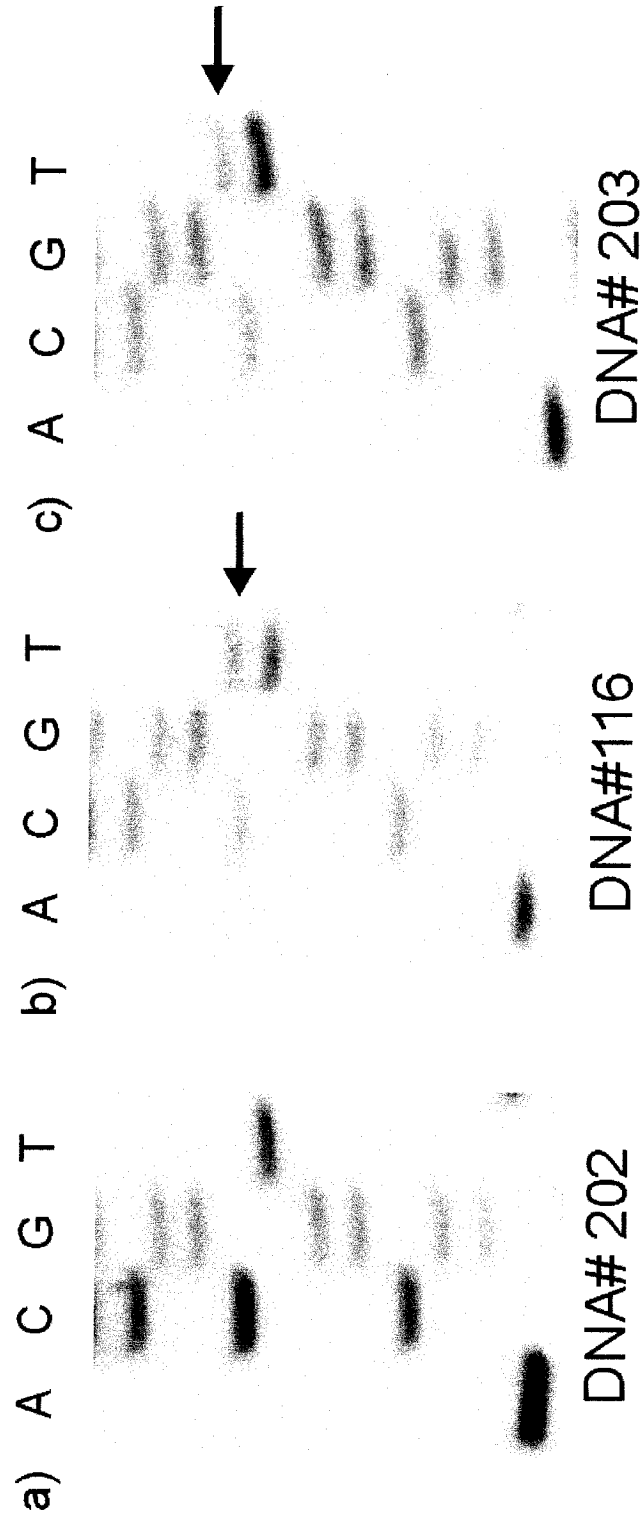
4.2.4 *IHH* Mutations in Descendants of Drinkwater's Families - Pedigrees 2 and 3

Recently Gao *et al.* reported that BDA1 was caused by mutations of the *IHH* gene in 3 large families with the malformation (122). To assess if BDA1 was caused by mutation of the same gene in the Drinkwater kindreds, the complete *IHH* coding region was sequenced in an affected and an unaffected member from each family. In doing so, a G→A transition was identified in the affected individuals from both kindreds, at position 298 of the coding sequence (Figure 17). The missense mutation was predicted to cause an Asp100Asn amino acid substitution. Comparison of the human *IHH* protein to hedgehog proteins from other species showed that the position of this amino acid is highly conserved (Figure 18). Using ASOH and sequence analysis, the G→A transition was identified exclusively in the affected individuals (Figure 19). Each affected individual carried both the variant and wild type alleles. Moreover, the G298A allele was not detected among 194 chromosomes from unrelated, non-BDA1 control individuals (data not shown).

4.2.5 Exclusion of Linkage to Chromosome 5 in Pedigrees 4 and 5

To determine if BDA1 was linked to chromosome 5p in individuals from pedigrees 4 and 5 a blind study was performed. Coded DNA from both families was genotyped at five loci in the critical region. Genotyping results were then sent to researchers in Texas, who decoded the samples and drew haplotypes accordingly. Linkage to the BDA1 locus on chromosome 5p was subsequently excluded based on inheritance of identical haplotypes in three affected and one unaffected individuals from pedigree 4, and three affected and four unaffected individuals in pedigree 5 (Figure 20). *IHH* mutational status in these

Figure 17: G→A transition at position 298 of the *IHH* gene. Shown are reverse complement exon 1 sequences of the *IHH* gene from a) individual 202, b) individual 116 and c) individual 203. Individuals 116 and 203 are from pedigrees 2 and 3, respectively, and have both been diagnosed with BDA1. Individual 202 is from pedigree 3 and is unaffected by BDA1. The black arrows indicate the reverse complement position of the G→A transition at position 298 of the coding sequence.



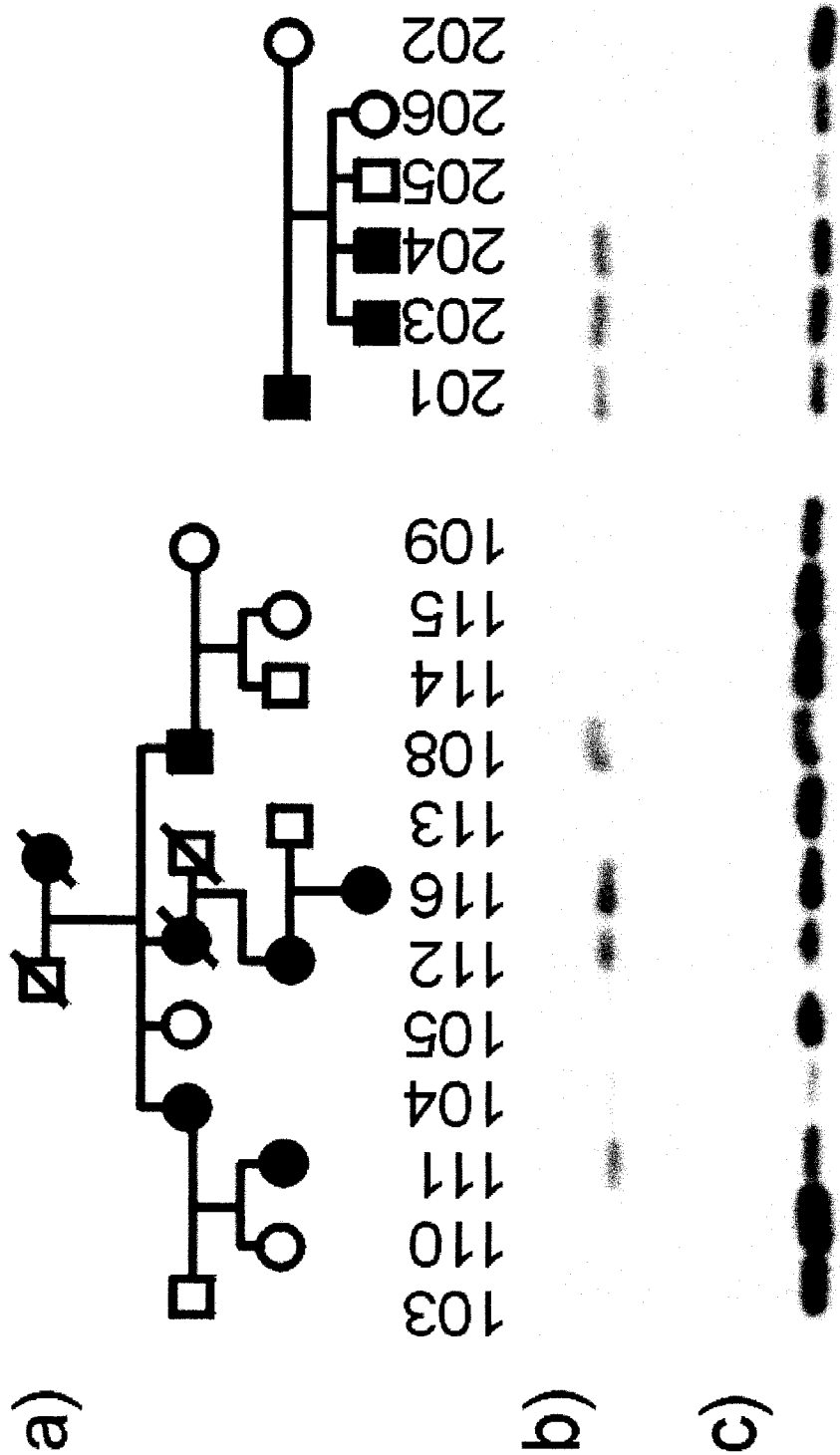
Reprinted with permission from McCready ME, Sweeney E, Fryer AE, Domai D, Baig A, Racacho L, Warman ML, Hunter AG, Bulman DE. 2002. A Novel Mutation in the IHH Gene Causes Brachydactyly Type A1: A 95-year-old mystery resolved, Hum. Genet., 111: 368-375. Human Genetics copyright Springer, Germany.

Figure 18: Conservation of Hedgehog Asp100 and substitution in BDA1. Shown are the protein alignments of 60 amino acid residues from the human IHH protein with IHH proteins from a number of organisms. The human IHH protein was also aligned to the human sonic hedgehog (SHH), fruit fly hedgehog (HH) and zebra fish echidna hedgehog proteins. Finally, alignment to the mutant proteins reported herein, by Gao *et al.* (2001) and Kirkpatrick *et al.* (2003) are shown. The three different amino acid substitutions reported by Gao *et al.* have been listed on the same row. Amino acids are shown using their single letter codes. “-“ indicates that the amino acid at that position is the same as that in the same relative position in the human IHH sequence.

<i>Homo sapiens</i>	41	PRKLVPLAYK	QFSPNVPEKT	LGASGRYEGK	IARSSERFKE	LTPNYPDII
Gao et al.	41	-----	-----	-----	-----	-----
Pedigree 2,3,7,10,14	41	-----	-----	-----	-----	-----
Pedigree 4	41	-----	-----	-----	-----	-----
<i>Mus musculus</i>	41	-----	-----	-----	-----	-----
<i>Gallus gallus</i>	37	----I----	-----	-----	---N-----	-----
<i>Xenopus laevis</i>	36	-T--S--S--	-----	-----	-S-N-----	-----
<i>Danio rerio</i>	36	----T-----	-----A---	-----	VTP-----	-----
<i>H. sapiens</i> SHH	36	-K--T-----	--I--A---	-----	-S-N-----	-----
<i>D. melanogaster</i> HH	86	A-N-Y--VL-	-TI--LS-Y-	NS---PL--V	--A-D-PK-D	-V----R--L
<i>Homo sapiens</i>	91	FKDEENTGAD	RLMTQRCKDR	LNSLAISVMN	QWPGVKLRVT	EGWDEDGHHS
Gao et al. (122)	91	----K----E	-----	-----	-----	K-----
Pedigree 2,3,7,10,14	91	-----N	-----	-----	-----	-----
Pedigree 4 (24)	91	----G----	-----	-----	-----	-----
<i>Mus musculus</i>	91	-----	-----	-----	-----	-----
<i>Gallus gallus</i>	87	-----	-----	-----	-----	-----
<i>Xenopus laevis</i>	86	-----I----	-----	-----	-----	-----F
<i>Danio rerio</i>	86	-----	-M-----K	-----	L----R----	-----L--
<i>H. sapiens</i> SHH	86	-----	-----K	--A-----	-----	-----
<i>D. melanogaster</i> HH	146	-R---G----	G--SK---EK	--V--Y----	E---IR-L--	-S-----Y--G

Modified with permission from McCready ME, Sweeney E, Fryer AE, Donnai D, Baig A, Racacho L, Warman ML, Hunter AG, Bulman DE. 2002. A Novel Mutation in the IHH Gene Causes Brachydactyly Type A1: A 95-year-old mystery resolved, Hum. Genet., 111: 368-375. Human Genetics copyright Springer, Germany.

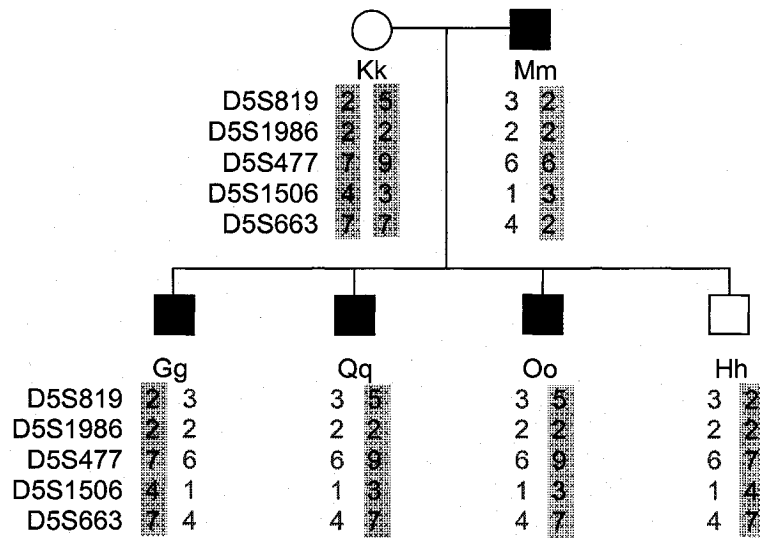
Figure 19: ASOH analysis of the IHH G298A mutation in individuals from pedigrees 2 and 3. a) from left to right, pedigree 1 and 2 respectively. DNA numbers for each sample are shown below the symbol. b) Hybridization of the oligonucleotide specific for the mutant allele, IHHx1T, and c) the wildtype allele, IHHx1C, to the Southern blot. DNA samples were amplified, electrophoresed in the order specified by a), and transferred to the Southern blot used for b) and c).



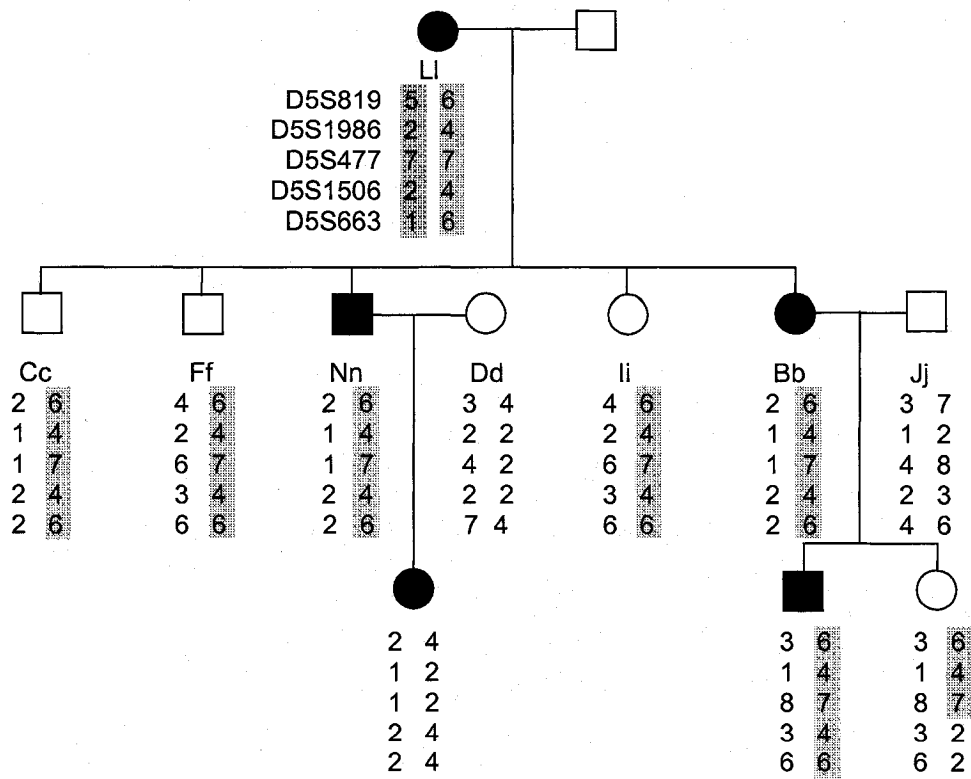
Reprinted with permission from McCready ME, Sweeney E, Fryer AE, Donnai D, Baig A, Racacho L, Warman ML, Hunter AG, Bullman DE. 2002. A Novel Mutation in the IHH Gene Causes Brachydactyly Type A1: A 95-year-old mystery resolved, Hum. Genet., 111: 368-375. Human Genetics copyright Springer, Germany.

Figure 20: Exclusion of linkage to Chromosome 5 in pedigrees 4 and 5. Haplotype analysis of markers from the chromosome 5 BDA1 locus indicate neither of the haplotypes cosegregate with the disease, thus excluding linkage. a) In pedigree 4, the yellow haplotype is inherited by affected and unaffected individuals. b) In pedigree 5, the blue haplotype is inherited by affected and unaffected individuals.

a)



b)



pedigrees was subsequently assessed by our collaborators and they reported an A→G transition at position 284 of the coding sequence in affected individuals from pedigree 5 (24). The mutation causes a glutamate to glycine amino acid substitution at codon 95. Mutations of the *IHH* coding sequence were not identified in individuals from pedigree 4 (24).

4.2.6 *IHH* Mutation Analysis and Evidence for Common Ancestry

To assess if BDA1 was caused by mutations of *IHH* in BDA1 families other than pedigrees 2 through 5, genomic DNA samples were prepared from probands in pedigrees 6 through 14, and the complete *IHH* coding regions were amplified and sequenced from each sample. While mutations were not observed in the probands from pedigrees 6, 8, 9, 11 or 12, a G→A transition was observed in sequences from pedigrees 7 (sample p7-01), pedigree 10 (sample p10-01), and pedigree 14 (sample p14-01) (Figure 21). This was the same mutation previously identified in Drinkwater's first and third families (refer to Chapter 4.2.4). Consistent with an autosomal dominant trait, a graduate student within our laboratory showed that the mutation cosegregated with the malformation in the rest of the individuals from pedigree 14 (Allison Grimsey, manuscript in preparation) (Figure 22). Inheritance of the mutation was not assessed in pedigree 7 and 10 as additional family members were not willing to participate in the study.

Identification of a single mutation in five separate pedigrees, pedigrees 2, 3, 7, 10 and 14, can occur as a result of independent mutational events at the same nucleotide position, or because of a single mutation in a common founder. To determine if the five kindreds were related, despite a lack of common surnames, haplotypes of polymorphic markers in and

Figure 21: *IHH* sequence analysis and identification of the G298A mutation in probands from three pedigrees. *IHH* coding sequences were sequenced from the DNA of probands in pedigrees 2-14. Shown are portions of the exon 1 sequence from five different probands. Arrows indicate the location of the G298A mutation. a) Reverse complement sequence from probands in pedigree 6 (P6-03), pedigree 7 (P7-01), and pedigree 8 (P8-01). b) Forward sequence from the proband in pedigree 10 (P10-01) and an unaffected, unrelated control. c) Forward sequence from the proband in pedigree 14 (P14-01) and an unaffected, unrelated control.

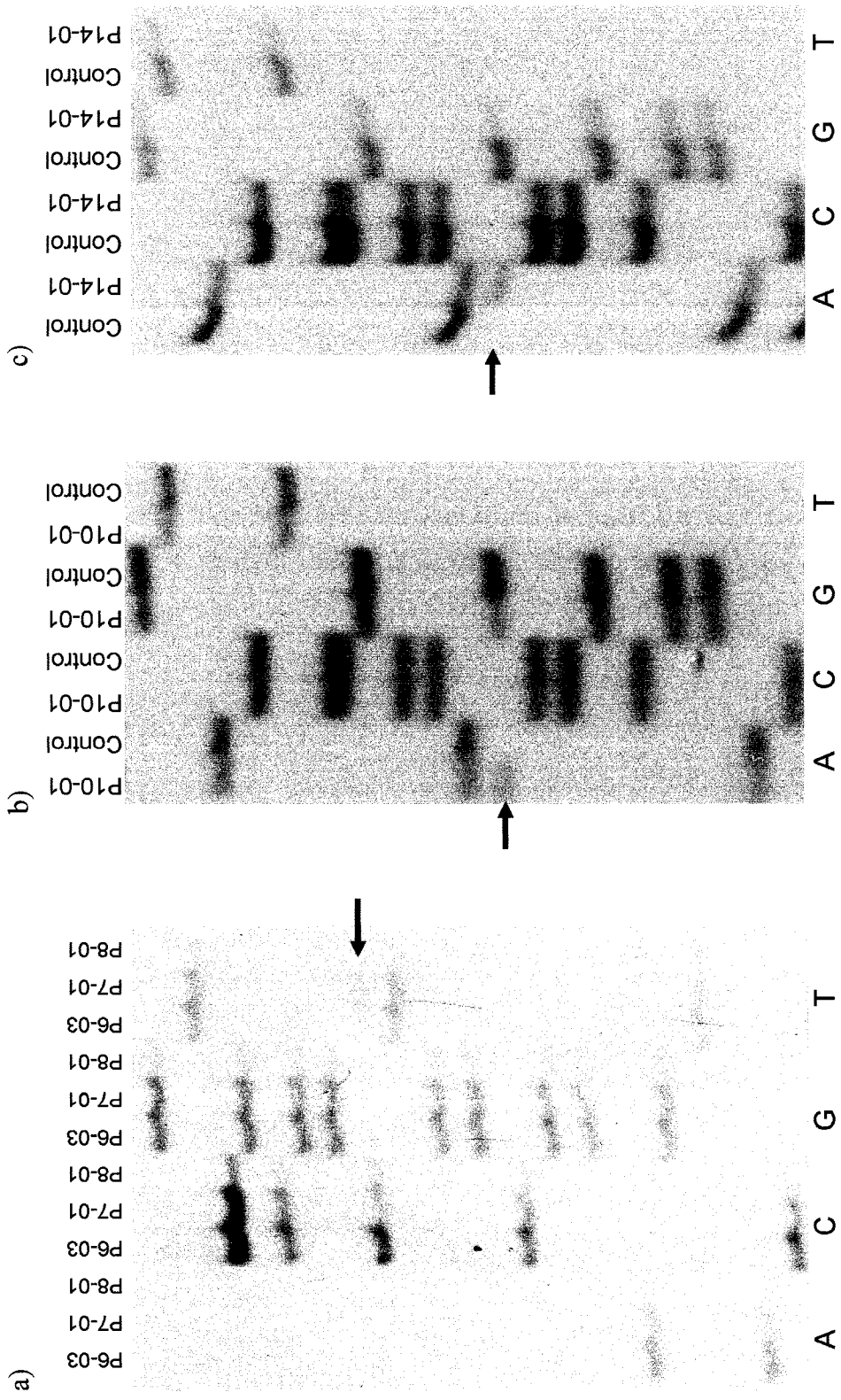
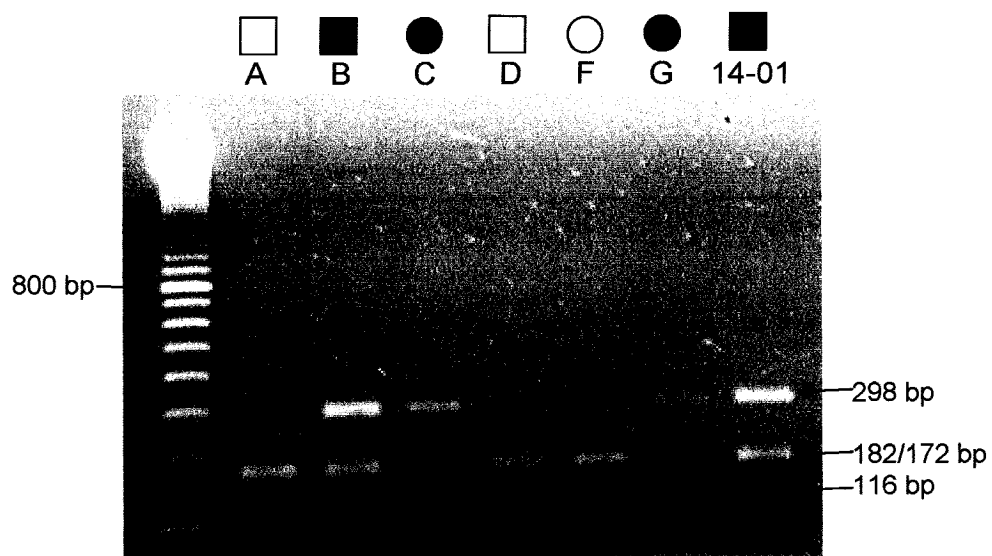


Figure 22: Genotyping of the *IHH* G298A mutation in individuals from pedigree 14. *IHH* exon 1 PCR products were amplified from DNA of pedigree 14 individuals including four affected individuals (B,C, G and 14-01) and three unaffected individuals (A, D and F). The PCR products were digested with *BsiEI*, generating three fragments (182, 172 and 116 bp) if there is a “G” at position 298, and two fragments (298 and 172 bp) if there is an “A”. The first lane on the left hand side of the gel contains a 100bp ladder. Data was provided by Allison Grimsey (manuscript in preparation).



around the *IHH* locus were assessed. By comparing the haplotypes from each family, excluding pedigrees 7 and 10, we were able to demonstrate a common haplotype among the affected individuals from pedigrees 2 and 3 that spanned 5 cM of DNA between D2S2250 and D2S1323 and included the *IHH* gene (Figure 23).

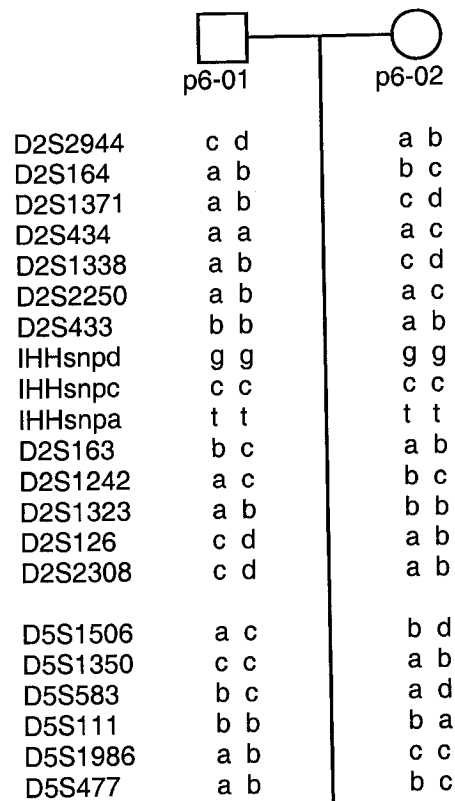
4.2.7 Assessment of Contiguous Gene Deletions in Pedigree 6

Based on the absence of disease in both parents from pedigree 6, and the presence of numerous developmental malformations in the proband, physicians hypothesized that the proband may have a contiguous gene deletion syndrome involving a number of developmental genes, including one that causes BDA1. To assess if such a macro-deletion had occurred, homozygosity mapping studies were conducted. A total of fifteen markers from chromosome 2 and six markers from chromosome 5 were assessed. Homozygosity was observed at 8/21 loci in the proband (Figure 24).

Figure 23: Haplotypes of affected individuals from pedigrees 2, 3 and 14. Marker names are listed to the left of the haplotypes, whereas the symbol and DNA number for each sample are listed above the haplotype. The map positions listed are based on the Marshfield Sex-Averaged Genetic Map. The common haplotype is highlighted by open rectangles. Solid squares represent affected males, and solid circles represent affected females. “-” indicates that the marker was not examined.

		14	01	4 4 4 3 4 3 5 2 1 9 g c - g a t t t 4 5 6 4 2 3 - 1 4 4
		14	G	4 5 4 3 4 5 1 3 g g c c - g a t t t 5 3 4 6 3 2 2 2 4 4 4 4
		14	C	4 5 3 3 3 2 1 9 g c c - g a t t t 4 5 4 2 2 3 2 2 3 2 2 4
		14	B	4 4 4 3 4 5 2 1 g g c c - g a t t t 4 5 4 4 3 2 2 1 4 4
		3	204	4 3 3 3 5 4 1 3 g g c c c g a t t t 5 5 4 3 3 3 2 1 4 3 5 1
		3	203	4 5 3 3 5 2 1 3 g g c c c g a t t t 5 2 4 1 3 2 2 1 4 3 5 4
		3	201	3 4 4 3 3 5 2 1 g g c c c g a t t t 5 5 5 4 2 3 2 2 2 4 4 5
		2	116	5 1 4 5 5 1 1 3 g c c c t g a t t t 1 3 3 3 1 3 2 2 4 3 4 6
		2	111	5 3 5 4 3 5 3 1 g g c c c g a t t t 3 5 2 4 1 3 2 2 5 8 4 4
		2	108	3 5 2 4 4 5 3 1 g g c c c g a t t t 3 5 3 4 2 3 1 2 7 8 4 4
		2	104	3 5 2 4 4 5 4 1 c g t c c g a t t t 4 5 4 4 3 3 1 2 5 8 2 4
Pedigree			Map	
DNA#			Position	
Marker				
D2S2944			210.43 cM	
D2S434			215.78 cM	
D2S2250			216.31 cM	
D2S433			216.31 cM	
SNPd			N/A	
SNPc			N/A	
SNPe			N/A	
GAC100AAC			N/A	
SNPb			N/A	
SNPa			N/A	
D2S163			218.45 cM	
D2S1242			218.45 cM	
D2S424			218.45 cM	
D2S1323			221.13 cM	
D2S126			221.13 cM	
D2S2308			227.00 cM	

Figure 24: Homozygosity mapping in the proband from pedigree 6. Fifteen polymorphic markers from chromosome 2 and six polymorphic markers from chromosome 5 were examined in individuals from pedigree 6. The name of each marker is shown to the left of the haplotypes. Homozygous markers that are uninformative for LOH are highlighted in the proband. Squares represent males; circles represent females; black symbols represent affected individuals.



D2S2944	c b
D2S164	a c
D2S1371	b d
D2S434	a a
D2S1338	a d
D2S2250	a a
D2S433	b b
IHHsnpd	g g
IHHsnpb	c c
IHHsnpa	t t
D2S163	b b
D2S1242	c b
D2S1323	a b
D2S126	b d
D2S2308	b d
D5S1506	a b
D5S1350	c b
D5S583	b a
D5S111	b b
D5S1986	a c
D5S477	a b

4.3 Discussion

To our knowledge, the prevalence of brachydactyly type A1 has not been reported, however, incidences of the trait are thought to be rare. The reports of affected individuals that do exist span a century of publications and includes less than forty kindreds (1;2;4-10;12-23;122;139). The hallmark feature in each of these reports has been shortening of the middle phalanges in digits 2 through 5, which can be mild, as with the pedigree from Chapter 3, or can result in loss of the middle phalanx entirely. A range of skeletal and developmental conditions have also been reported in association with BDA1 including clinodactyly (5;12;13;15;22), premature epiphyseal closure (9;13;14;22), short stature (4-6), nystagmus (16;17) and developmental delay (16-18;139). To examine the genetics of BDA1 in affected individuals with different manifestations of the same trait, we have studied individuals from 12 kindreds with BDA1, in addition to that described in Chapter 3. These additional kindreds reside in locations across North America, Europe, and New Zealand and include both sporadic and familial cases of BDA1.

All twelve families examined have characteristics typical of classical BDA1 including pronounced shortness of the middle phalanges in all their digits. Phenotypes varied from short, square-shaped middle phalanges in each digit, to a complete loss of middle phalanges in both the hands and feet. As with previous reports, BDA1 was clearly transmitted as an autosomal dominant trait in pedigrees with a family history of the condition. In the remaining pedigrees, for which there is only one incident of BDA1, it was not possible to determine whether the trait was caused by a *de novo* dominant mutation, or by recessive mutations. Although the predominant cases of BDA1 are autosomal dominant conditions, Mortier *et al.* recently described two families with an autosomal recessive skeletal dysplasia, that was characterized by short stature, short limbs, cone-shaped epiphyses, and

brachydactyly type A1 (123;124). Autosomal recessive inheritance was also suspected in a report of a young girl born to consanguineous parents, who had a complex syndrome including BDA1, dwarfism, ptosis, mixed partial hearing loss, microcephaly and mental retardation (17). Despite these reports, we suspect that the symptoms in each sporadic case is likely due to *de novo*, rather than recessive genetic changes since most of the BDA1 reports have described autosomal dominant inheritance patterns, and there are no incidents of consanguinity to our knowledge amongst these pedigrees. The precise nature of the underlying molecular causes, in each case, will only be deciphered when the mutations are identified experimentally.

Despite the common feature of brachydactyly, considerable inter- and intrafamilial heterogeneity was observed between the twelve kindreds examined. In some individuals BDA1 occurred as an isolated malformation. Others also experienced a variety of more complex symptoms including musculoskeletal abnormalities, scoliosis, and/or developmental delay. Variability between affected individuals, with respect to stature, metacarpal shortening and the occurrence of terminal symphalangism, were also noted. The presence of this inter- and intrafamilial heterogeneity suggests that there may be a modifier gene that can modulate the severity of the observed phenotype. Alternatively, symptoms such as scoliosis, developmental delay, renal dysfunction, nystagmus, and musculoskeletal problems may be due to independent mutations that cosegregate with the disease, or variances in environmental factors. We think that this is unlikely as many of these symptoms were observed in multiple pedigrees and have also been described elsewhere in association with BDA1 (17-19;22;139).

The most common feature reported among affected individuals from our study, other than brachydactyly, was musculoskeletal abnormalities, and in particular knee, joint and

spinal problems. Abnormal or painful knees were described in four pedigrees, including pedigrees 2, 3, 10, and 14. The frequent occurrence of joint, knee and spinal conditions in individuals with BDA1 suggests that, despite differences in penetrance, each of these phenotypes may be caused by the same gene. This is further supported by a report describing a three generation family in which BDA1, abnormal knee menisci (a crescent-shaped cartilaginous structure of the knee), and scoliosis segregated together in an autosomal dominant fashion (22). It was shown that while brachydactyly was highly penetrant, abnormal menisci and scoliosis had lower penetrance and variable expressivity. The types of knee problems varied and included arthritic pain, degenerative menisci and/or discoid menisci (disc-shaped, rather than crescent-shaped menisci). Based on the variable involvement of the spine and knees in their family, the authors speculated that other families with “isolated” forms of BDA1, may also have subtle knee and spinal conditions, and that these should be examined in addition to the hands and feet when considering a diagnosis of BDA1 (22). Scoliosis and BDA1 have also been described in a four-generation family in combination with tall stature and club feet (19).

Other than musculoskeletal abnormalities, the only other feature that had recurred in multiple kindreds was developmental delay. However, because of the high prevalence of developmental delay and mental retardation in the natural population, and because only one incident was reported in each family (pedigrees 3 and 6), it is likely that the occurrence of developmental delay is coincidental. If a contiguous gene deletion in the proband from pedigree 6 is found, it could further support involvement of different genes in the etiology of BDA1 and developmental delay in this individual. There have, however, been other reports of developmental delay and BDA1 (17;18;139), which would support a common gene in the etiology of the two traits. For example, Tsukahara *et al.* described a young girl with a

combination of BDA1, dwarfism, ptosis, ocular anomalies, mixed partial hearing loss, microcephaly and mental retardation (17). In a second report, Grange *et al.* described four of nine siblings with variable degrees of fibromuscular dysplasia, hypertension, bicuspid aortic valve, osteogenesis imperfecta, brachydactyly, learning disabilities and mental retardation (18). In this family, only those individuals with brachydactyly showed signs of learning disabilities or mental retardation, suggesting that these traits are part of the same syndrome, and were likely caused by the same genetic defect. Identification and characterization of the BDA1 gene will be necessary to better assess whether the same gene also contributes to normal neurological development.

Recently Gao *et al.* (122) described mutations in the *IHH* gene in three unrelated families with BDA1, however identification of a second BDA1 locus on chromosome 5p13.3-13.2 suggest that there are other genes that can also cause this disorder (121). To determine the mutational defect in some of the earliest reports of BDA1, we have examined these two loci in descendants from Drinkwater's first and third families (5;6). Mutational analyses of the *IHH* gene in descendants from Farabee's family (4), and from nine other pedigrees with BDA1 were also performed.

Although it is not known whether they are related, we have identified a common mutation in five pedigrees, including Drinkwater and Farabee's families that cause an aspartate to asparagine amino acid substitution at position 100. The mutation was present in the heterozygous state for all the affected individuals examined, but in none of their unaffected relatives, nor was it detected among 194 chromosomes from BDA1-unaffected, unrelated controls. Conservation of this amino acid between hedgehog proteins further supports the hypothesis that this alteration may be the disease-causing mutation in these families.

In his original reports, Drinkwater speculated that the two families he described in 1908 and 1915 may be related through a distant relative since they shared a similar phenotype and were residents of the same area in England (6). The identification of a common mutation between the two families supports this hypothesis but, despite having partial genealogy records across eleven generations, a common surname could not be identified between the two families. By comparing the haplotypes from each family, we were able to show a common haplotype among the affected individuals that spanned 5 cM of DNA between D2S2250 and D2S1323 and included the *IHH* gene. These results are consistent with a common founder from whom both families inherited the BDA1 trait, but do not prove that they were related. It remains possible that the same mutation arose independently in each family, although this alternative seems unlikely.

Drinkwater also proposed that the family represented in Figure 9B was related to the American kindred described by Farabee, through an individual named Benjamin Hyde (6). Subsequent reports, however, indicated that the information provided to Drinkwater was erroneous, and that the individual's surname from Farabee's pedigree was "Ride", and not "Hyde" (7). To address whether these families were also related, we examined polymorphic markers from chromosome 2. We were able to show that the common haplotype shared by Drinkwater's families was also shared with the descendants from Farabee's family, thus supporting the hypothesis that the three kindreds share a common founder. The haplotype analysis further suggests that Farabee's pedigree is more closely related to the second family described by Drinkwater than the first, as affected individuals from pedigrees 3 and 14 shared a common haplotype > 6 cM in size, compared to 5 cM shared between pedigrees 2 and 14.

The identification of an *IHH* mutation in five kindreds from this thesis provides support to Gao *et al.*'s recent findings implicating *IHH* in BDA1 (122). In their report, they described mutations in three unrelated families resulting in amino acid substitutions at Glu95, Asp100 and Glu131 (122). The mutation in codon 100 is of particular interest as it is the same codon altered in the families examined herein. Gao *et al.* described a C→A transversion at nucleotide position 300 of the coding region in the affected individuals from their third family, which is predicted to result in an Asp100Glu substitution. In contrast, the G298A transition, identified in individuals from Drinkwater's and Farabee's families, as well as two others, is predicted to cause a substitution of an aspartate residue by an asparagine at codon 100. The same mutation, G298A, was also recently identified in a kindred of Italian descent, with mild BDA1 (20). Independent mutations in at least three unrelated families with BDA1, which result in amino acid substitutions at the same protein residue, suggests that Asp100 is important for the normal function of *IHH* during skeletal development and that it may be a hot spot for mutation in BDA1. Asp100 is highly conserved between species and among human hedgehog proteins, supporting the importance of this residue in normal *IHH* function. Similar to Asp100, Glu95 may also represent a hot spot for mutations in BDA1, as mutations that alter this amino acid have now been identified in at least two reports (24;122). In an independent examination of *IHH* in individuals from pedigree 5, Kirpatrick *et al.* identified a novel A→G transition at position 284 of the coding sequence (24). This affects the same codon mutated in Gao *et al.*'s report, but is predicted to cause a different amino acid substitution (Glu95Gly versus Glu95Lys) (24;122). Gao *et al.* predicted that Asp100, along with Glu95 and Glu131, are necessary for *IHH* binding to the Patched receptor based on similar functions of the same region in murine *Shh* (58;122;140). Based

on the central role of IHH in regulating chondrocyte proliferation and differentiation during endochondrial ossification (56), attenuation of IHH signaling may decrease the growth potential of developing bones by reducing the number of proliferating chondrocytes, and/or increasing the number of cells differentiating from the proliferative to hypertrophic stages. As the middle phalanges are the last phalanges to ossify, they may be more affected by reduced chondrocytes than other phalanges (3). Further experimentation is required to assess the significance of Glu95, Asp100 and Glu131 for normal IHH function during skeletogenesis and to determine how these changes contribute to BDA1 pathogenesis.

BDA1 is one of two conditions known to be caused by mutations of the *IHH* gene. Mutations affecting the amino-terminal signaling domain of the IHH protein were recently identified in patients with acrocapitofemoral dysplasia (ACFD) (123), an autosomal recessive disorder characterized by short stature, brachydactyly, cone-shaped epiphyses, premature epiphyseal closure, an egg-shaped femoral head with a short femoral neck, a narrow thorax and a relatively large head (124). Homozygosity mapping of individuals from two consanguineous families was used to define a 3.74 cM critical region on chromosome 2q35-q36. This led to the identification of a C→T transition at nucleotide 137 (P46L) in family one, and a T→C transition at nucleotide 569 (V190A) in family two (123). Despite being heterozygous for these mutations, the parents of each affected individual did not show signs of ACFD. The authors did note, however, that the parents from family one had short middle phalanges (up to -2.5 SD in digits 2-4 and up to -5 SD in digit 5), which were neither rudimentary nor fused to the terminal phalanges (123). The parents from family two were asymptomatic except for mild shortening of the metacarpals and proximal phalanges (> 2 SD) in two individuals (123). Hellemans *et al.* predicted that the differences in phenotype

between individuals with ACFD and BDA1 may be in part due to the differences in location of the respective mutations (123). They showed that while the mutations causing BDA1 occur clustered together in a single pocket along the IHH protein surface, the mutations identified in individuals with ACFD are predicted to affect amino acids on the opposite face of the protein (123). Due to the lack of *in vitro* or *in vivo* data, it is difficult to determine what effect these mutations have on normal IHH function. The mild phenotype observed in some carriers of the ACFD mutations, and lack of phenotype observed in others, suggests that either the mutant protein retains some activity, or that haploinsufficiency of the IHH protein is not sufficient to greatly affect bone developmental processes. In contrast, the *IHH* mutations identified in individuals with BDA1 are inherited in an autosomal dominant pattern, indicating that the wild type IHH gene can not complement for these mutations. Gao *et al.* predicted that they may disrupt normal endochondrial ossification in the digits through haploinsufficiency of the wild-type IHH protein (122). However, if the recessive mutations identified in ACFD carriers also cause haploinsufficiency, it is more likely that the dominant BDA1 mutations disrupt IHH signaling through a dominant-negative effect.

Although evidence indicates that mutations of the *IHH* gene can cause BDA1 in a subset of affected individuals, we were unable to identify mutations within the IHH coding regions from five probands. Likewise, independent analysis of *IHH* in individuals from pedigree 4 showed that these coding sequences were wildtype. This is not surprising given the identification of a second BDA1 locus on chromosome 5p13.3-13.2 (121) (Chapter 3). By analysis of microsatellite markers from the BDA1 locus on chromosome 5p, we were able to exclude linkage to this locus in pedigree 4, as well. This suggests that there may be a third locus that can cause BDA1 in a subset of individuals, and provides further evidence for the genetic heterogeneity of this condition (24). It should be noted however, that linkage to

the *IHH* locus on chromosome 2q was not assessed in pedigree 4, thus macrodeletions of this region, or mutations in the promoter or regulatory sequences could not be formerly excluded. Likewise, based on a lack of additional DNA samples from other family members, or on the sporadic nature of the condition, linkage analysis of the remaining five pedigrees was not possible. Consequently, we were unable to confirm which locus was involved in the etiology of BDA1 in these families. The presence of a contiguous gene deletion on either chromosome 2q or 5p was examined in the proband from pedigree 6 using homozygosity mapping, but without definitive findings. Despite these set backs, the lack of mutations in the *IHH* coding regions from each of the five probands increases the likelihood that the chromosome 5p locus is involved. Only by identifying the disease-causing gene from this region, and screening it for mutations in the five families, will we be able to better understand the underlying genetic abnormality.

In summary, we have identified the cause of brachydactyly type A1 in three of the original kindreds described with the disorder, as well as in two additional kindreds. The mutation is predicted to cause an aspartate to asparagine amino acid substitution at residue 100 of the *IHH* protein. As this is the same residue effected by a mutation in a Chinese kindred with BDA1 (122), it suggests that Asp100 is important for *IHH* function during normal bone and limb development. Moreover, we have shown that, based on a common haplotype at the *IHH* locus, it is probable that the two Drinkwater families shared a common ancestor from whom the *IHH* mutation was inherited in both families.

Chapter 5 – Candidate Gene Analysis at the Chromosome 5p BDA1 Locus

In the last two decades, a paradigm shift has occurred in the way molecular geneticists identify disease-associated genes. In as recent as 1980, specific disease genes were cloned almost exclusively using a “functional cloning” strategy (141). The most important factor using this strategy was the identification of biochemical defects related to the disease, rather than the gene’s chromosomal location. Today the most common strategy used for identifying disease genes is “positional candidate cloning”, which relies on analysis of candidate genes according to their position within the human genome and either their predicted function or expression pattern (141-143). It has been popularized in recent years by publication of the draft human genome sequence (144;145), and more recently by the completion of the Human Genome Project (April 14, 2003 press release at www.genome.gov). Annotation of the predicted 30,000 – 40,000 protein-coding human genes is ongoing and remains one of the key objectives for the National Human Genome Research Institute (NHGRI) (143;144). Meanwhile, human genome sequences are available through private and public databases such as the Celera, National Center for Bioinformatics (NCBI) and UCSC Genome Bioinformatics (Genome Browser) databases. These databases permit easy access to information about known and predicted genes at a specific chromosomal location and minimizes the amount of resources required to build transcriptional maps of a disease interval compared to costly, labour-intensive techniques used only a few years ago (141;142). Moreover, the publication of genome sequences from mice (146), worms (147), *Drosophila melanogaster* (148;149) and other model organisms (reviewed in 150;151), has opened up the field of comparative genomics, and permitted comparison of sequences between species. These comparisons provide a powerful tool by allowing investigators to identify regions of similarity between genomes and make inferences about gene structure, function and regulatory elements (151).

The extraordinary efforts required to sequence the human genome were coordinated by two independent groups, the publicly funded International Human Genome Sequencing Consortium and the privately funded research group at Celera Genomics (144;145). The Human Genome Project (HGP) was officially initiated in 1990 under the direction of the National Institute of Health and the United States Department of Energy. Collaborations were subsequently made with 20 groups from the United States, the United Kingdom, Japan, France, Germany and China, with the ultimate goal of producing a high quality, contiguous sequence of each human chromosome. To do this, the HGP used a hierarchical shotgun sequencing approach. BAC libraries were generated that covered the genome, and shotgun sequencing was then used to characterize the overlapping clones (144). Because this approach requires assembly of a clone contig prior to shotgun sequencing, it can prove costly and labour intensive. Gaps in the sequence are also inevitable, as some regions of the human genome are difficult to clone and may not be represented in the BAC libraries (152). However, because the sequence assembly is anchored to the genome by a physical map, many of the problems introduced by repetitive and duplicated elements could be avoided, thus increasing the accuracy of the assembly (144;152). By contrast, Celera Genomics used a whole genome shotgun sequencing (WGS) approach, in which genomic fragments were sequenced directly without initially generating a clone contig. This approach was first used successfully to sequence the *Haemophilus influenzae* genome (153) and has since been used to sequence the genomes from a number of prokaryote and multicellular organisms (reviewed in 150). Although the WGS approach does not require assembly of a BAC library, or ordering of clones into overlapping fragments, it is more prone to errors as a consequence of misaligning sequences at sites of repetitive DNA, or genome duplications (144;152).

Celera Genomics at least partially addressed these issues, by incorporating data from the HGP as it became available (145).

The first assembled, draft versions of the human genome sequence were published in February 2001, but were not ideal (144;145). Consistent with a “draft” sequence, they were plagued by gaps, errors, redundancy and incomplete annotation (154;155). The sequence generated by the HGP, for example, covered approximately 90% of the gene-rich, euchromatic portion of the genome. Only 28% of this sequence was in a finished format with a sequence accuracy of > 99.99%, which is contiguous and with the only remaining gaps corresponding to regions that cannot be sequenced using current techniques. In addition, there were about 150,000 gaps (155;156). Similarly, the assembled draft sequence from Celera Genomics covered only 88-93% of euchromatic DNA (145;157). A computational comparison of the two draft sequences, by Aach *et al.*, showed that although both drafts were similar in size and in the amount of unique sequence, they varied in their assembly and had differences in the size and distribution of gaps (154). Katsanis *et al.* further evaluated five draft versions of the public genome sequence, released between April 2000 and April 2001 (157). By comparing the annotated location of 138 expressed sequence tags (ESTs) to experimentally determined positions they found discrepancy rates that ranged from 36% in the April 17, 2000 release to 12% in the April 5, 2001 release (157). Inconsistencies of annotated data were also observed in our own laboratory when comparing different releases of the draft sequence. The chromosome 5 BDA1 locus, for example, was very dynamic and information regarding the number of known genes and physical size of the region frequently varied between different versions of the genome sequence. Moreover, genes that had been assigned to the critical interval in one version of the genome were often reassigned to other chromosomal locations only months later. In October 2000, the Genome

Browser website (version 3) indicated that the BDA1 critical region was approximately 6.7 Mb in size and contained ten known genes. By the November 2002 release, the size of the region had been reduced to only 3.8 Mb, and the number of known genes had doubled from 10 to 20.

Despite the above shortcomings, access to the draft genome sequence has provided investigators with a wealth of information about the human genome and its genes. When the HGP was initiated, fewer than 100 disease genes had been identified. Today scientists have discovered more than 1580 disease genes (142) (LocusLink, January 23, 2004 (search “disease_known AND has_seq”)). This number is sure to increase as the amount of biological and genetic information about our genome continues to be deposited into public databases at a phenomenal rate. Since the first releases of the draft human genome sequence, considerable efforts have been made to convert the “draft” sequence to “finished” sequence, and on April 14, 2003 the International Human Genome Sequencing Consortium announced its successful completion of the HGP (156). The finished human genome sequence covers about 99% of the euchromatic regions and has been sequenced to an accuracy of 99.99%. It is highly contiguous and contains less than 400 defined gaps (156). The BDA1 locus on chromosome 5p13.3-p13.2 is 3.0 Mb in size and contains 15 known genes. Seventeen predicted genes have also been mapped to the BDA1 locus (according to the July 2003 release) based on alignment of ESTs, mRNA or information about protein homologies from other translated sequences.

Key to identification of disease genes using the positional candidate cloning strategy is the ability to recognize transcriptional units within a defined locus and to assess their candidacy as the disease-causing gene (141). This process again has been simplified through the efforts of the HGP and the generation of draft transcriptional maps for each chromosome.

Where previously investigated, the same public databases that provide these maps also provide internet links to information about each gene's expression pattern or potential function. This enables researchers to assess the likelihood that genes within a disease interval can cause the disease, and to prioritize analysis based on interesting functions or expression in relevant tissues. We choose to use this method when looking for the BDA1 gene, rather than a traditional "positional cloning" strategy that considers only a gene's position. This was done to focus our efforts on those genes that are more likely to cause the trait, and to ultimately identify the mutation. Since approximately 99% of the reported mutations underlying disease phenotypes have been found in the coding regions and at splice sites (142), we further decided to focus our attention only on the transcribed regions, and flanking intronic sequences from each gene. This chapter details this effort and describes the exclusion of the transcribed regions from fifteen genes of carrying the disease mutation.

5.1 Materials and Methods

5.1.1 Assessment of Gene Candidacy

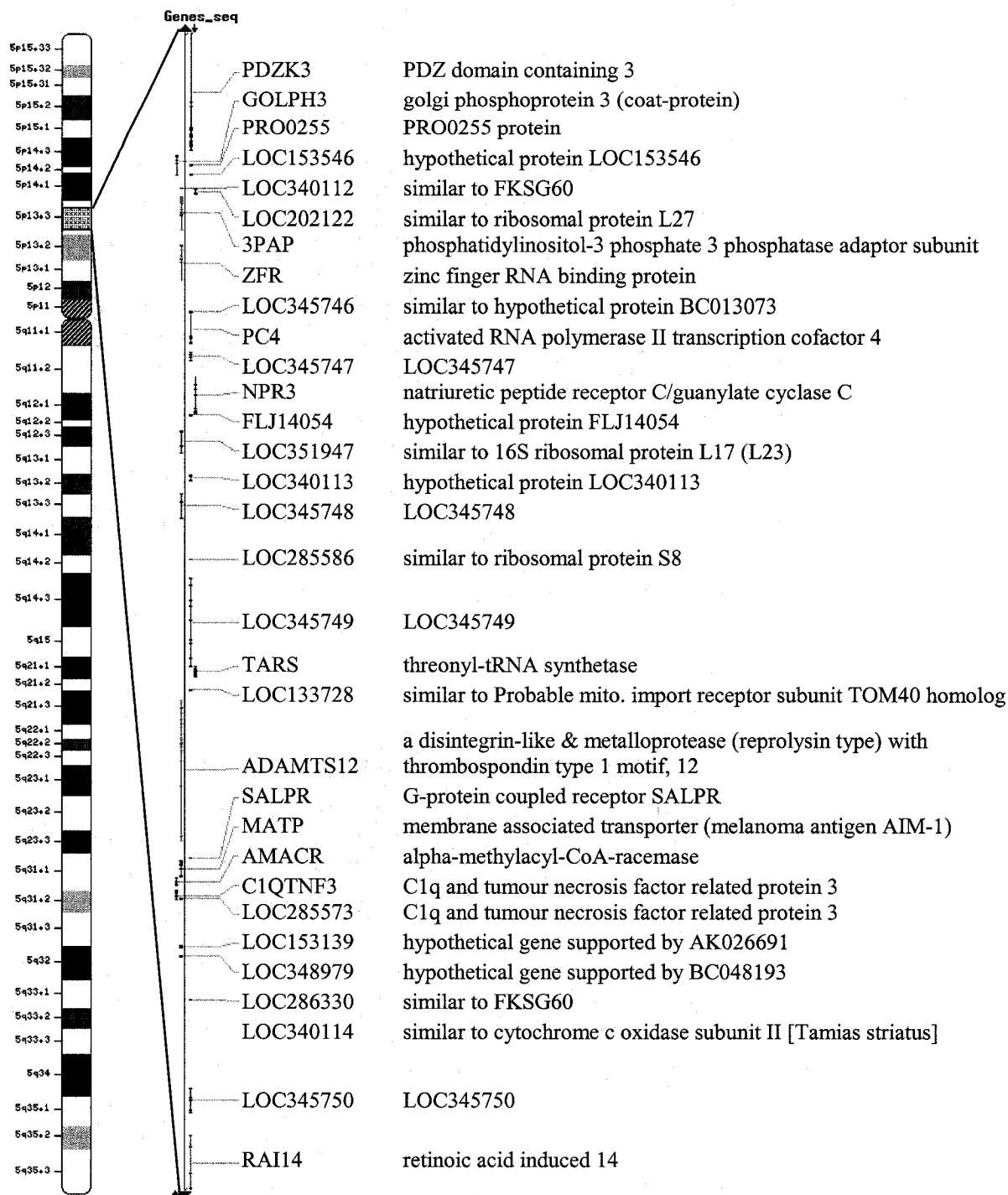
Physical maps of the BDA1 locus on chromosome 5p were examined for the presence of known genes between D5S1986 and D5S426/D5S663 by consulting two separate resources – the NCBI and the Genome Browser websites (www.ncbi.nlm.nih.gov and genome.ucsc.edu, respectively) (Figures 25 and 26). Priorities for gene analysis were subsequently based on known or predicted functions, involvement in skeletogenesis, expression patterns, and physical position within the critical region in at least two versions of the draft genome sequence. Genes that are necessary for normal endochondrial ossification were given the highest priority for sequence analysis, followed by genes that have predicted functions related to cell signaling, transcriptional regulation, or maintenance of the extracellular matrix. Finally, genes without known functions, but whose expression has been identified in mesenchymal, cartilaginous or osseous tissue were examined. Known genes that are not expressed in skeletal tissue were given the lowest priority for sequence analysis.

5.1.2 Mutation Screening through Cycle Sequencing

In total, fourteen known genes and one predicted gene (Table 12) were screened for mutations within the transcribed regions. The promoter region of one of these genes, NPR3 (158), was also analyzed, as well as a portion of a fifteenth known gene, cadherin-18. Analysis of cadherin-18 was not completed however, due to repositioning of the gene outside of the critical region in more recent drafts of the human genome sequence.

To determine the structure of each gene, and the arrangement of individual introns/exons, annotated mRNA and EST sequence alignments were referenced through the Genome

Figure 25: NCBI physical map of genes at the Chromosome 5p13.3-13.2 BDA1 critical region. On the left side of the figure is an ideogram of the human chromosome 5. The BDA1 critical region is highlighted in red and expanded to the right. The position of each gene along the map is represented by colored lines on either side. Each color indicates what evidence is available to support the gene model. Blue denotes a confirmed gene model based on alignment of mRNA and/or ESTs to genomic DNA, dark brown denotes a gene model predicted by Gnomon that has EST support, light brown denotes a gene model predicted by Gnomon without EST support and orange denotes discrepancies between the mRNA sequence and the gene model. The name or locus designation of each gene is listed at the right-hand side of the figure. Source: NCBI Map Viewer (www.ncbi.nlm.nih.gov/mapview) (October 5,2003).



NCBI Map Viewer October 5, 2003

Figure 26: UCSC Genome Browser physical map of genes at the Chromosome 5p13.3-13.2 BDA1 critical region. This window depicts the BDA1 critical region from the UCSC Genome Browser site (July 2003 release) and includes the nucleotide positions of the critical region on the chromosome 5 map, cytogenetic bands, the position of STS markers, known and predicted genes, and human mRNA that maps to the genomic sequence. Also shown is a tiled list of the known genes from the RefSeq database. The position of the flanking recombinant markers, D5S1986 and D5S426 are shown by red arrows.

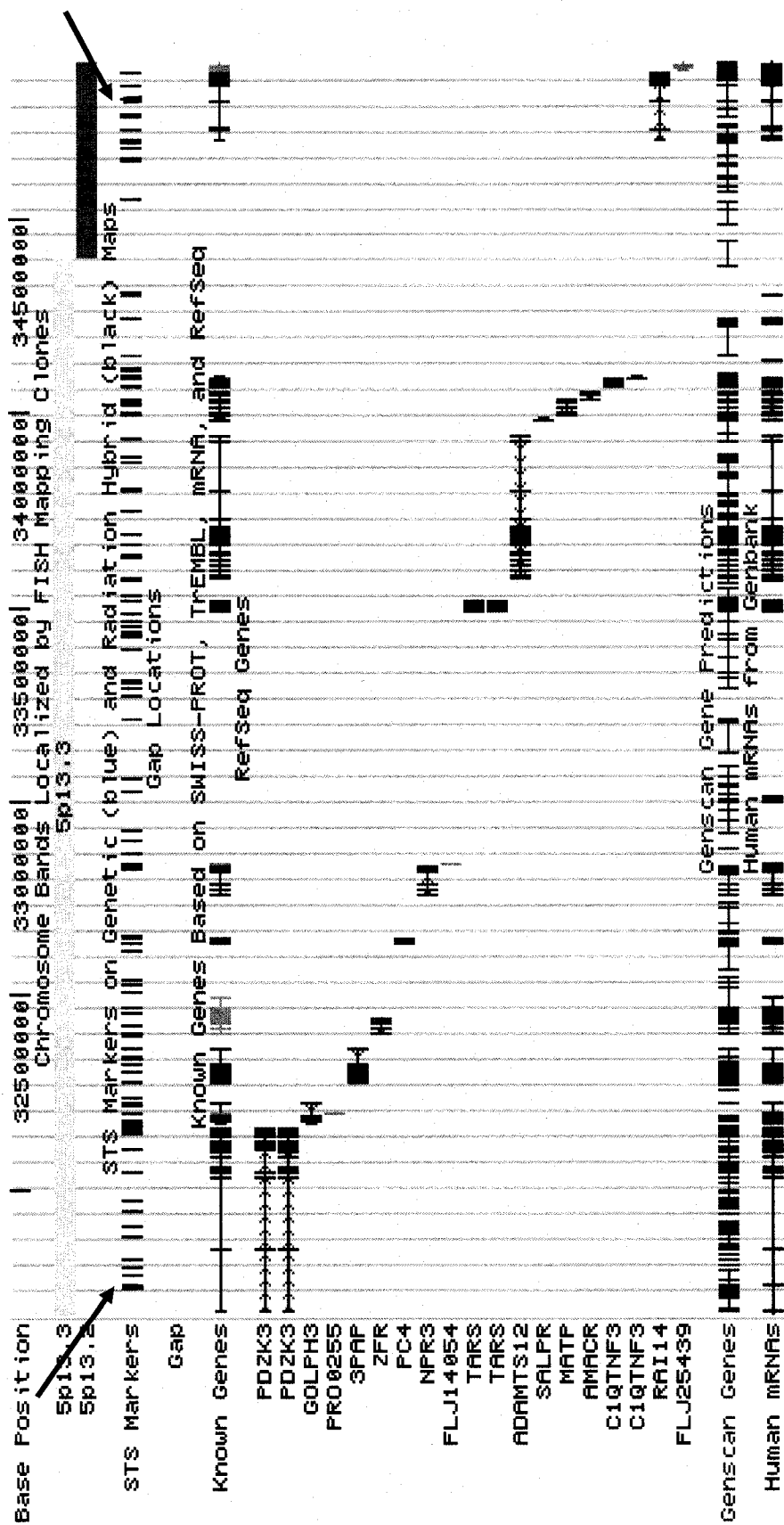


Table 12: Candidate genes from the BDA1 critical region examined for mutations.

Gene Name (Gene Symbol)	Model mRNA Accession	Accession used in Primer Design	RefSeq Gene Status	Transcript Size (bp)	No. of Exons
PDZ containing 3 (PDZK3)	a-NM_178140 b-NM_015022	NM_1718140 AF338650 estBX648242	a-Reviewed b-Reviewed	a – 11705 b – 11108	a – 25 b – 21 (& 1 alternative exon)
Golgi phosphoprotein 3 (coat protein) (GOLPH3)	NM_022130	BC012123 AK057544	Reviewed	2676	4
Phosphatidylinositol-3-phosphate associated protein (PIP3AP)	NM_019061	AK000483 AY028703	Reviewed	5064	17
Zinc finger RNA binding protein (ZFR)	NM_016107	AJ314790 BC000476 AC008949	Validated	4667	20
Activated RNA polymerase II transcription cofactor 4 (PC4)	NM_006713	BC022339 estB1546512 estBG770891 estB1755295	Provisional	1312	5 (& 5 alternative exons)
Natriuretic peptide receptor C/ guanylate cyclase C (NPR3)	NM_000908	M59305 (158) estBG400846 estBG427953 estBU626326	Provisional	1752	8 (& 4 alternative exons)
Hypothetical protein FLJ14054 (FLJ14054)	NM_024563	AK024116	Predicted	2896	1
Threonyl-tRNA synthetase (TARS)	a-NM_003191 b-NM_152295	BC101578 estB1546605	a-Reviewed b-Provisional	a-2644 b-2662	a-19 b-19 (& 1 alternate exon)
A disintegrin-like & metalloprotease (reprolysin type) with thrombospondin type 1 motif, 12 (ADAMTS12)	NM_030955	AJ250725	Reviewed	5115	25
G-protein coupled receptor SALPR (SALPR)	NM_016568	D88437	Provisional	1857	1
Membrane associated transporter (MATP)	NM_016180	AF172849	Reviewed	1686	7
Alpha-methylacyl-CoA-racemase (AMACR)	NM_014324	AF047020 estAK022765	Provisional	2068	5 (& 1 alternate exon)
C1q and tumour necrosis factor related protein 3 (C1QTNF3)	a-NM_030945 b-NM_181435	AF326976 AK094895	a-Provisional b-Provisional	a-1710 b-3003	a-6 b-1
Retinoic acid induced 14 (RAI14)	NM_015577	AF155135 BC028681 AY354204 AY317139 estBX475171	Provisional	4925	18 (& 5 alternatively spliced exons)
Similar to hypothetical protein BC013073 (LOC254411)	N/A	XM_172455	N/A	618	3

Browser website (<http://genome.ucsc.edu>) (Table 12). Intronic primers were subsequently designed using the Oligo software package (version 4.0) to allow amplification of the complete exon and at least 30 base pairs of flanking intronic sequence (Appendix 2 – list of sequencing/ PCR primers). In cases where the locus of interest exceeded 350 base pairs, additional exonic primers were designed to allow generation of shorter, overlapping amplicons that span the entire region. Alternatively, primer pairs were designed to amplify a single PCR product up to 1 kb in size. Nested primers, spaced approximately 150-200 bp apart, further allowed mutation analysis across the entire amplicon. It should also be noted that primers were only designed to amplify 3 of 18 exons from RAI14, and five alternatively spliced exons, since the remaining transcribed regions were positioned outside of the critical region on the physical map.

To screen for mutations, each exon was amplified individually from genomic DNA of BDA1-unaffected (sample 33 from pedigree 1), or unrelated, unaffected controls, and BDA1-affected (samples 19, 74, 61 or 70 from pedigree 1 and/or the probands from pedigrees 6, 8 and 9) individuals. PCR products were subsequently sequenced with the same primers used for amplification. Larger PCR products were also sequenced with nested primers, to allow analysis of the complete exon. Autoradiographs from each sequence were assessed individually to verify that the complete transcribed region had been sequenced, and to determine whether there were any nucleotide differences between affected and unaffected individuals. Nucleotide differences were characterized as “naturally occurring” polymorphisms or sequence variants, and not the disease-causing mutation, if both alleles were present in unaffected individuals, if affected individuals were not heterozygous, or if the difference had been reported previously in public databases. Novel nucleotide changes from the wildtype sequence that occurred exclusively in BDA1-affected individuals, were

further characterized using either RFLP or sequence analysis to assess whether the change cosegregated with the disease, and whether it was absent from the natural population (Chapter 5.1.3). All PCR and sequencing reactions were performed as previously described (Chapter 4.1.3), except for sequencing of the PDZDx15a45, C1Q-CA and C1Q-BH PCR products (Appendix 2), which were performed by the OHRI Core Sequencing Facility. Portions of the transcribed regions from *TARS*, *ADAMTS12*, *GOLPH3*, *PC4*, *FLJ14054*, *AMACR*, *RAI14* and *PDZK3* were amplified and sequenced with technical assistance by individuals within the laboratory.

5.1.3 Genotyping of *PDZK3* Variants

To assess whether two variants from *PDZK3* cosegregated with the disease allele, exon 13 and a portion of exon 15 were amplified individually from the 38 DNA samples in pedigree 1 using the PDZDx13 and PDZDx15d primer sets (Appendix 2). Genotypes at the exon 13 locus were subsequently determined by sequencing the exon 13 PCR products using the PDZDx13.3-F primer (Appendix 2) and the protocol described in Chapter 4.1.3. Restriction digests using the restriction endonuclease, *HinP1I* (New England Biosystems Inc., Beverly, Massachusetts), were further used to confirm each genotype. This enzyme is specific for the “G”, but not the “A” allele and generates fragments that are 608 and 452 bp in size. Genotypes at the exon 15 locus were assessed by RFLP analysis using the restriction endonuclease *DdeI* (New England Biosystems Inc., Beverly, Massachusetts). This enzyme is specific for the “A”, but not the “G” allele, and generates DNA fragments 533 and 432 bp in size. All of the digests were prepared with 10 units of either *HinP1I* or *DdeI* enzyme, and 1x digest buffer as suggested by the manufacturer. The digests were subsequently incubated at 37°C for 2 hours.

To determine the allele frequency of the above variants in the natural population, *PDZK3* exons 13 and 15 were amplified from DNA of 93 and 89 unaffected, unrelated controls respectively, using the primers listed above. They were then examined by RFLP analysis. As with pedigree 1 samples, *PDZK3* exon 13 PCR products were digested using the restriction endonuclease *HinPII* while the exon 15 PCR products were digested using the restriction endonuclease *DdeI* (New England Biosystems Inc., Beverly, Massachusetts) as described above.

5.1.4 Mutation Screening by PCR Amplification from Monoallelic Hybrid Cell Lines

To assess whether the candidate genes were present on the maternal and paternal copies of chromosome 5 in an affected individual from pedigree 1, haploid cell lines were prepared by GMP Genetics Inc. (Waltham, MA, USA) (159;160). Lymphoblastoid cells from individual 74 (in Figure 4) were fused with HAT-sensitive, geneticin-resistant mouse E2 cells. The hybrids were then selected on a HAT plus geneticin medium (159), and screened by GMP Genetics Inc. for clones containing only one copy of human chromosome 5. Two hybrid cell lines were received, EC4 and EC12. To determine which hybrid line carried the maternal haploid chromosome and which one carried the paternal chromosome, DNA from each hybrid was genotyped using the markers D5S1986, D5S477, D5S583, and D5S663, as described in Chapter 3.1.3. DNA from samples 74 (diploid), 12 (father, affected) and 13 (mother, unaffected) were also included in the genotyping reactions as positive controls.

To screen the monoallelic hybrids for the presence of each candidate gene, individual exons from DNA of each hybrid, sample 74 (positive control), mouse DNA and dH₂O (negative controls) were amplified using the primers described in Chapter 5.1.2. Optimization of the PCR conditions were frequently required to better promote primer

annealing to the human, and not mouse DNA. Consequently some of the reaction conditions used to analyze the GMP hybrids varied from those used in previous sections.

5.2 Results

5.2.1 Prioritization of Candidate Genes

With the information provided by NCBI (Figure 25) and UCSC's Genome Browser (Figure 26), we identified a series of genes which are known to map within the critical region (Table 12). Based on the identification of *IHH* mutations in a subset of individuals with BDA1 (20;24;122;137), it is reasonable to assume that other BDA1 genes are part of the same biological pathway. Despite this, according to the information currently available, none of the genes identified within the chromosome 5p critical region meet this criterion. Other factors were subsequently used to prioritize analysis of candidate genes, including the proteins known or predicted function, and its expression pattern (Table 13). The most important factor considered was whether the gene, or its homolog in another species, is known to mediate skeletogenesis. Only two genes were identified that fit this criteria, *NPR3* (161-163) and *CIQTNF3* (164). Mutations of *Npr3* in mice cause a skeletal overgrowth phenotype characterized by an exceptionally long body, and delayed endochondrial ossification (161). *Cors-pending*, the mouse homolog of *CIQTNF3*, is expressed at high levels in prechondrocytic mesenchymal condensations from mouse embryos at 13-15 days postcoitus, and promotes growth of the mesenchymal cell line, CH310T1/2 (164). Genes that encode transcription factors, signaling molecules, and extracellular matrix proteins were also considered strong candidates (assuming that they were also expressed in skeletal tissues) since these type of proteins can have direct effects on differentiation processes (165). For example, it has been demonstrated that PC4 can both mediate transcriptional activation of some genes (166;167) and repress transcription of others (168;169). It was

Table 13: Prioritization of known genes at the Chromosome 5 BDA1 locus.

Gene Name (Gene Symbol)	Locus Link Accession	Priority	Comments
PDZ containing 3 (PDZK3)	23037	++++	-contains 6 PDZ protein interaction domains(170;171) -is proteolytically cleaved to generate a secreted product with two PDZ domains (171) -interacts with prenilin-1 in the Wnt/ β -catenin pathway (170)
Golgi phosphoprotein 3 (coat protein) (GOLPH3)	64083	++	- peripheral membrane protein of golgi stack (172) - numerous cDNA sources include osteosarcoma, chondrosarcoma and Ewing's sarcoma tissue (UniGene Cluster Hs.18271)
PRO0255 protein (PRO0255)	29042	+	-only cDNA source identified thus far is liver (UniGene Cluster Hs.278935)
Phosphatidylinositol-3-phosphate associated protein (PIP3AP)	54545	++++	-cDNA sources include an osteosarcoma cell line, subchondral bone, and metastatic chondrosarcoma (UniGene Cluster Hs.93872)
Zinc finger RNA binding protein (ZFR)	51663	++++	- contains a Zn-finger, C2H2 type motif (173) - numerous cDNA sources include bone, an osteosarcoma cell line, and chondrosarcomas (UniGene Cluster Hs.173518)
Activated RNA polymerase II transcription cofactor 4 (PC4)	10923	++++	- has a single-strand DNA binding motif; transcriptional cofactor (158) - can activate or repress transcription of various promoters <i>in vitro</i> (169) - numerous cDNA sources include osteosarcoma cell line, osteoarthritic cartilage, chondrosarcomas, & Ewing's sarcoma (UniGene Cluster Hs.229641)
Natriuretic peptide receptor C/ guanylate cyclase C (NPR3)	4883	+++++	- point mutations in mice result in a skeletal phenotype with generalized increased bone length, and a kinked tail (161) -cDNA sources include chondrosarcomas and subchondral bone (UniGene Cluster Hs.237028)
Hypothetical protein FLJ14054 (FLJ14054)	79614	++	-cDNA sources include chondrosarcomas, bone marrow stroma, bone and whole embryos (UniGene Cluster Hs.13528)
Threonyl-tRNA synthetase (TARS)	6897	+++	-catalyzes the aminoacylation of Thr-tRNA by threonine amino acid residues (174) -numerous cDNA sources include bone marrow stroma, osteosarcoma cell lines and chondrosarcomas (UniGene Cluster Hs.84131)
A disintegrin-like & metalloprotease (repolysin type) with thrombospondin type 1 motif, 12 (ADAMTS12)	81792	++++	-encodes a protein that is part of the ADAMTS protein family (175) -family members each contain a propeptide region, a metalloprotease domain, a disintegrin-like domain, and a thrombospondin, type1 motif (175). -cDNA sources include osteosarcoma cell lines and a chondrosarcoma (176)(UniGene Cluster Hs.155824)
G-protein coupled receptor SALPR (SALPR)	51289	+	-somatostatin and angiotensin-like protein receptor -expression not yet identified in skeletal tissue (176)(UniGene Cluster Hs.170146)
Membrane associated transporter (MATP)	51151	+	-encodes a melanocyte differentiation antigen -mutations of this gene have been identified in oculocutaneous albinism (OMIM #606574) (177) -expression not yet identified in skeletal tissue (UniGene Cluster Hs.278962)
Alpha-methylacyl-CoA-racemase (AMACR)	23600	+++	-converts pristanoyl-CoA and C27-bile acyl-CoAs to (S)-stereoisomers (178) -may be a marker for tumour progression (179) -cDNA sources include a chondrosarcoma and a metastatic prostate bone lesion (UniGene Cluster Hs.49598)
C1q and tumour necrosis factor related protein 3 (C1QTNF3)	114899	+++++	-mediates mesenchymal condensation in CH310T1/2 cells (164) -expression of murine ortholog (Cors26) is restricted to fibroblasts, proliferating cartilage and kidney cells (164) - cDNA sources include a chondrosarcoma and an 8 week old embryo (UniGene Cluster Hs.171929)
Retinoic acid induced 14 (RAI14)	26064	++	-numerous cDNA sources include bone, chondrosarcomas, subchondral bone and bone marrow stroma (UniGene Cluster Hs.368605)

+++++ - highest priority; homologs mediate skeletogenesis in mice

++++ - high priority; gene product has predicted function in regulation of transcription, cell signaling or maintenance of the extracellular matrix

+++ - medium priority; other known function (compared to ++++); EST and/or mRNA has been detected in skeletal tissue

++ - low priority; unknown function; EST and/or mRNA has been detected in skeletal tissue

+ - lowest priority; gene is not known to be expressed in skeletal tissue

therefore considered a good candidate as it may be important for regulating expression of genes during skeletogenesis. *ZFR*, another known gene from the critical region, encodes a protein containing DNA and double stranded RNA (dsRNA) binding motifs (173). Although it does not have a transactivation domain, knock out experiments in mice indicate that it is absolutely required for normal growth and development during embryogenesis (180). Three other genes within the critical region were identified that meet the above criterion, *PDZK3*, *PIP3AP* and *ADAMTS12*. *PDZK3* and *PIP3AP* have potential roles in cell signaling based on the presence of PDZ protein-interacting domains (170) and interactions with the second messenger phosphatidylinositol-3-phosphate (181), respectively. *ADAMTS12* encodes a disintegrin and metalloproteinase with thrombospondin-type repeats. It is a member of the ADAM-TS (a disintegrin and metalloproteinase domain, with thrombospondin type-1 modules) family of proteins, which mediate cell-cell interactions and proteolysis of the extracellular matrix (175). Although the function of *ADAMTS12* is unknown, studies of other members in this family have shown that they are important for normal cellular differentiation (reviewed in 175).

As only seven of the fifteen known genes that mapped to the BDA1 critical region, had functions related to developmental processes, other factors were required to prioritize analysis of the remaining genes. The most important factor used to do this was expression within skeletal tissues. Genes not expressed in mesenchymal, chondrocytic, or osteocytic cells, were given the lowest priority for sequence analysis. The remaining genes were categorized as medium or low priority based on whether information was available regarding their function. Often a clear relationship between the gene product's function and skeletal development was not apparent, but knowledge of what a protein does in normal conditions may provide clues to how it contributes to the pathology of BDA1 if mutations are identified.

Five genes were identified in the critical region that are expressed in skeletal tissue but do not appear as strong candidates for the BDA1 gene. These are *TARS*, *AMACR*, *GOLPH3*, *FLJ14054*, and *RAI14*. The first two were classified with a medium priority for mutation analysis, while the latter three were classified with a low priority. *TARS* catalyzes the aminoacylation of threonyl-tRNA by threonine amino acid residues, and therefore is necessary for normal protein synthesis (174). Incorrect or perturbed protein synthesis could have a profound effect on normal development, but would also affect other cellular processes, making this gene a less likely candidate for BDA1 than those discussed above. According to the UniGene database, *TARS* mRNA is expressed almost ubiquitously and has been isolated from over 140 sources including bone marrow stroma, osteosarcoma cell lines, and chondrosarcomas (UniGene Cluster Hs.84131 *Homo sapiens*). Similar to *TARS*, a cellular function for *AMACR* has been identified, but it is not clear whether this gene contributes to normal skeletal development. The *AMACR* gene product converts pristanoyl-CoA and C27-bile acyl-CoA to (S)-stereoisomers (178). It may be a marker for tumour progression (179), and mutations have been identified in individuals with adult-onset sensory motor neuropathy (178). High levels of *AMACR* expression have been identified in liver, kidney, lung, gall bladder and salivary glands (182) but mRNA has also been found in normal bone and a chondrosarcoma (182) (UniGene Cluster Hs.49598 *Homo sapiens*). The function of the remaining three genes, *GOLPH3*, *FLJ14054* and *RAI14*, are unknown, although evidence suggests that *GOLPH3* encodes a peripheral golgi membrane protein (172), and that *RAI14* expression is induced by retinoic acid (183). *GOLPH3* and *RAI14* are both expressed in a large number of tissues, while expression of *FLJ14054* appears more restricted (Table 13).

The remaining three known genes identified in the BDA1 critical region, *PRO0255*, *SALPR* and *MATP*, were classified with the lowest priority for sequencing as there is no evidence that they are expressed in skeletal tissues. ESTs and cDNA sequences corresponding to the *PRO0255* gene have been isolated from a single fetal liver cell library (Unigene Cluster Hs.278935 *Homo sapiens*), while expression of *SALPR* and *MATP* appears limited to tissues of the central nervous system (176) and melanoma cells (UniGene Cluster Hs.278962 *Homo sapiens*), respectively. Although *PRO0255* remains unanalyzed, *SALPR* and *MATP* were sequenced early in this doctoral thesis based primarily on their predicted functions rather than their expression patterns. *SALPR* is a G-coupled protein receptor, and is therefore likely involved in intercellular signaling (176), while studies of AIM-1 (common name of *MATP* until recently) indicated that it is necessary for proper cytokinesis (184). Unfortunately, the AIM-1 protein discussed in the latter study is encoded by a gene on chromosome 17p13.1, *aurora kinase B (AURK)*, and not *MATP*.

The only predicted gene sequenced as part of this thesis work, was a gene temporarily designated as LOC254411 (model mRNA: Accession XM_172455). Although it has since been removed from the NCBI database as a consequence of standard annotation processes, it was examined based on its position in the chromosome 5p critical region, and sequence similarity to a 35 bp region of *IHH* spanning the start codon (identity of 32/35 bp) (Figure 27). The sequence similarity was identified during a standard BLASTn search of *IHH* exon 1 and its flanking intronic sequence (performed on August 26, 2002; Score = 46; E-value = 0.095). An untranscribed pseudogene, LOC391775, is located at the same position of the physical map in a recent release (NCBI, March 16, 2004).

Figure 27: LOC254411 gene structure. Structure of the predicted gene LOC254411 based on BLAST comparisons of the model mRNA (Accession XM_172455) to the genomic clone AC034240. Bold, capital letters represent exonic sequence. Underlined sequences are identical to sequences from *IHH* exon 1.

104881	atcactacag	agagcggtaa	gtcctatgat	gaaacaatag	ttcagatgag	agatgaagat
104941	gtgaaatcaa	gtatatgggg	aaaaggggaa	aacttaggtt	taaaaatcga	cgaatttggc
105001	caggcatagt	ggctcatggg	agcctgaggc	gggaggattg	cttaagctta	ggagtttgag
105061	gccagcctgg	gcacagggca	aaaccccgtc	tctacaaaaa	atacaaaaaa	tagccagttg
105121	tgggagagtt	agcggcctcc	ggtgtgggat	ggccacggag	ccggggccgga	<u>gacATGGCCC</u>
105181	<u>GGGGGCCCCG</u>	<u>CCAGCTAGGC</u>	<u>AGGCCCTCGCC</u>	<u>CCTATATGGT</u>	<u>CGCCATGCCC</u>	<u>AAGAGAGGAA</u>
105241	<u>AGCGACTCAA</u>	<u>GTTCTGGGCC</u>	<u>CACGACGCCT</u>	<u>GCTCTGGACA</u>	<u>AGTGACTGAG</u>	<u>GTGGATTACG</u>
105301	<u>CCAACTCGGA</u>	<u>TCCAGCGGTC</u>	<u>GTGAG</u> gtctg	gatgagtcaa	gaaagcctta	gccaacgctg
105361	ttcagcagga	agtaaaatct	ctttgtggct	tggaagcctc	tcaggttcct	gcagaggaag
105421	ctctttctgg	ggctgggtgag	ccctgtgaca	tcacgcacag	cggatgatgag	acgatgcca
105481	ggaggaaaagc	atctgtgaga	gaactgtctc	<u>CAGAAAAAAG</u>	<u>AAAAGCAAGA</u>	<u>GGCACAAAGA</u>
105541	<u>AGAACTGGAC</u>	<u>GGGGCTGGAG</u>	<u>GAGAAGAGTA</u>	<u>TCCCATGGAT</u>	<u>ATTTGGCTAT</u>	<u>TGCTGGCCTC</u>
105601	<u>CTATATCAGT</u>	<u>CCTGAGGACA</u>	<u>TTGTGAATTT</u>	<u>TTCCCTGATT</u>	<u>TGTAAGAATG</u>	<u>CTTGGAAGTG</u>
105661	<u>CACTTGCACT</u>	<u>GCTGCCTTTT</u>	<u>GGACAAGGTT</u>	<u>GTACCGAAGG</u>	<u>CACTACACGC</u>	<u>TGGATGCTTC</u>
105721	<u>CCTGCCTTTG</u>	<u>CGTCTGCGAC</u>	<u>CAGAGTCAAT</u>	<u>GGAGAAGCTG</u>	<u>CGCTGTCTCC</u>	<u>GGGCTTGTGT</u>
105781	<u>GATCCGATCT</u>	<u>CTGT</u> accata	tatgtatgag	ccatttgctg	ctccaatctc	caagaatcca
105841	agcaccacca	gcacattaaa	gaattccaaa	tgcttacttt	tctggtgcag	aaatattggt
105901	gggaacagac	aggaaccaat	gtggaaattc	aacttcaagt	taaaaaaaca	<u>gGTTAAAGAG</u>
105961	<u>CAAGTGTACA</u>	<u>GGAGGATTGC</u>	<u>AGCCCCCTGT</u>	<u>TCAGTACGAA</u>	<u>GATGTTTCATA</u>	<u>CCAATCCACA</u>
106021	<u>CCGGGACTGC</u>	<u>TGCCTACTGC</u>	<u>AGGCCACCAC</u>	<u>CCTCAATTTT</u>	<u>ATCTTCATTC</u>	<u>CGATTGTCAT</u>
106081	<u>GAGAATGATA</u>	<u>TTTACTCTCT</u>	<u>TTACTATCAA</u>	<u>TGTGAGCAAG</u>	<u>GACATACGGC</u>	<u>Atcattgagt</u>
106141	gagactgggtg	tttcaagatt	cccctgtcca	tggtgggtcgg	aaactgtgca	gtgaacaggg
106201	tgtgcaagtc	atcctggacc	cagtgcacag	ctttcggctc	tttgactgat	ggcaccctca
106261	gtacccattc	tccttgagag	cgtagttact	gcttcccatc	ccttgggggc	agccccgatt
106321	ctagtccatt	agtaatcaga	ttccagtttg	gacagggcgg	ctggattata	tatctgggta

5.2.2 Exclusion of Transcribed Regions from Fifteen Genes

To determine whether there were any mutations in the transcribed regions of candidate genes, each exon was amplified individually from DNA of BDA1-affected and unaffected individuals, and then sequenced. The transcribed regions from fourteen known genes and one unknown gene were assessed, but no mutations were found (refer to Figures 28 through 35). Two novel sequence variants were identified in affected individuals from pedigree 1 (samples 19 and 74) that caused amino acid substitutions of the PDZK3 gene product, but were not observed in unaffected individuals during initial mutation screens (Figure 36). The first variant, found in exon 13, was a G to A transition at nucleotide 3044 (of isoform b), and is predicted to cause an alanine to threonine substitution at amino acid position 885. Analysis of exon 13 from other pedigree 1 family members indicated that the “A” allele cosegregated with the disease and was absent from each unaffected individual within the family (Figure 36a). Examination of DNA from unaffected, unrelated controls indicated that the “A” allele occurs in the natural population at a frequency of 1.6% (4/186 alleles). The second variant, identified in exon 15 sequences, also results in a G to A transition, and occurs at nucleotide 6531 (isoform b). It is predicted to cause an arginine to glutamine amino acid substitution at position 2048. RFLP analysis of PDZK3 exon 15 PCR products, using the restriction endonuclease *DdeI*, showed that the “A” allele cosegregated with the disease allele, but that it was also present in DNA of an unaffected individual, sample 23 (Figure 36b). Moreover, sample 22, who is affected with BDA1, was homozygous for this allele. When DNA from unrelated, unaffected controls was examined, the “A” allele occurred at a frequency of 7.9% (14/178 alleles examined). Since these studies, the exon 15 SNP (nucleotide 6531) has been catalogued in the NCBI – SNP database (Accession #10066063), although an allele frequency was not reported.

Figure 28: Polymorphisms and variants identified in *PDZK3* during mutational analysis. a) The genomic structure of *PDZK3* showing the approximate location of each exon and two microsatellite markers, D5S1986 and D5S477. Exons are represented by grey boxes, UTRs are represented by dashed lines, and exons exclusive to isoform a are in black. The exon numbers are based on isoform b. Exons in blue represent alternatively spliced exons. b) The physical map of exons 4-20 is enlarged to show the location of each polymorphism/variant (arrows). Listed are the variation type, the SNP database accession if available (in brackets), the nucleotide position (based on *PDZK3* isoform b – Accession NM_015022) and the predicted amino acid change, if applicable. Red text indicates that the variant has not been reported elsewhere and was not identified in unaffected controls during the initial mutation screens, while italics indicates that the locus was genotyped for each individual from pedigree 1. Figures are not drawn to scale.

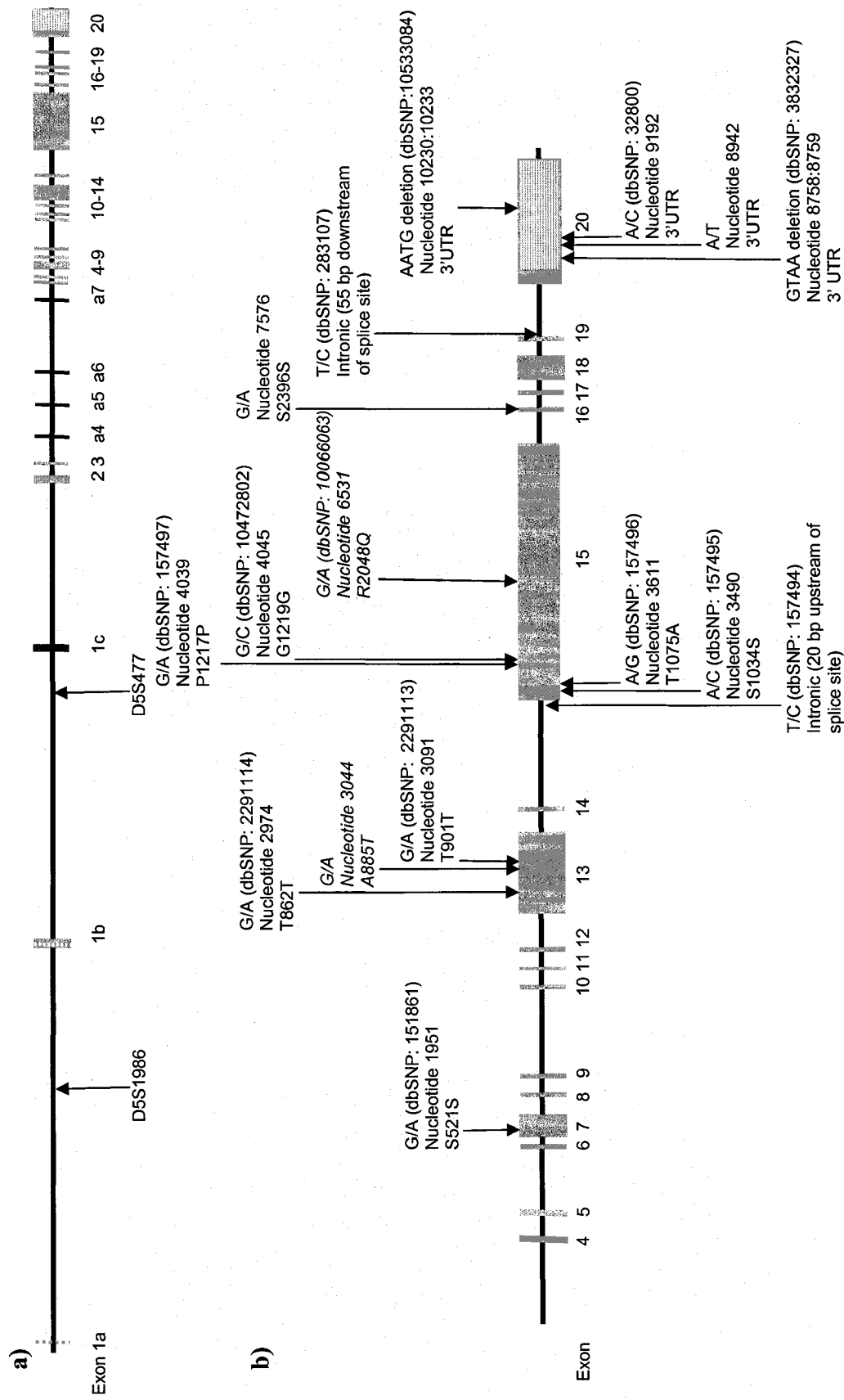
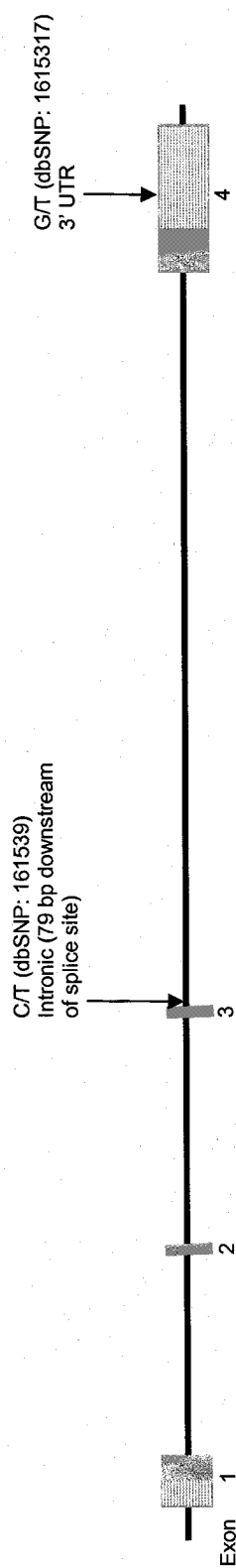
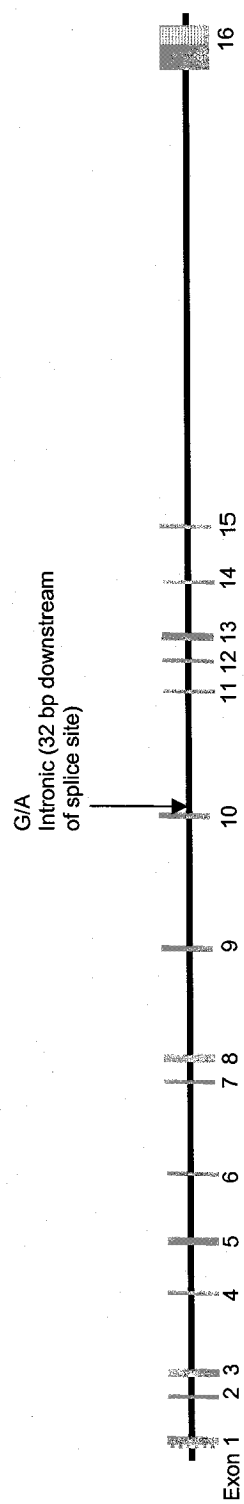


Figure 29: Polymorphisms identified in the *GOLPH3* and *PIP3AP* genes during mutational analysis. The genomic structure of *GOLPH3* and *PIP3AP* are shown. Exons are represented by grey boxes, UTRs are represented by dashed lines. Each SNP observed during the mutation screen is indicated by an arrow. Listed for each SNP is the variation type, the SNP database accession if available (in brackets), the nucleotide position (based on *GOLPH3* nucleotide accession NM_022130 and *PIP3AP* nucleotide accession NM_019061) and the predicted amino acid change, if applicable. Red text indicates that the variant has not been reported elsewhere and was not identified in unaffected controls during the initial mutation screens. Figures are not drawn to scale.



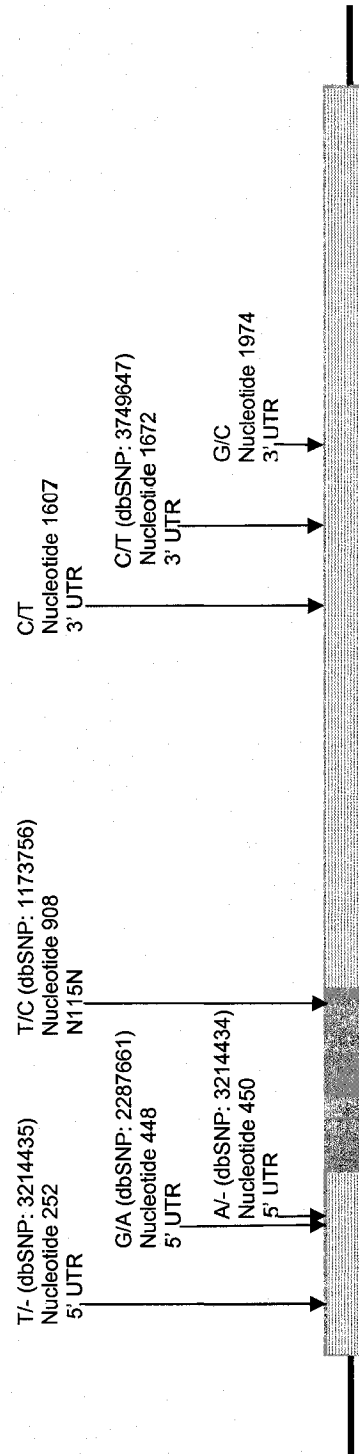
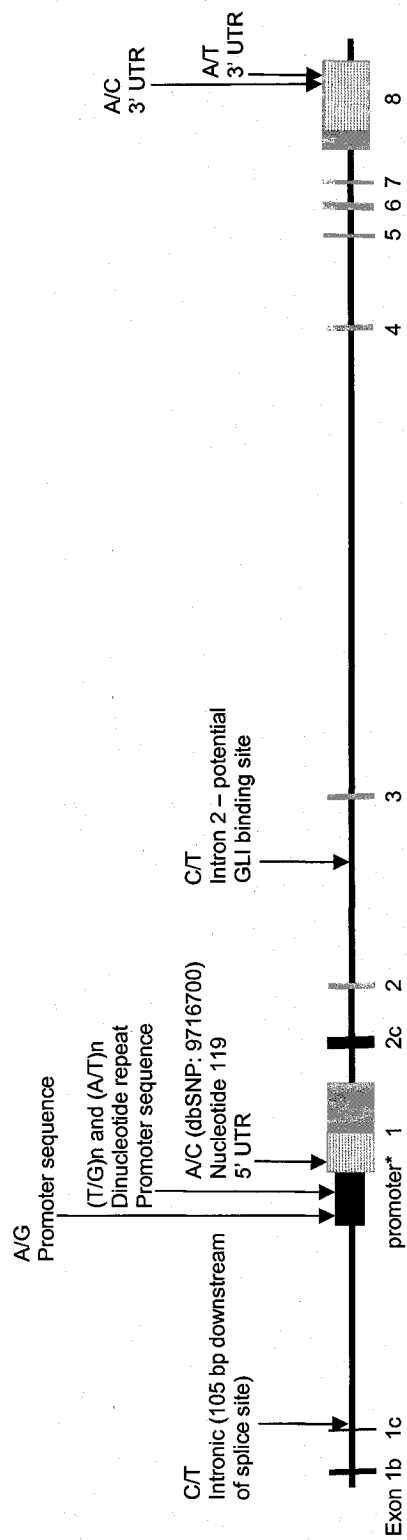
GOLPH3



PIP3AP

Figure 30: Polymorphisms and variants identified in the *ZFR* and *PC4* genes during mutational analysis. The genomic structure of *ZFR* and *PC4* are shown. Exons are represented by grey boxes, alternate exons are represented by blue exons, and UTRs are represented by dashed lines. Each SNP observed during the mutation screen is indicated by an arrow. Listed for each SNP is the variation type, the SNP database accession if available (in brackets), the nucleotide position (based on *ZFR* nucleotide accession NM_016107.2 and *PC4* nucleotide accession NM_006713) and the predicted amino acid change, if applicable. Red text indicates that the variant has not been reported elsewhere and was not identified in unaffected controls during the initial mutation screens. Figures are not drawn to scale.

Figure 31: Polymorphisms and variants identified in the *NPR3* and *FLJ14054* genes during mutational analysis. The genomic structure of *NPR3* and *FLJ14054* are shown. Exons are represented by grey boxes, alternate exons are represented by blue exons, promoter sequence (158) is represented by a black box and UTRs are represented by dashed lines. Each SNP observed during the mutation screen is indicated by an arrow. Listed for each SNP is the variation type, the SNP database accession if available (in brackets), the nucleotide position (based on *NPR3* nucleotide accession M59305 and *FLJ14054* nucleotide accession NM_024563) and the predicted amino acid change, if applicable. Red text indicates that the variant has not been reported elsewhere and was not identified in unaffected controls during the initial mutation screens. Figures are not drawn to scale.



* Promoter region characterized by Yanaka *et. al.* (158)

Figure 32: Polymorphisms and variants identified in the *TARS* and *ADAMTS12* genes during mutational analysis. The genomic structure of *TARS* and *ADAMTS12* are shown. Exons are represented by grey boxes, alternate exons are represented by blue exons, and UTRs are represented by dashed lines. Each SNP observed during the mutation screen is indicated by an arrow. Listed for each SNP is the variation type, the SNP database accession if available (in brackets), the nucleotide position (based on *TARS* nucleotide accession NM_152295 and *ADAMTS12* nucleotide accession NM_030955) and the predicted amino acid change, if applicable. Red text indicates that the variant has not been reported elsewhere and was not identified in unaffected controls during the initial mutation screens. Figures are not drawn to scale.

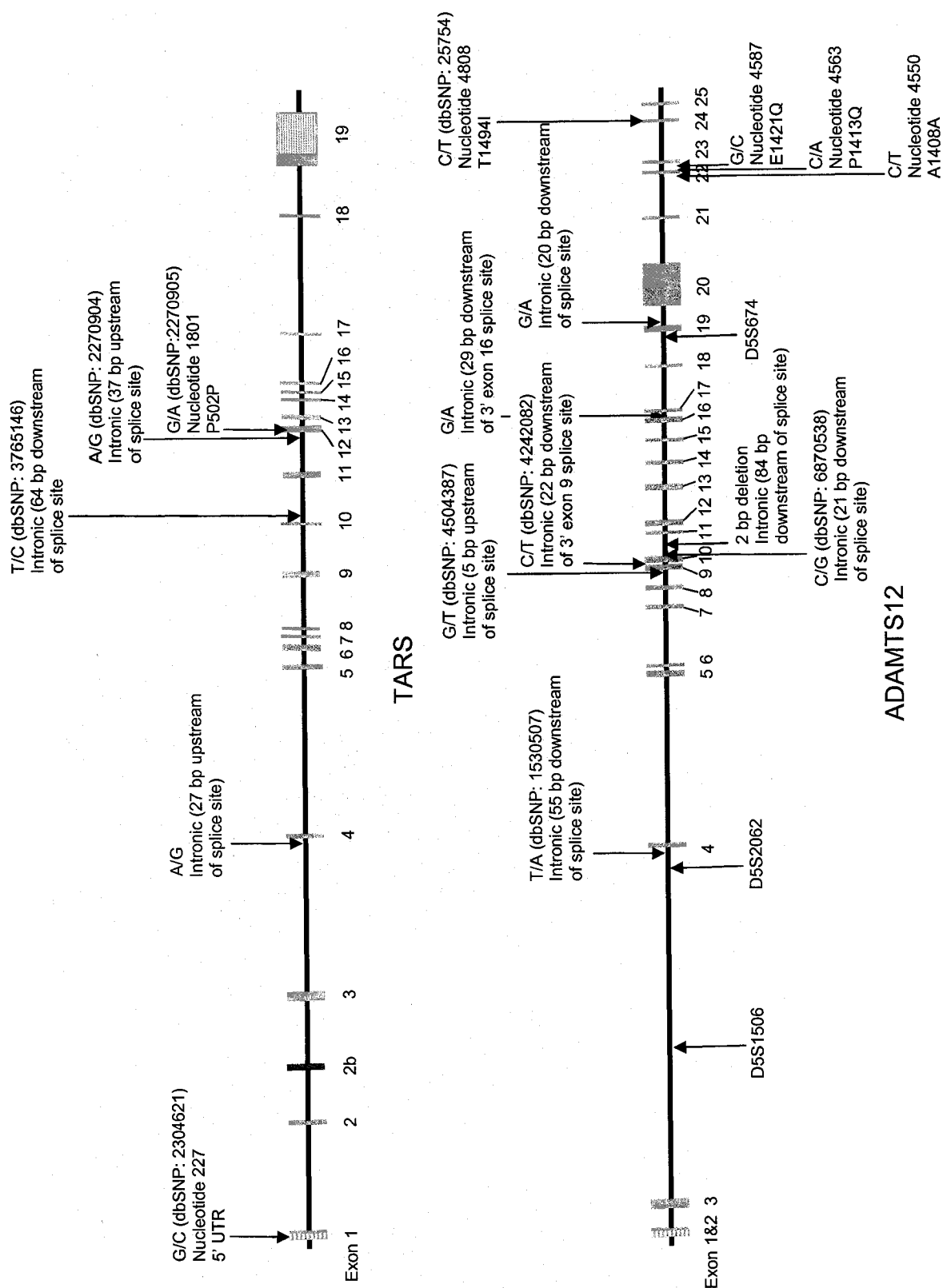


Figure 33: Polymorphisms and variants identified in the *SALPR* and *AMACR* genes during mutational analysis. The genomic structure of *SALPR* and *AMACR* are shown. Exons are represented by grey boxes, alternate exons are represented by blue exons, and UTRs are represented by dashed lines. Each SNP observed during the mutation screen is indicated by an arrow. Listed for each SNP is the variation type, the SNP database accession if available (in brackets), the nucleotide position (based on *AMACR* nucleotide accession AF047020) and the predicted amino acid change, if applicable. Red text indicates that the variant has not been reported elsewhere and was not identified in unaffected controls during the initial mutation screens. Figures are not drawn to scale.

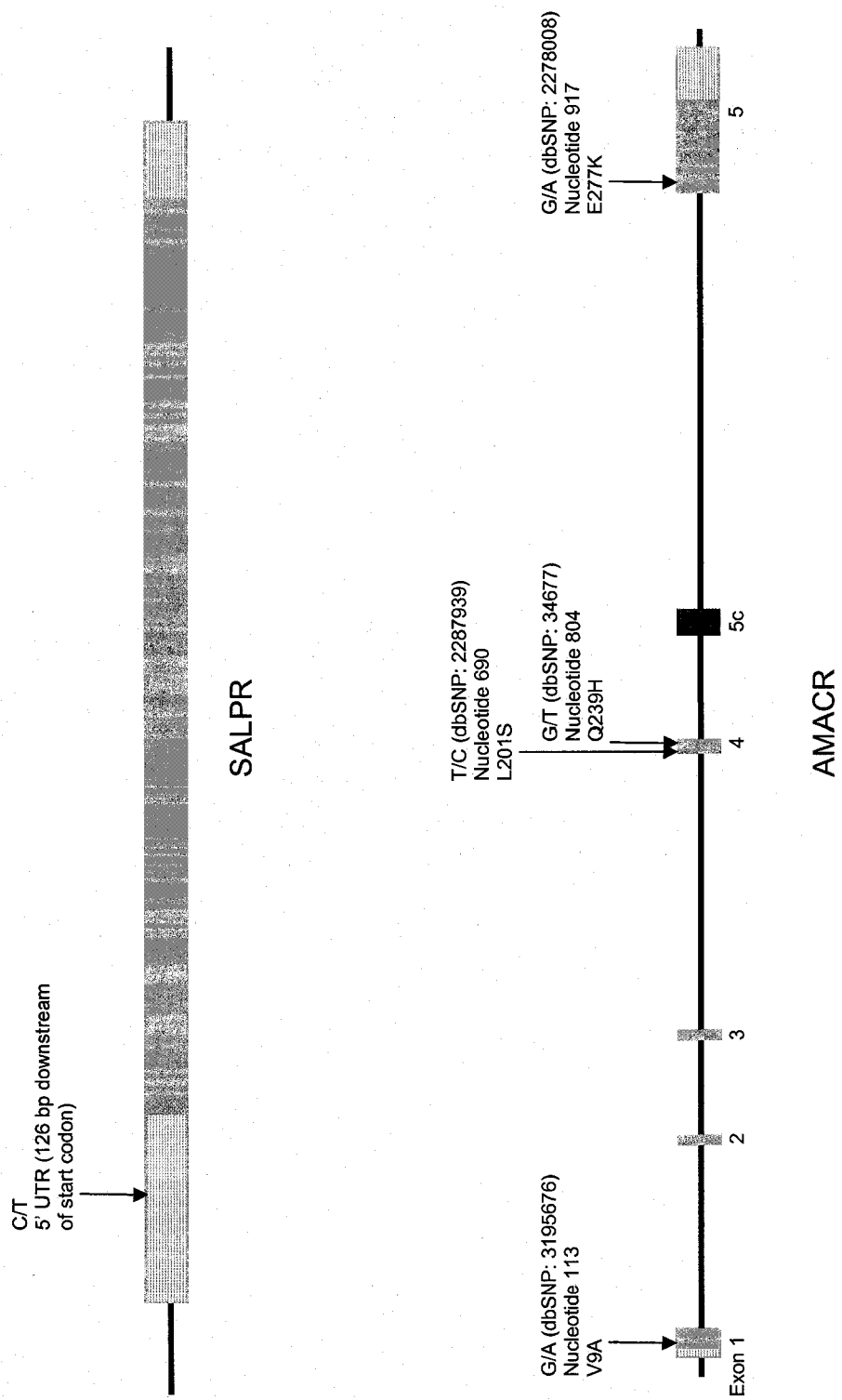


Figure 34: Polymorphisms and variants identified in the *CIQTNF3* and *RAI14* genes during mutational analysis. The genomic structure of *CIQTNF3* and the 5' end of *RAI14* are shown indicating the position of each exon and the two microsatellites, D5S426 and D5S663. Exons are represented by grey boxes, alternate exons are represented by blue exons, and UTRs are represented by dashed lines. Exon 1 from *CIQTNF3* – isoform a is denoted by “1a”, while the single exon from isoform b is denoted by “1b”. Each SNP observed during the mutation screen is indicated by an arrow. Listed for each SNP is the variation type, the SNP database accession if available (in brackets), the nucleotide position (based on *CIQTNF3* (isoform a) nucleotide accession NM_030945, *CIQTNF3* (isoform b) nucleotide accession NM_181435 and *RAI14* nucleotide accession AY317139) and the predicted amino acid change, if applicable. Red text indicates that the variant has not been reported elsewhere and was not identified in unaffected controls during the initial mutation screens. Figures are not drawn to scale.

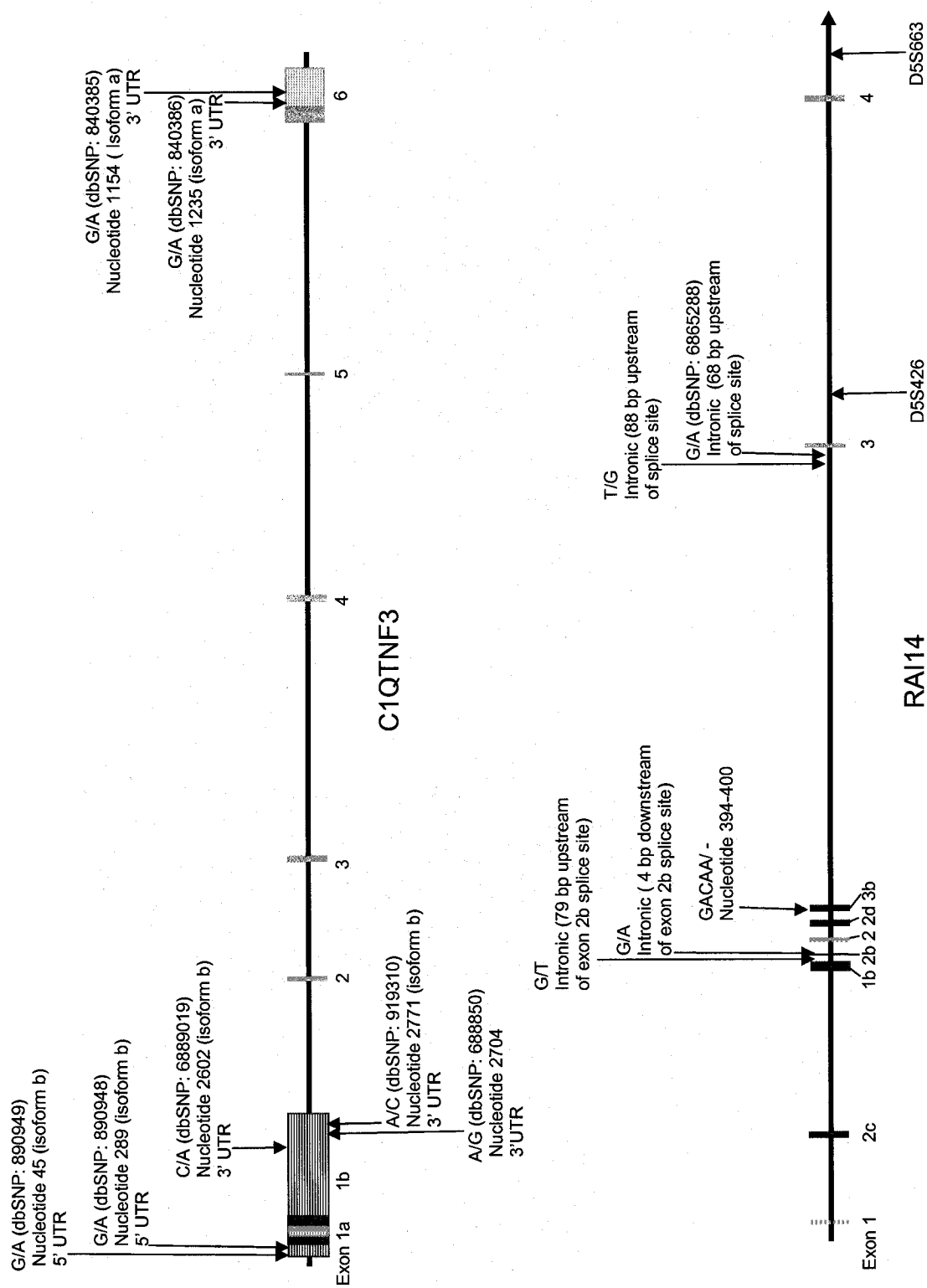


Figure 35: Polymorphisms and variants identified in the *LOC254411* predicted gene during mutational analysis. The genomic structure of *LOC254411* is shown. The predicted exons are represented by grey boxes. Each SNP observed during the mutation screen is indicated by an arrow. Listed for each SNP is the variation type, the SNP database accession if available (in brackets), the nucleotide position (based on nucleotide accession XM_172455) and the predicted amino acid change, if applicable. Red text indicates that the variant has not been reported elsewhere and was not identified in unaffected controls during the initial mutation screens. Figures are not drawn to scale.

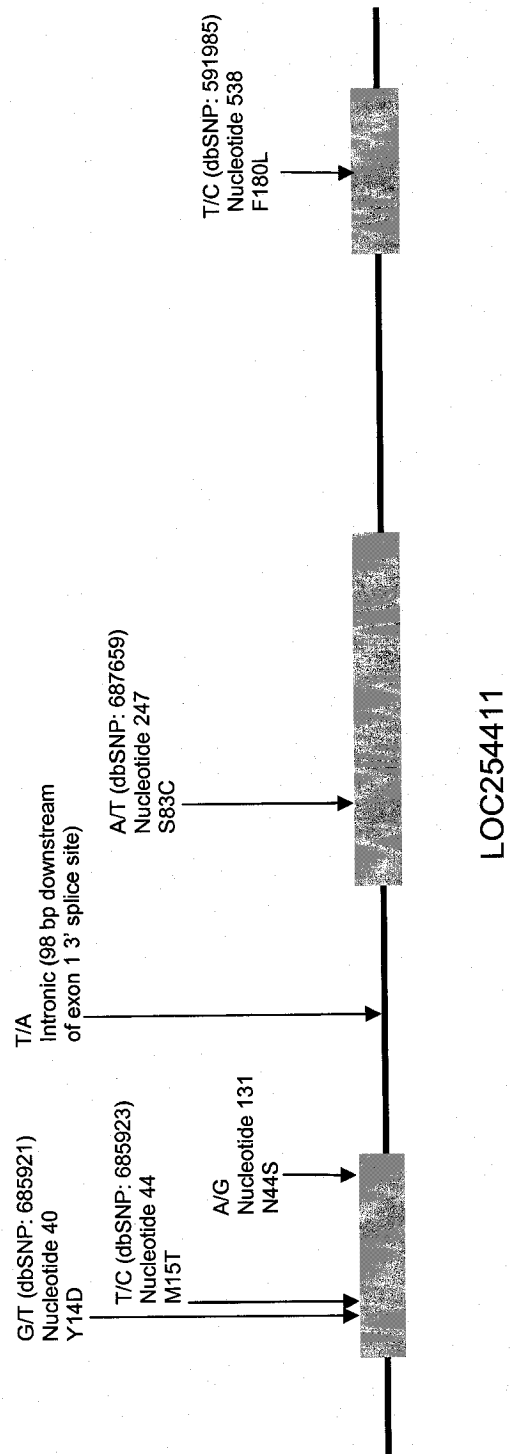
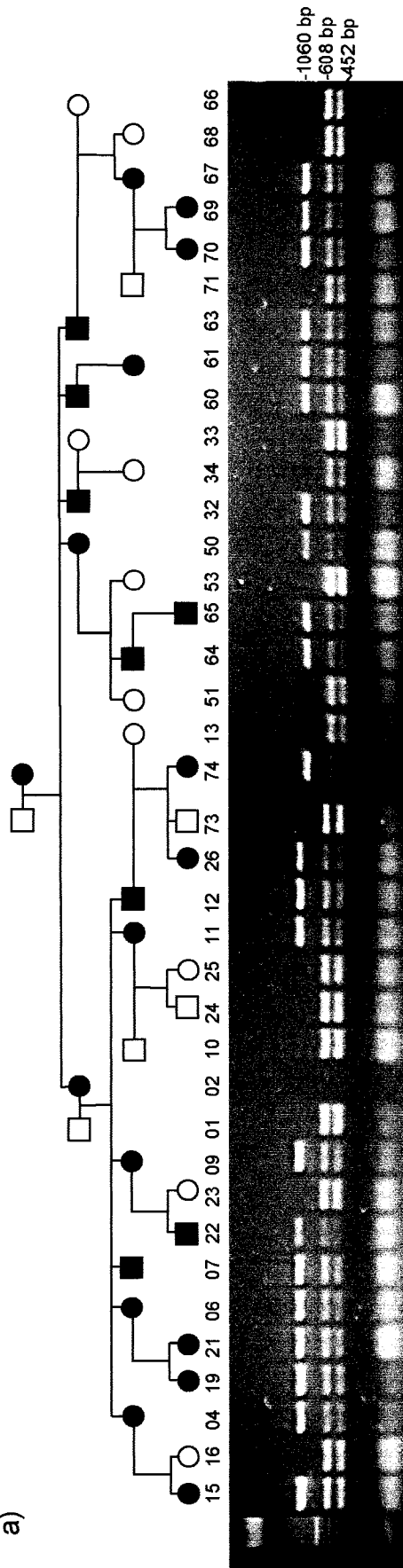
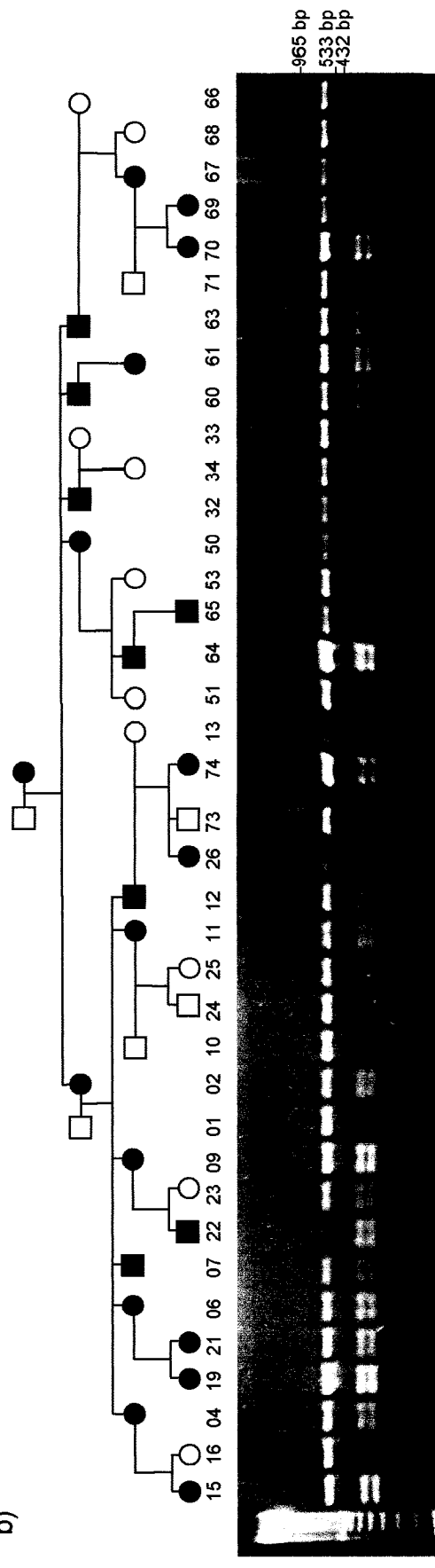


Figure 36: RFLP analysis of sequence variants in the PDZK3 coding sequence. Shown are the restriction endonuclease digests of two sequence variants, at nucleotide positions 3044 and 6531 of the PDZK3 isoform b transcript. The pedigree is drawn at the top of each agarose gel and the digest of individuals from the pedigree are shown under their symbol. a) *HinP1I* digest of PDZK3 exon 13 PCR products. *HinP1I* is specific for the “G”, but not the “A” allele, and generates two fragments 608 and 452 bp in size. The “A” allele cosegregates with BDA1 in affected individuals from this pedigree. b) *DdeI* digest of PDZK3 exon 15 PCR products (amplified with the PDZDx15d primer set from Appendix 2). *DdeI* is specific for the “A”, but not the “G” allele, and generates DNA fragments 533 and 432 bp in size. The “A” allele cosegregates with the BDA1 in affected individuals from this pedigree. The first lane on the left hand side of the gel contains a 100bp ladder.

a)



b)



In addition to the two novel polymorphisms identified in *PDZK3*, four other variants were identified in the probands from pedigrees 6, 8 or 9, that could not be classified as “naturally occurring”, according to the definition in section 5.1.2. These include one variant in the 5’UTR of *PC4*, one intronic variant upstream of exon 4 in *TARS*, and two variants in the 3’UTR of *FLJ14054* (Table 14). Frequencies of these variations in the natural population were not examined. A fifth variant was also identified in *PDZK3* exon 16 from DNA of two affected individuals in pedigree one. Although this variant is in the coding sequence, it occurs at the third position in codon 2396 (isoform b) and does not cause an amino acid substitution.

Table 14: List of novel sequence variants identified during mutation screens. The sequence variants listed were identified during the initial mutation screens of candidate genes, but are not catalogued in the SNP database. In addition, they were not observed in sequences from unaffected controls, or in the homozygous state. For each variant, the sample it was identified in is listed in the last column. The pedigree number of these samples is indicated by a “p” followed by the pedigree number. The number after the dash represents the DNA sample number of the individual examined during mutation screens.

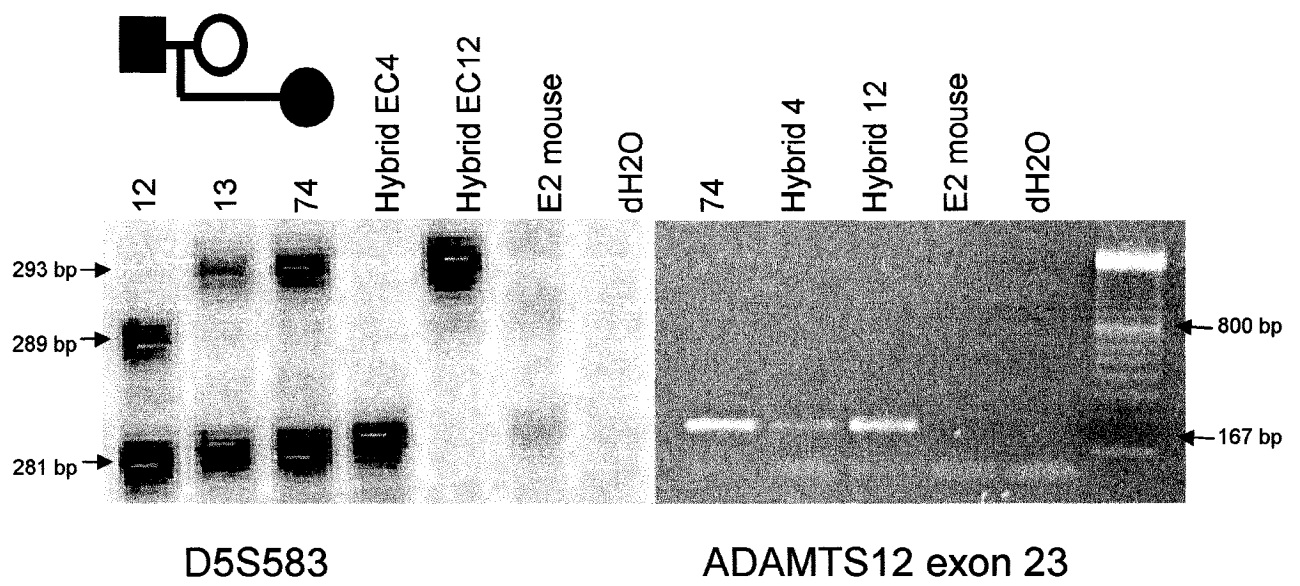
Gene	Variant	Variant Location	Sample Identified In
<i>PDZK3</i>	G→A	Nucleotide 7576	p1-19, p1-74
<i>PC4</i>	C→T	5’ UTR; Nucleotide 39	p9-01
<i>FLJ14054</i>	C→T	3’ UTR; Nucleotide 1607	p8-01
<i>FLJ14054</i>	G→C	3’ UTR; Nucleotide 1974	p6-03, p8-01
<i>TARS</i>	A→G	27 bp upstream of exon 4	p9-01

5.2.3 Haploid Analysis for Detection of Macrodeletions

Although the procedures described in the previous sections are useful for detecting point mutations or small insertions and deletions, they are incapable of detecting larger deletions in which either primer binding site is missing. To compensate for these insufficiencies, we generated monoallelic human-mouse cell lines derived from the lymphoblast tissue of an affected individual (pedigree 1, sample 74). Two haploid cell lines were examined, each carrying different copies of chromosome 5. Genotype analysis of microsatellite markers from the critical region showed that the EC4 cell line carried the paternal copy of chromosome 5, and thus also carried the mutant copy of the disease gene. The EC12 cell line carried the maternal copy of chromosome 5 (refer to Figure 37), and was used in subsequent experiments as a wild type control.

To assess if there are any macrodeletions in the candidate genes described in section 5.2.1 and 5.2.2, each exon was amplified individually from EC4 and EC12 DNA. Due to the presence of mouse DNA in these lines, PCR amplification frequently required additional optimization to allow for specific amplification from human DNA within each hybrid cell line. Despite these complications, PCR products of the correct sizes were amplified from both EC4 and EC12 samples at all of the loci examined.

Figure 37: Mutation screening using monoallelic hybrid cell lines. Genotyping of microsatellite markers was used to determine which monoallelic hybrid cell line carried the maternal copy of chromosome 5 and which one carried the paternal copy. Genotyping of the D5S583 marker is shown as an example. DNA from the two hybrid lines was amplified along with DNA from the affected individual used to generate the hybrid (74), DNA from his parents (12 and 13), E2 mouse DNA (negative control), and dH₂O (negative control). The gel confirms that hybrid EC12 carries the maternal allele, while hybrid EC4 carries the paternal allele. Also shown is an example of PCR amplification to assess whether a specific locus is present on the maternal and paternal copies of chromosome 5. Exon 23 of ADAMTS12 was amplified from DNA of both hybrids, individual 74 (positive control) but not E2 mouse DNA (negative control) nor dH₂O (negative control).



5.3 Discussion

The long term goal of this project is to identify the gene on chromosome 5p13.3-13.2 that causes BDA1. Analysis of recent physical maps indicates that there are between 15 to 32 genes within the critical region, any of which may contain the BDA1 mutation. To facilitate analysis of these genes, candidates were prioritized for analysis based on their expression and potential function. In total, 14 known genes and one predicted gene were analyzed. These included genes that are necessary for normal limb development in mice (ie. *NPR3* and *CIQTNF3*), genes with functions that may facilitate cellular differentiation (ie. *PDZK3*, *PIP3AP*, and *ADAMTS12*), and genes that have unknown functions, but are expressed in limb tissues (ie. *FLJ14054*).

Of the fifteen genes analyzed, *NPR3* and *CIQTNF3* were considered the strongest candidates. Mutations identified in the mouse homolog of *NPR3* result in a skeletal overgrowth phenotype characterized by exceptionally long bodies and delayed endochondral ossification (161). This is in contrast to the short stature and premature epiphyseal closure commonly observed in patients with BDA1. Despite these apparently contradictory phenotypes, one could argue that if mutations which blocks normal *NPR3* function can cause skeletal overgrowth, then mutations that result in *NPR3* overexpression may have the opposite effect and stunt growth. As such, the *NPR3* promoter sequence (158) was assessed for mutations in addition to the transcribed regions, since changes here could affect gene expression. *CIQTNF3* was also considered a very strong candidate since analyses of the mouse homolog, *Cors26*, have shown that it is expressed in prechondrocytic cells of developing cartilage, and can promote growth of the mesenchymal cell line, CH310T1/2, when overexpressed (164), suggesting that the *Cors26* protein may have a role in skeletal development and in the regulation of mesenchymal condensation. If *CIQTNF3* has similar

functions in humans, then mutations of the gene may cause brachydactyly by reducing the size of digital mesenchymal condensations during development.

In addition to NPR3 and C1QTNF3, five other genes from the critical region were considered good candidates based upon predicted functions related to cell signaling, gene regulation, and/or maintenance of the extracellular matrix. Mutations that directly affect either of these processes may perturb normal bone development by altering the cell's ability to detect and respond to differentiation signals. The five candidate genes identified include *PDZK3*, *PIP3AP*, *ZFR*, *PC4*, and *ADAMTS12*. The first of these, *PDZK3*, has six protein-protein interacting domains that can mediate intracellular signaling by providing a scaffold for protein complexes (170). Using a yeast two-hybrid system, Chaib *et al.* showed that the *PDZK3* gene product interacts with the presenilin1 protein, thus linking it to the Wnt/ β -catenin signaling pathway (170). *PIP3AP* has also been implicated in intracellular signaling, and acts as an adapter subunit in a protein complex with a phosphatidylinositol (3)P 3-phosphatase (181). Both the Wnt/ β -catenin and phosphoinositide-3-kinase signaling pathways have been implicated in osteoblast differentiation (185;186), while studies of Wnt have also shown that it can regulate chondrocyte differentiation (187). Although models of *PDZK3* or *PIP3AP* mutations do not exist, we speculated that they may affect bone development through alterations of their signalling pathways.

Unlike *PDZK3* or *PIP3AP*, the *ZFR* and *PC4* genes may mediate differentiation by regulating gene expression, and not intracellular signaling. *ZFR* is a zinc-finger protein that is highly conserved between species (173). It contains three C₂H₂ zinc fingers that can bind to double-stranded RNA and DNA (173). Although its function *in vivo* has not been ascertained, null mutations in mice caused a dramatic phenotype including growth impairment, dysmorphogenesis and embryonic lethality (180). *PC4* has been better

characterized than ZFR. Studies have shown that it acts as a transcription cofactor that stimulates transcription of class II genes (166). It can also act as a strong transcriptional suppressor (168;169). Based on the lethal phenotype observed with *Zfr*^{-/-} mice, and the central role *PC4* plays in expression of class II genes, one might suspect that mutations of these genes affect a number of systems in addition to the skeleton. However, depending on the nature of the mutation, it may not alter protein function in all of the tissues that it is expressed. *IHH*, for example, is involved in mammalian pancreatic (188), colonic (189) and skeletal development (56), however only skeletogenesis appears to be affected in BDA1 patients with *IHH* mutations.

The final gene from the critical region that was considered a good candidate, and given a high priority for mutation analysis, was the *ADAMTS12* gene. This gene encodes a protein that belongs to the ADAMTS family of proteins, which are characterized by disintegrin-like, metalloprotease and thrombospondin type 1 domains (190). Similar to the closely-related ADAM family of proteins, ADAMTS proteins are involved in the proteolysis of extracellular proteins, and may also mediate cell-cell and cell-extracellular interactions (190). Both functions are vital during development and allow for tissue remodeling, cell migration, and cell lineage decisions during embryogenesis. As such, several ADAM and ADAMTS proteins have been implicated in mammalian and invertebrate development (191-194). Although a specific function for *ADAMTS12* has not been identified, a role in development and even skeletogenesis, seems plausible.

The remaining eight genes assessed were given medium to low priorities for analysis due to a lack of data supporting roles in development. For three of these genes, *GOLPH3*, *FLJ14054*, and *RAI14*, candidacy was based solely on evidence that they are expressed in skeletal tissues (Unigene Cluster Hs.18271, Hs.13528, and Hs.368605, respectively), since

little is known about their function (172;183). Two other genes, *TARS* and *AMACR*, were also considered candidates primarily based on their expression in skeletal tissues (Unigene clusters Hs.84131 and Hs.49598, respectively). They were given a slightly higher priority for analysis than either *GOLPH3*, *FLJ14054*, or *RAI14*, because they have been better characterized and more information is available about their predicted functions (174;178;179;182). Consequently, if mutations were found in these genes, it would be easier to design experiments that would allow us to characterize differences between the mutant and wildtype proteins.

Three genes were analyzed as part of this study that, in retrospect, were not good candidates for the BDA1-causing gene. The first of these, *cadherin-18*, encodes an extracellular matrix protein, and thus may be important during development of various tissues. However, as the draft genome sequences were improved and brought closer to completion, the physical position of this gene was repositioned outside the BDA1 critical region. Consequently, analysis of the gene was halted and it was removed from the candidate list entirely. The other two genes, *SALPR* and *MATP*, were initially examined based on their functions. *SALPR* is a member of the large G-protein coupled receptor family of transmembrane proteins, which transduce extracellular signals to the nucleus through GTP-binding proteins (176). *MATP* (previously *AIM1*) is a transporter protein that is mutated in individuals with oculocutaneous albinism (177). We had mistaken it for a protein by the same name that is required for cytokinesis (184). Based on potential roles in intracellular signaling and cytokinesis we thought that *SALPR* and *MATP* may be important for bone development, however data suggests that expression of these genes is limited. Expression in skeletal tissue has not been reported (176) (Unigene cluster Hs.170146, and

Hs.278962, respectively). Consequently, they are not likely involved in BDA1 pathophysiology.

The final candidate gene analyzed was a predicted gene, *LOC254411*, that was identified through a BLAST search of the *IHH* exon 1 sequence and its flanking intronic sequence. A BLAST hit to *LOC254411* was significant since the predicted gene was positioned in the chromosome 5p BDA1 critical region and was identical to *IHH* at 32/35 bases that span the start codon of each gene. We hypothesized that mutations of *LOC254411* that make it more similar to *IHH*, may disrupt *IHH* expression through RNA interference.

Through mutational analysis of the transcribed regions of fifteen genes, we have been able to exclude these sequences from the portion of the critical region that may contain the BDA1-causing mutation. Absence of a mutation in these sequences suggests that the mutation may be at a different chromosomal location, it may be in a portion of the BDA1 locus that we did not analyze, or it may be undetectable using the methods described. Although the first of these explanations is plausible, it is an unlikely reason a mutation was not identified. In Chapter 3, a maximum LOD score of 7.81 ($\theta = 0.0$) was obtained at D5S477, indicating that the odds are only 1 in $10^{7.81}$ that the mutation is at a different locus. Consequently, the second or third scenario seems more probable.

Although the transcribed regions of fifteen genes were assessed, non-transcribed regulatory sequences, and intronic sequences were not. The decision to focus on the translated portions of each gene for analysis was based on previous observations that 99.2% of all known mutations causing hereditary disorders occur in these portions of genes (142). However, the relatively high frequency of disease-causing mutations that occur inside translated portions of genes may in part reflect a bias towards analysis of these regions. We,

therefore, also examined the untranslated regions (UTRs) of each gene as it required minimal extra effort and provided a more complete mutational analysis. Mutations within the UTR have been previously reported and have the potential of causing disease by altering gene or protein expression. For example, a study of four thalassaemia mutations identified in the 5'UTR of the human β -globin gene, showed that the mutations caused reduced steady state levels of the mRNA that were between 61-85% of wildtype levels (195). Mutations within regulatory and intronic sequences of genes also have the potential of causing hereditary disease by a similar mechanism to UTR mutations, or by generating alternative splice sites. In an atypical case of chronic granulomatous disease, mutations in the *CYBB* promoter were shown to cause the disease by drastically reducing gene expression resulting in low levels of the gene product and subsequent low levels of O_2^- production in neutrophils (196). By contrast, mutations in intron 2 of the *CDKN2A* gene were shown to predispose individuals to melanoma by creating a false GT donor site 105 bp 5' of exon 3, and causing aberrant splicing of the transcript (197). Unfortunately, based on the relative infancy of the human genome map, it is unclear whether we analyzed the complete UTR for each gene, although they were assessed based on information available at the time. With the exception of the *NPR3* promoter, regulatory and intronic sequences were not examined. This was largely due to the relative infrequency of mutations here and because of time and resource restraints inherent with sequence analysis of the complete regulatory and intronic regions (assuming that the regulatory regions have even been characterized). Due to a lack of skeletal samples from affected individuals we could not properly assess gene expression of candidate genes in the relevant tissues, either. Despite such concerns, these regions of the gene may need to be analyzed at some time in the future to identify the disease causing mutation.

Additional portions of the chromosome 5p BDA1 locus that were not examined, but may contain mutations, include the predicted genes. In the October 5, 2003 version of the NCBI human genomic map, there were seventeen predicted genes within the BDA1 critical region. These numbers will likely change as genes are removed from the list either through ongoing genome annotations, or by validating the existence of genes and reclassifying them as “known genes”. The value of predicted genes as candidates is difficult to assess, primarily because there is a lack of sufficient information about them. If available, evidence that may be considered includes expression profiles or homology to other species. The importance of examining predicted genes is emphasized by the fact that, because brachydactyly is a congenital trait that occurs due to anomalous bone development, expression of the disease-causing gene may be restricted to developmental tissues. As such, ESTs or mRNA sequences may be difficult to find in standard cDNA libraries, particularly if the library is made from adult tissue. Proof of the gene may be limited to *in silico*, rather than physical data, making classification as a known gene impossible. This, however, does not preclude the gene’s existence, or the chance that it contains a mutation. Although the predicted genes within the BDA1 critical region were not examined as part of this doctoral thesis, this line of investigation is being pursued by others in the laboratory.

Finally, the BDA1-causing mutation may not have been identified during this doctoral work because of limitations associated with the methodologies used. Two main techniques were used during mutational analysis, cycle sequencing of PCR products and amplification from monoallelic hybrids. Although cycle sequencing is often described as the “gold standard” for mutation analysis, it is limited in its ability to detect macrodeletions or chromosomal rearrangements. Heterozygous point mutations and small insertions/deletions are easily detected by sequence analysis and will appear as double bands of half the intensity

at the same position on the sequencing gel (whereby one band corresponds to the wild type allele and one corresponds to the variant allele). Homozygous mutations are also easy to detect if the mutant and wildtype sequences are compared. However, if the point mutation occurs at the primer annealing site, or if a macro deletion or chromosomal rearrangement has excised one or both primer annealing sites, then amplification will occur from the wild type, but not the mutant allele. Consequently, when the PCR products are sequenced they will appear normal, and the mutation will go undetected. To partially address this issue, we had monoallelic hybrids prepared from a lymphoblast cell line of an affected individual. This allowed amplification of the maternal (unaffected) and paternal (affected) alleles separately. Using this technique we were able to amplify the transcribed regions of all fifteen genes from both alleles, indicating that BDA1 was not caused by macrodeletion of these loci in the individual from whom the monoallelic lines were made. Despite this, analysis of the monoallelic hybrids does not allow detection of complex chromosomal rearrangements in which a portion of the gene is translocated to another chromosomal location. Southern blot analysis can address this latter issue, although preliminary attempts to do so were unsuccessful (data not shown).

Another methodological hurdle that we had to address in our search for the BDA1 gene was the limitations inherent in the analysis of individuals from a single family. Although one may be very successful at identifying the disease-causing gene by studying only one family, analysis of multiple families increases the possibility of detecting different mutations in a gene. While we were able to confirm linkage to the BDA1 locus on chromosome 5 in only one family, the absence of *IHH* mutations from five individuals with sporadic incidences of BDA1 suggests potential involvement of the chromosome 5 locus in these cases. Consequently, mutation analysis conducted subsequent to October 2002

included DNA from three of these individuals (probands from pedigrees 6, 8 and 9). Although seven genes, *NPR3*, *MATP*, *SALPR*, *ZFR*, *CTRP3*, *3PAP*, and *ADAMTS12*, were screened for mutations prior to this period, we do not feel that the lack of sequence data from the additional probands hindered our ability to find a mutation in these genes. Rather, we feel that the sequence data from these individuals enhanced our ability to search for a mutation in the remaining genes.

Despite our efforts to identify a mutation in fifteen genes, the alternative remains that a traditional mutation may not exist. In our analysis of *PDZK3* two alleles were identified that cosegregate with the family, and cause amino acid substitutions. Because each of these alleles occur in the natural population (at frequencies of 1.6% to 7.9%), they were classified as “naturally occurring” polymorphisms, rather than mutations, according to the definition provided in section 4.1.2. However, one can speculate that inheritance of these two polymorphisms in *cis* may alter protein function, and ultimately affect normal bone development. Polymorphisms have been previously implicated as factors in complex diseases such as obesity, hypertension (198), and sensorineural hearing loss (199), although we are not aware of reports indicating involvement in simple traits. Despite this, personnel in our laboratory have examined the affect of these amino acid changes *in silico* and have shown that both these changes have the potential of altering protein phosphorylation. The G→A transition from exon 13 (A886T, isoform b) may result in the gain of a Protein Kinase C recognition site (X-S/T-X-R/K). By contrast, the G→A transition from exon 15 (R2048Q, isoform b) may result in the loss of a Protein Kinase A recognition site (R-X_{1,2}-S/T-X) (Lemuel Racacho, unpublished data). Analysis of protein secondary structure using the GOR-IV computer program further indicated that the R2048Q amino acid substitution may affect protein structure by changing a random coil to an extended strand (Lemuel Racacho,

unpublished data). Unfortunately, due to the lack of *in vivo* or *in vitro* data, we are unable to show what effect, if any, that these two changes have on protein function. Further epidemiology studies would also be required in order to assess if the two variants occur in *cis* among the natural population.

Although a number of genes occur in the BDA1 locus on chromosome 5 that can be classified as candidates based on their function or expression in skeletal tissue, the disease-causing gene remains elusive. The transcribed regions of fifteen genes were analyzed and subsequently excluded. Further analysis of predicted genes, as well as the regulatory and intronic regions of candidates, will be required as part of future studies to identify the mutation. Studies that examine gene expression or the occurrence of complex chromosomal rearrangements may also be useful. By identifying and characterizing the mutation on chromosome 5 that causes BDA1, we hope to better understand its molecular pathology, and thus learn more about processes involved in normal digital development.

Chapter 6 – Conclusions

In summary, we have presented strong evidence for a gene that causes BDA1 on chromosome 5p13.3-p13.2. This is supported by a maximum LOD score of 7.81 ($\theta = 0.0$) at D5S477. As mutations have also been identified in the *IHH* gene on chromosome 2, characterization of a second locus on chromosome 5p indicates that the trait is genetically heterogeneous. By saturating the region with polymorphic markers and recruiting additional family members, we were able to define a 7 cM (3.0 Mb) critical region flanked by the markers D5S1986 and D5S426. Analysis of recent physical maps indicates that there are between 15-32 genes within this region. Many of these were considered good candidates based on their predicted functions and their expression in skeletal tissues. Through mutation analysis, we were able to exclude the transcribed regions of fifteen of these genes, including fourteen known genes, and one predicted gene. Exclusion of these regions suggests that the mutation may be within a predicted gene, or in regulatory/intronic sequences that were not examined. Alternatively, the trait may be caused by a chromosomal rearrangement that could not be detected using either sequence analysis or amplification from monoallelic hybrids.

In addition to characterization of the chromosome 5p locus, we examined BDA1-affected individuals from twelve additional families and showed that there was considerable inter- and intrafamilial variation. Although interfamilial variability may be partially explained by the involvement of different loci, the intrafamilial heterogeneity suggests that there is a modifier gene that can modulate the severity of this disorder. To assess whether the *IHH* or chromosome 5p loci were associated with BDA1 in each family, mutation analysis of the *IHH* coding region was performed. *IHH* mutations were identified in six of the twelve families, reaffirming that mutations of this locus are frequent, but only account for a subset of the BDA1 incidences. Five of these kindreds carried a common G→A transition

that caused an aspartate to asparagine amino acid substitution at codon 100. As this is the same codon affected in a Chinese family with BDA1 (122), we propose that it may represent a hot spot for BDA1 mutations. The mutation at codon 100 was of further interest since three of the kindreds identified with this change are descendants from the earliest reports of BDA1 inheritance (4-6). The identification of a disease-causing mutation in these kindreds is therefore of historical relevance, particularly since one of these kindreds includes descendants of the family described by Farabee in 1903 (4). One century after he described the first example of Mendelian inheritance of an autosomal dominant trait in humans, we now know the causative mutation. Haplotype analysis of individuals in the three kindreds further showed that they likely shared a common ancestor.

The remaining six families analyzed, which do not carry *IHH* mutations, are of particular interest since there is an increased likelihood that they carry a mutation on chromosome 5. Identification of such individuals is important since mutations of the same gene in different families supports a role for that gene in the etiology of BDA1. Haplotype analysis of these families may also be useful by identifying recombination events that can narrow the critical region. Unfortunately, linkage analysis of individuals from the only familial case of BDA1 among this group clearly showed that, despite lacking mutations in the *IHH* coding region, the trait was not linked to chromosome 5 either. This indicates that there may be a third BDA1 locus, and while it further supports genetic heterogeneity in this trait, it also confounds our ability to ascertain the BDA1 locus involved in each family. More studies are required to identify additional BDA1 families in which the trait is linked to chromosome 5, and to ultimately identify the disease-causing gene.

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Contribution of Collaborators

This thesis could not have been possible without valuable contributions by our many collaborators. First and foremost was a collaboration with Drs. Alisdair Hunter and Christine Armour, who provided clinical evaluations and DNA from a large Canadian kindred with BDA1 (pedigree 1). Dr. Christine Armour played a further key role in the study by recruiting additional family members, and by providing the initial evidence of a BDA1 locus on chromosome 5p. DNA samples and clinical evaluations from other pedigrees were provided by Dr. Elizabeth Sweeney, Allan E. Fryer (pedigree 2), Dr. Dian Donnai (pedigree 3), Dr. Hope Northrup (pedigrees 4 and 5), Dr. Alisdair Hunter (pedigree 6), Dr. Angus Dobbie (pedigree 7), Dr. Judith Allanson (pedigrees 8 and 9), Jamie Brown, Dr. De-Ann Pillars (pedigree 10), Dr. W.T. Keng (pedigree 11), Dr. Jacqueline Bartelt (pedigree 12), and Tim Styer and Dr. William C. Meade (pedigree 14). Technical collaborations were also established with a number of individuals from within the laboratory. Heather MacDonald provided invaluable technical assistance and was responsible for amplification and sequence analysis of the transcribed regions of *PC4*, *FLJ14054*, *GOLPH3*, *ADAMTS12* and *AMACR*, as well as a portion of the sequences from *C1QTNF3*, *RAI14*, and *PDZK3* (isoform a). Jingang Yin and Kathryn Traulsen amplified and sequenced the transcribed regions of *TARS*. Sequence analysis of the *IHH* coding region from probands in pedigrees 11 and 12 was further provided by Andrew Seto, while genotyping of individuals from pedigree 14 at loci within *IHH* was provided by Allison Grimsey. Amanda Kenney also assisted in the electrophoresis analysis of several DNA sequences. Lemuel Racacho and Akeel Baig completed genotyping of several loci from chromosomes 2 and 5 in pedigrees 2 and 3. Lemuel also provided valuable *in silico* research with respect to positions of potential Gli binding sites and the affects of various PDZK3 polymorphisms on protein structure and post-translational modification. Finally, under the supervision of Dr. Hope Northrup in Texas,

Tim Kirkpatrick sequenced the *IHH* coding regions of individuals from pedigrees 4 and 5.

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Appendix 1 – Consent Forms

Appendix 2 – Primer Sequences Used in PCR Amplification and Cycle Sequencing

Gene	Exon	Exon Length	Amplimer Length	Primer Name	Primer Sequence (5' to 3')
PDZK3 (isoform b)	2	502 bp	731 bp	PDZDx2-F	AAGGGTGTGTGGGGTTCTAA
				PDZDx2-R	TTGGCTTCTCAATGCGAGGT
				PDZDx2.1-R*	AGTCCGTCCTCCACCTTTT
				PDZDx2.2-F*	GCTCAATACCTCAGTCCATCG
				PDZDx2.2-R*	CGTTTCCAAGTTCAGGGTCA
	3	155 bp	337 bp	PDZDx3-F	GCCGTGTGTCTGTCAACCT
				PDZDx3-R	GTTTCCTCCTGTGCTCCA
	4	121 bp	325 bp	PDZDx4-F	AAAGTCTGAGTGTATTTTCTGC
				PDZDx4-R	GGCAACAGAGCAAGAACCT
	5	117 bp	326 bp	PDZDx5-F	TAGGGAGGGCACTTCAGTTA
				PDZDx5-R	TGGCATTCACTCACATCTTG
	6	72 bp	627 bp	PDZDx67-F	GGTATCCCAAAATAAGTCTGAC
				PDZDx67-R	ATTCATAGAACAACAAGGAAGAA
				PDZDx6-R	AGAAGGAAATAAAGAAAGGAAAA
	7	72 bp	627 bp	PDZDx67-F	GGTATCCCAAAATAAGTCTGAC
				PDZDx67-R	ATTCATAGAACAACAAGGAAGAA
				PDZDx7-F	TGTTTTTTTTTTTAAACCCTTTG
	8	118 bp	244 bp	PDZDx8.1-F	TCTGTTTCAAATCAGTTACACATT
				PDZDx8.1-R	TTTATGAACACTCCAGAACCTT
	9	88 bp	360 bp	PDZDx9-F	TGTATGCCAACGGGAAAAGA
				PDZDx9-R	GGAAAACCATCTCACCAAAG
	10	87 bp	288 bp	PDZDx10-F	GGGAGAAATAAATAAATAAAACC
				PDZDx10-R	CAGCCAAGAGAAAGCATAGA
	11	30 bp	313 bp	PDZDx11.2-F	TGAGCCTATTGTGACTTGAG
				PDZDx11.2-R	TGTTGCTCTGTTCTTTTCCTG
	12	159 bp	301 bp	PDZDx12-F	TAACCCTGAAAGTCTGCTTC
				PDZDx12-R	GTAGCCGCCAGCACAGAG
	13	804 bp	1060 bp	PDZDx13-F	ATGTGTAATGATGGGGTCAGA
				PDZDx13-R	TCCAAGCAGTCCAGGTATTTA
				PDZDx13.1-F*	CAGGTGACAGTTGCCAGAC
				PDZDx13.2-F*	GGATGACCCGAGGCGTGT
				PDZDx13.3-F*	GCAGCCAACCTCACGGAC
				PDZDx13.4-F*	GCTCCAGGCACAGACCAG
	14	152 bp	312 bp	PDZDx14-F	CCTTTGCCTTTTCCTGAAC
				PDZDx14-R	TGCTTTCATTAGGGTATCTCC
	15	4035 bp	a - 993 bp	PDZDx15a-F	ATTGAGGGGCTGCTTTCTTAT
				PDZDx15a-R	ATAGGGAGGTGCTGTCTTCAT
				PDZDx15a-1F*	TGACCCCAGCAAGACCTC
				PDZDx15a-2F*	TCCCCCTGACTACAGCAA
				PDZDx15a-3F*	CATCCAGGAGCAGAGACA
				PDZDx15a-4F*	GCCATCCACTGACAACACC
				PDZDx15a-5F*	GGGACAGTGGCTCTCAG
				PDZDx15a-5R*	CTGAGAGCCACTGTCCCC
			b - 940 bp	PDZDx15b-F	CAGCCTTCGGTTTTAGATTCAA
				PDZDx15b-R	GTGTGCCTTGTTATGTTTATT
				PDZDx15b-1F*	GGCGATGCTGAGGATTCTTC
				PDZDx15b-2F*	CGAGTCCTGCTCATCTTCC
				PDZDx15b-3F*	ACTCTTCCCAGCCATCATC
				PDZDx15b-4F*	TCACAGTCCGCCCATCATT

* - Primer used for cycle sequencing only, and not for PCR

Gene	Exon	Exon Length	Amplimer Length	Primer Name	Primer Sequence (5' to 3')
PDZK3 (isoform b)	15	4035 bp	c - 1009 bp	PDZDx15c-F	AAAACCTCCACATCTCTGAAAG
				PDZDx15c-R	GGCTTGTACCACATACAGTCT
				PDZDx15c-1F*	TCTAATGCCGTGCCAGAAGTC
				PDZDx15c-2F*	AATGGTCAGAAGGCAAAGTG
				PDZDx15c-3F*	TCCCACAAAACACTTTCTAAGG
				PDZDx15c-4F*	GACACGAGCATCAGGACATT
				PDZDx15c-5F*	ACCACCCCTAAATCTCCTAA
			d - 965 bp	PDZDx15d-F	CCGACTGGCGAAAAAGGAG
				PDZDx15d-R	CTTGCTGCTGCGTCACCT
				PDZDx15d-1F*	GGCAACTGTCAGGAGAAGA
				PDZDx15d-2F*	CTCCCTTCAAACAGCCATT
				PDZDx15d-3F*	CCAGCCCAGTTCTCAAGC
				PDZDx15d-4F*	GCCGCTGCTGGACACAT
			e - 892 bp	PDZDx15e-F	CCAGACGACACTACTGCTATG
				PDZDx15e-R	CTAAAACTATTCCAAAATGAAACA
				PDZDx15e-1F*	CATTGGGAGGCGGTCTT
				PDZDx15e-2F*	CTGACCATCTCTCGGCAGA
				PDZDx15e-3F*	CCGTGTCCCGCTCCAAG
				PDZDx16-F	CAAAAGAGAGCAGAAAATACCT
	16	117 bp	307 bp	PDZDx16.1-R	GCTGTGAAACTCGCAACC
	17	102 bp	322 bp	PDZDx17-F	GTGAAAAGCCCCTCCAAGA
				PDZDx17-R	TCTCTGAACCCGCTGATTG
	18	271 bp	469 bp	PDZDx18-F	CCCTCTCAAAAAATAAAAAATAAA
				PDZDx18-R	ATGTTCAACCAGAATCTCCTC
			443 bp	PDZDx18.2-F	TCCCTTTTCCTTTCCTCATC
				PDZDx18.2-R	TAAGCAGCATCCAAACACAC
	19	135 bp	325 bp	PDZDx19-F	CAACCCTGTCATTTTCATCTT
				PDZDx19-R	ATGTCCATACAGAATAAACCTTG
	20	2962 bp	a - 966 bp	PDZDx20a-F	TTTTAGTTTACTTAGGATTTTGTAA
				PDZDx20a-R	ATGGCTTACACTTTTGAGATAGA
				PDZDx20a-1F*	ACCTCTGGTTGGGCTCAT
				PDZDx20a-2F*	CAGTTCTCTTCTTTCTTTAGCAA
				PDZDx20a-3F*	GGGTCCCTTCGTTCCAGT
				PDZDx20a-4F*	CAGGGAATAAAAGTCTGAGG
			b - 997 bp	PDZDx20b-F	CCAAAAAGGAAAAAGCAAAATAAT
				PDZDx20b-R	CTCCAACATAAAATCTTAGTGTCAT
				PDZDx20b-1F*	GCTTCACTGCTTAGAGGGAG
				PDZDx20b-2F*	AAGTATCTTTCTAAGTGTCCTTG
				PDZDx20b-3F*	AAGTTTTTGCTGTTTTCGTTT
				PDZDx20b-4F*	ATTCCGTTTCGTCGTCACA
			c - 892 bp	PDZDx20c-F	AAGGTAAAGGAGCAGAAATGTAG
				PDZDx20c-R	CTTGAACACTGAAGACAAATAAAT
				PDZDx20c-1F*	CTTGGGTCTATGGCTGTGA
				PDZDx20c-2F*	ATTTCTCATCAACTGGCAAT
			d - 660 bp	PDZDx20c-3F*	TGATTGTTTTTCAGTCTCTGG
				PDZDx20c-4F*	ACAGCCCTATGTGAACAGACA
				PDZDx20d-F	CTACATCTTGCCAGAAGGTTTCC
				PDZDx20d-R	TAGGTGACAATAAACATTTCTCATC
				PDZDx20d-1F*	GTGAACACTAATGGTATTGTCCT

* - Primer used for cycle sequencing only, and not for PCR

Gene	Exon	Exon Length	Amplimer Length	Primer Name	Primer Sequence (5' to 3')
PDZK3 (isoform b)	20	2962 bp	d - 660 bp	PDZDx20d-2F*	AGTAGGCTGGGTTTCATTTC
PDZK3 (isoform a)	a4	133 bp	311 bp	PDZDxa4-F	GTATCCTCTTTAGCCCAATCTT
				PDZDxa4-R	CAACCCCAACTCACCCTG
	a5	153 bp	354 bp	PDZDxa5-F	GCTTGCTTCTGGGGAGTAAT
				PDZDxa5-R	TCCGAGAAAAACATAGAAAGAA
	a6	112 bp	311 bp	PDZDxa6-F	GGAGGGGGACAGCATAAGT
				PDZDxa6-R	CCATCCAAGGTAAAAATGATT
	a7	156 bp	273 bp	PDZDxa7-F	TGCTTACGATTCTTTTGTCC
				PDZDxa7-R	ATGGATGGGCAGTTCTTCTTA
	a14	45 bp	260 bp	PDZDxa14-F	GCTGGCTGCTAACACAGAG
				PDZDxa14-R	GGAAAACCATCTCACCAAAG
GOLPH3	2	132 bp	334 bp	GOLPH3x2-F	AGGAAAGGTAAAAGGCACCA
				GOLPH3x2-R	CCACTTTAATTTTGAAGGCTA
	3	115 bp	327 bp	GOLPH3x3-F	ATCAACTCAGAAAAGGCATTC
				GOLPH3x3-R	AAACCATTACTTCCCCACTCT
	4	1921 bp	a - 1251 bp	GOLPH3x4a-F	CTGTTTTCTTGGGGCAAA
				GOLPH3x4a-R	CAAAATACTAAGATGGGAGCAG
				GOLPH3x4a-1F*	GCTAAAAACCTGGTGGAAAA
				GOLPH3x4a-2F*	TCTTGACAAATGGGTGAATG
				GOLPH3x4a-3F*	GCCAACACCAATGAGGTT
				GOLPH3x4a-4F*	GGTGGCTTTTCTTTTTTGT
				GOLPH3x4a-5F*	TCAGACAGAAGTCACAAAAAGG
				GOLPH3x4a-6F*	GCAGATAATACTCATTTTCTAAC
				GOLPHx4a-1R*	TGTTATTGGTGAGGGGATGT
			b-934 bp	GOLPH4Bb-F	TGTCCCTTGATTTTGCTTC
				GOLPH4Bb-R	AACTAAATGAGAATACAAAAACACA
				GOLPH3x4b1-F*	CTGCTCCCATCTTAGTATTTTG
				GOLPH3x4b2-F*	CATCCCCCTTKTTAGATTTTTTTTA
				GOLPH3x4b3-F*	GGGGAAAAAATCAAAATGTCAG
				GOLPH3x4b4-F*	TCACTTTATTGTATCATCACCATT
PIP3AP	2	60 bp	277 bp	3PAPx2-F	GCTCCTGAGGGTATGTGATAT
				3PAPx2-R	TGTGAGAAGATGAAAACAAAGG
	3	139 bp	327 bp	3PAPx3-F	AAAGTTCTAAAGCCCTGTGTTG
				3PAPx3-R	ACGGAACCACAACCACACT
	4	73 bp	358 bp	3PAPx4-F	GAATGGTGATTTACTCTGCCTA
				3PAPx4-R	GTTCCAAGTCAGGTCCAAA
	5	131 bp	298 bp	3PAPx5-F	CTTCATTTCTTTAGCATTCACTT
				3PAPx5-R	CCTCCCTCAACCTTCAT
	6	94 bp	368 bp	3PAPx6-F	TGCCATTATTCCAAAAAGC
				3PAPx6-R	GAAAAAACAACCAACCCATAG
	7	130 bp	337 bp	3PAPx7-F	TGGGCTTAGTGGAGGAAAC
				3PAPx7-R	TTGACTGTGGTGATGACTGC
	8	76 bp	233 bp	3PAPx8-F	CACTCTGCTTGTATTCATCGTA
				3PAPx8-R	CATTTTGCCAGGATAGACC
	9	107 bp	233 bp	3PAPx9-F	GCTTATGTGTCCCCTGTTG
				3PAPx9-R	GCTGTTCCCTGCTTCTTG

* - Primer used for cycle sequencing only, and not for PCR

Gene	Exon	Exon Length	Amplimer Length	Primer Name	Primer Sequence (5' to 3')	
PIP3AP	10	124 bp	303 bp	3PAPx10-F	GAATGCCTACTGAGCCCTTA	
				3PAPx10-R	GCACCAACTTTTCACTGAT	
	11	79 bp	241 bp	3PAPx11-F	CACAGTTGAGGAGCACAGGA	
				3PAPx11-R	TAAATTATTTGTCTGGTTTGATCT	
			396 bp	3PAPx11.1-F	GAAATGACCCTTACTCAAAATAGA	
				3PAPx11.1-R	GCACAGAAATAAGTAGCAAATA	
	12	71 bp	324 bp	3PAPx12-F	GGAAAAAAAAAAAAAAAAATAAGAGAGAA G	
				3PAPx12-R	GGAGAAAGGAATAGGAAGGAT	
	13	173 bp	347 bp	3PAPx13-F	GCAAGTGAGAGCATTCTGAAA	
				3PAPx13-R	GCAAGCAGCAATCCTACTACA	
	14	168 bp	377 bp	3PAPx14.1-F	AGAGAATGAAGTCCTCAGAAGTG	
				3PAPx14.1-R	GAGCACCATTTTATTGTAGCA	
	15	162 bp	308 bp	3PAPx15-F	TATGTGTTTGCCTTCCTCAG	
				3PAPx15-R	GCAGACAGAATCCGTAAGTA	
	16	708 bp	861 bp	3PAPx16-F	CAGGGTTATGCTCTTTTTGTTAT	
				3PAPx16-R	TACTACAGATGCGGTTTAAATTG	
				3PAPx16.1-F*	AGAGGATTTTTCAGGGAAGA	
				3PAPx16.2-F*	GCCCGAAATCAAAGTCTG	
				3PAPx16.3-F*	AGACACCACAGCCAGCAG	
3PAPx16.4-F*				TTGGGAGATGAAGACGATTT		
ZFR	1	100 bp	273 bp	ZFRx1&2-F	TCGGAGGCGGCGGTCTGT	
				ZFRx1-R	TCGGAGGCGGCGGTCTGT	
	2	70 bp	206 bp	ZFRx2-F	GCGTCTCGGCTCCTCTGTG	
				ZFRx1&2-R	GGGAGAGCAAGGGGCGAAC	
	3	283 bp	a - 291 bp	ZFRx3-F	ATGTGCTTTTTATTTCAGTTG	
				ZFRx3.1-R	GGGGTAGCCTCCATAAG	
				b - 355 bp	ZFRx3.2-F	AGTTACTGCTGCCTATGC
	ZFRx3-R	TGTCCCAAACCTACCAAGG				
	4	145 bp	321 bp	ZFRx4-F	TTTCCTTCCACTTGTCTCATAGC	
				ZFRx4-R	AGAACCTGCCTCCTAAGATGATG	
	5	209 bp	313 bp	ZFRx5-F	TGGCTTACTCTTCTAATTTTCTG	
				ZFRx5-R	AAGTCTCTTCTAATTTGTTTACTC	
	6	244 bp	435 bp	ZFRx6-F	AAATCTATCTTTATTGGCAGGA	
				ZFRx6-R	TCTTTTAGTAGGCTCAGGAGTTAG	
	7	192 bp	284 bp	ZFRx7-F	AAAGAAAATTAGTGAAGCAAAATGT	
				ZFRx7-R	AGATAACAGAATCAAGGCATAAGG	
	8	292 bp	a - 282 bp	ZFRx8.1-F	TTCAAAGCCGACTGCCTC	
				ZFRx8-R	ACAGGGAGAAGGGTTAGTGCT	
				b - 262 bp	ZFRx8-F	ATGTTCCAGGCAGACTTTTCA
					ZFRx8.2-R	CCTGTAGTCGTAAGACCCTTCA
	9	196 bp	364 bp	ZFRx9-F	CAGTTAGGAGTTTGACTATTAGCAG	
				ZFRx9-R	GCACTTCTTCCCTTTATCCAAT	
	10	120 bp	285 bp	ZFRx10-F	TATGTGGGGGTTTATGAATGAT	
				ZFRx10-R	GCTGTGTTTAGATGCTTTCTTTTAC	
	11	140 bp	265 bp	ZFRx11-F	GATTGTTTTGGGGCTGCT	
				ZFRx11-R	ATGCTCAGAATGGCTAAGTT	
	12	162 bp	313 bp	MPHx3-F	TTATTGCTTTTTATGTCATTG	
				MPHx3-R	TTGTTTTCTTCTAATGCTGA	

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Gene	Exon	Exon Length	Amplimer Length	Primer Name	Primer Sequence (5' to 3')
ZFR	13	205 bp	359 bp	MPHx4-F	ATTGCTTTTGCTCAGGAA
				MPHx4-R	CACACCAGAGTTACCAGTCAT
	14	150 bp	325 bp	MPHx5-F	AATGGCACAAAAGTTATTTAC
				MPHx5-R	CCGAAGCACAGTCAGTCC
	15	141 bp	361 bp	MPHx6-F	TAAGAGAGTGAAAGAGCCATAAT
				MPHx6-R	GCTAAATAAGTCAAATCTATCAAAT
	16	97 bp	360 bp	MPHx7-F	AAGTGTCTTGCTCACCTGGT
				MPHx7-R	TGGGCAGTCTTTTGAAATGT
	17	95 bp	350 bp	MPHx8-F	TACTAACCCTGGCATAAAATA
				MPHx8-R	ATGTAAACTGAGAGGATAAAAGC
	18	111 bp	722 bp	MPHx910-F	GTTTGTCCATCTTGCCC
				MPHx910-R	ACTACAGAAGCAGAAGAACGAATA
	19	97 bp	722 bp	MPHx910-F	GTTTGTCCATCTTGCCC
				MPHx910-R	ACTACAGAAGCAGAAGAACGAATA
	20	1590 bp	a - 798 bp	MPHx11.1-F	TTGGAGGGAGGTAAATGTAGTA
				MPHx11.1-R	CTTTTGTGTTGTAATCCTTCA
				MPHx11.1b-F*	CCAAAAAAAAAAAAATGTGCTC
				MPHx11.2-F	GAGGTTTTGTTTGCTTTTGAC
		b - 355 bp		MPHx11.2b-R	TTATCAATTATGCAGAGGCTTT
				MPHx11.2c-F	CAAGTTTGCTGTTAGTTGTGC
		c - 414 bp		MPHx11.2-R	TGATGGGGTGTGTGGAGA
				MPHx11.3-F	CAGTTCCTTATGGTGTGTGTG
		d - 483 bp		MPHx11.3-R	AATTATGTGCTTAGACAACAAACA
PC4	1	104 bp	359 bp	PC4x1-F	AGAGATGGTGGAGGGGAAT
				PC4x1-R	TCAGCCAGAAAGCAAATCAT
	2	87 bp	361 bp	PC4x2-F	ACTGGTTTTTGTGTCTCTGTT
				PC4x2-R	CCAGATTAAGCAGCAAAGAAT
	3	60 bp	300 bp	PC4x3-F	GGTCTAAGTGTGGTTTCTGTG
				PC4x3-R	AGGGGAAGGGGGAGGTAA
	4	133 bp	303 bp	PC4x4-F	CGTGTGCCCTTCAGTCCT
				PC4x4-R	AGAATTTTTCAAAGAACCACCT
	5	14 bp	483 bp	PC4x5/6-F	CTGGAGAAAGGTTAGAGGTGT
				PC4x5/6-R	CCCAAAGAAGTAGGATAACAGG
	6	72 bp	483 bp	PC4x5/6-F	CTGGAGAAAGGTTAGAGGTGT
				PC4x5/6-R	CCCAAAGAAGTAGGATAACAGG
				PC4x6-F*	AGAATGAACCGTGGAACCTG
	7	123 bp	303 bp	PC4x7-F	CACTGGGGTATGTTTTATTTTA
				PC4x7-R	GAGAACTGCTTGAACCTAGGA
	8	252 bp	313 bp	PC4x8-F	TAGGCACAAAGGGTCAGTAA
				PC4x8.1-R	CGCAGTTCACAATAGGGTTC
			362 bp	PC4x8.2-F	GAGCGGTGTGTGAGTGAAC
				PC4x8-R	GGAGATTAGCAGCAAAAAATAG
	9	109 bp	324 bp	PC4x9-F	TTCAAGGGTCAGCAGTAGTCA
				PC4x9-R	TGCCAACATTTCAAGTAAGCT
	10	953 bp	1074 bp	PC4x10-F	TATGAAAATCCTCAACGCTTA
				PC4x10-R	AATGACATCTCTGGCTACATATAA
				PC4x10.1-F*	CGAGCCATATAAATAAACCTG

* - Primer used for cycle sequencing only, and not for PCR

Gene	Exon	Exon Length	Amplimer Length	Primer Name	Primer Sequence (5' to 3')
PC4	10	953 bp	347 bp	PC4x10.2-F	TTGTCTGCCCCACCACCTA
				PC4x10.2-R	CAAAGAAAAGAATAAAGAGTGAAGT
			330 bp	PC4x10.3-F	CAATGGTTTTTCACTCTTTTAT
				PC4x10.3-R	AAGCAAGTCAGACATCTCATCT
NPR3	pro-moter	N/A	a - 280 bp	NPR3PR.1-F	ACCGCTGCTGTGGCTATG
				NPR3PR.1-R	TTCAACTTCTGGAGGTGTCA
			b - 309 bp	NPR3PR.2-F	AACAAAGCCCCGAGGACG
				NPR3PR.2-R	TCAGGGAACAAGGACCAAGG
			c - 243 bp	NPR3PR.3-F	GGCGGGGTGTAGGTGACG
				NPR3PR.3-R	AGGACTGGGGGAAAGGGA
			d - 303 bp	NPR3PR.4-F	GAGCAAGCGGCACCTAAG
				NPR3PR.4-R	GCCCTCTGCCTTTTCCTTA
			e - 298 bp	NPR3pr5.1-F	AGAGCGTGGAAAGGGAGAGG
				NPR3pr5.1-R	AGCGGCTGAGCGTGTTAC
			f - 344 bp	NPR3pr6.1-R	GAGCCCTGATGTAAGAATGTT
				NPR3pr6.1-R	ATTGCCCCCTCCGTTTATACA
			g - 291 bp	NPR3PR7-F	TCAAATGCGAGGGGAGATG
				NPR3PR7-R	GCCAAAAGATAGAGAGGAAGC
	1b	117 bp	267 bp	NPR3x1b-F	TGTCCCTTCCCTATTATCTCCT
				NPR3x1b-R	GCTGGCAATGGCTGAACA
	1c	60 bp	311 bp	NPR3x1c-F	ACTGAGGCTTGTCTTCTTGA
				NPR3x1c-R	ACAGCAACCATTTATTTACTTAGA
	1	1152 bp	a - 262 bp	NPR3x1-F	AAGAAGCCACCTCTAAGCAAAA
				NPR3x1.1-R	CCTCCCCACTCTTCTCTCCA
			b - 327 bp	NPR3x1.2-F	GCATTAGTGAAGGGGTATT
				NPR3x1.2-R	GGCAGTAACACCAGCACCTC
			c - 295 bp	NPR3x1.3-F	GACGCCAGGAGAGAGAGG
				NPR3x1.3-R	GCATACTCGCACACTGG
			d - 248 bp	NPR3x1.4-F	CGCCAAGCCAGACCTTATCC
				NPR3x1.4-R	TGTCGTCGCTGTAGACCAG
			e - 264 bp	NPR3x1.5-F	AGATGATGCTCGCCCTGTTCC
				NPR3x1.5-R	GCGGAGAGCGGTTGGGTTAG
			f - 225 bp	NPR3x1.7-F	CGCTTCTCTCTATCTTTTGG
				NPR3x1.7-R	ACGCCAGCCGAGTAGTA
	2c	225 bp	376 bp	NPR3x2c-F	CAGTGGGGAGTGGGGTCT
				NPR3x2c.1-R	CCAGCACAAGGACTCTGAG
	2	113 bp	303 bp	NPR3x2-F	GGAGATACTTCAGGACCATA
				NPR3x2-R	GGCATCTGACAACCTCTCTT
	intron 2	Gli binding?	288 bp	NPR3i2-F	AAAATAACAGAAAAACCAGGGA
				NPR3i2-R	CAAGCCAGACTCCAGATACCA
	3	167 bp	394 bp	NPR3x3-F	TATGGTGGCTGGCTTGGAAT
				NPR3x3-R	TGGCTCAGAATGGGACACTC
	4	156 bp	248 bp	NPR3x4-F	TGAGTCACTTGGTGTGTTGG
				NPR3x4-R	TACAGAACCAGAAAAGCAGCAG
	5	95 bp	213 bp	NPR3x5-F	TTTTTGTGGCAGACAGAGG
				NPR3x5-R	CAGAGTCCCCACGAGCAGAA
	6	136 bp	243 bp	NPR3x6-F	GCTTTGTGCCTCCTTTGACT
				NPR3x6-R	ACTGAGGTTAGAGGTGATGACA

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Gene	Exon	Exon Length	Amplimer Length	Primer Name	Primer Sequence (5' to 3')
NPR3	7	88 bp	227 bp	NPR3x7-F	TCCAAATGAACCCTGATTATCC
				NPR3x7-R	GGTGAAAGAGGGGATGTGGAC
	8	1151 bp	a - 386 bp	NPR3x8-F	AAACCCAGATGTCCCTTGAG
				NPR3x8-R	TGGAGGCAGATGGTGATTA
			b - 361 bp	NPR3x8.2-F	GGAGATAGACGGGTTGAAAG
				NPR3x8.2-R	AGAATGCTGTACTTGTAATAATGA
			c - 300 bp	NPR3x8.3-F	CTTTGGGAGCATTTCACA
				NPR3x8.3-R	CCAGAGTAGCGTTTATGAGACAC
			d - 551 bp	NPR3x8.5-F	ACAGGTTTGTGGTTGAGGACT
				NPR3x8.5-R	AGAACTGGAAGGAGTGTACC
			1192 bp	NPR3x8.2-F	GGAGATAGACGGGTTGAAAG
				NPR3x8.5-R	AGAACTGGAAGGAGTGTACC
FLJ14054	1	2643 bp	a - 1117 bp	FLJ140-1F	GCTAAGCAACATCTTTGACAA
				FLJ140-1R	GTCCCTCTGAACACCATCA
				FLJ140-1.1F*	GGTCAAAATGCGTAGATGC
				FLJ140-1.2F*	CCATCTCTCCCTCAAGACTG
				FLJ140-1.3F*	ATGGAGGGAAGAGAGAAATG
				FLJ140-1.4F*	TTATGGCTGATTTTGGGTGT
				FLJ140-1.5F*	TGGGACACAAAGCACTCAG
			b - 1021 bp	FLJ140-2F	ATGAGGAAGTTGGTAATGAGC
				FLJ140-2R	TTTTTAGCCATCTCTGTCCA
				FLJ140-2.1F*	ATTTAGTGCCCCCTCAACAG
				FLJ140-2.2F*	ACTCCAAGGATGTGTCAGCA
				FLJ140-2.3F*	GCTCTCGGGGAAGTTCAAG
				FLJ140-2.4F*	TGCTGTCAGTGTTCAGTTTT
			c - 909 bp	FLJ140-3F	CCAAGTGTAATCACAACATAGG
				FLJ140-3R	AACAAAAGCCCTCAAAGAATA
				FLJ140-3.1F*	AACAATGAAGTAGCCCCTGAA
				FLJ140-3.2F*	CTTCTGGGTCACATTATTCATT
				FLJ140-3.3F*	ATTTATTGCTGTTTTGTGTAGAA
TARS	1	159 bp	311 bp	TARSx1-F	CCTTCCCCCGTTATCCATTG
				TARSx1-R	GCGAGCGTAGCGTCTGC
	2	81 bp	293 bp	TARSx2-F	CACTTCCTGGGGCATCACA
				TARSx2-R	TTCTTCCTGATTTTTCCAACCA
	2b	158 bp	319 bp	TARSx2b-F	TAACGATTGGTCTGAAAAAAG
				TARSx2b-R	AGAGCCTGTCATTCTACAACC
	3	191 bp	441 bp	TARSx3-F	ATAGTCCTGGGTTTCTGTATTGTT
				TARSx3-R	AAAAATCTCAAGTAGGCAAATTA
	4	124 bp	376 bp	TARSx4-F	GGTACATTTTGGAAATTTGTTGTT
				TARSx4-R	CAATCGCAGCAGGACTGAG
	5	122 bp	283 bp	TARSx5-F	TGTATGCCAAGACTCAGACCA
				TARSx5-R	ACCTGATGAAGAAGCAGTGAGAT
	6	118 bp	826 bp	TARSx79-F	AAAACCCTTGAAGTATTCCAAAT
				TARSx79-R	TGGTAAAGAGAAATCAGTGAAAGTT
				TARSx7-R*	ACACGGAACGCCAGTGGT
	7	65 bp	826 bp	TARSx79-F	AAAACCCTTGAAGTATTCCAAAT
				TARSx79-R	TGGTAAAGAGAAATCAGTGAAAGTT

* - Primer used for cycle sequencing only, and not for PCR

Gene	Exon	Exon Length	Amplimer Length	Primer Name	Primer Sequence (5' to 3')
TARS	7	65 bp	826 bp	TARSx89-F*	AGCAAACCATTCCTTCAACAT
	8	79 bp	826 bp	TARSx79-F	AAAACCCTTGAAGTATTCCAAAT
				TARSx79-R	TGGTAAAGAGAAATCAGTGAAGTT
	9	147 bp	395 bp	TARSx9-F	TGAGGATAGAAAACAGCCAAC
				TARSx9-R	AGCCACAACATCTCTTCAAAA
	10	99 bp	381 bp	TARSx10-F	TGGTTTCTTTGATTCAAGAGT
				TARSx10-R	GCCACAGACTTCTACTTTTACTTA
	11	166 bp	286 bp	TARSx11-F	CTTGAAGTATGAGCATTTATTTG
				TARx11-R	GGAAGAACCCAGGATGAAT
	12	163 bp	1457 bp	TARSx1216-F	TAGCATTACAGAAGCAGTTTTATT
				TARSx1216-R	CATTCTCCCTTTCCATCTGA
				TARSx12-R*	ATTTTCCTTGACTTTCTTGACTC
	13	138 bp	1457 bp	TARSx1216-F	TAGCATTACAGAAGCAGTTTTATT
				TARSx1216-R	CATTCTCCCTTTCCATCTGA
				TARSx13-F*	GAGTCAAGAAAGTCAAGGAAAAT
	14	78 bp	1457 bp	TARSx1216-F	TAGCATTACAGAAGCAGTTTTATT
				TARSx1216-R	CATTCTCCCTTTCCATCTGA
				TARSx14-R*	TCATCCACAAGTGAAAATCAT
	15	101 bp	1457 bp	TARSx1216-F	TAGCATTACAGAAGCAGTTTTATT
				TARSx1216-R	CATTCTCCCTTTCCATCTGA
				TARSx15-F*	AGCCCTAATCCAATATTGAG
	16	104 bp	1457 bp	TARSx1216-F	TAGCATTACAGAAGCAGTTTTATT
				TARSx1216-R	CATTCTCCCTTTCCATCTGA
				TARSx16-F*	TTTCTGGTGTCTGCTCTGAA
	17	5 bp	231 bp	TARSx17-F	GGCTTATGGCTTCTTCGT
				TARSx17-R	AACCTGTGCTGCTTTCAA
	18	71 bp	303 bp	TARSx18-F	GGTCTTTTCATTTTGGGGTTTT
				TARSx18-R	GAATACAGGGCTTATGTGATTT
	19	115 bp	1408 bp	TARSx1920-F	GGGGAATAATGGCAGTGAAT
				TARSx1920-R	AGGGACAGAGCAGTCTTGAA
				TARSx19-R*	CTCAAGGTTTCAGTAGATTTCC
	20	505 bp	1408 bp	TARSx1920-F	GGGGAATAATGGCAGTGAAT
				TARSx1920-R	AGGGACAGAGCAGTCTTGAA
				TARSx20.1-F*	TTCTTTTCAGATGTTTCAGTGTG
				TARSx20.2-F*	TCAAAGAGTTCCGCAGCAAA
				TARSx20.3-F*	TGGCAAAGTCTGAATAGGTCA
ADAMTS12	3	362 bp	a - 284 bp	DMTSx3.1-F	CTCCCTCACTGTTCTTCCATT
				DMTSx3.1-R	TTCCCATATCTCTTCTCCATGA
			b - 320 bp	DMTSx3.2-F	GCTCAGAGGACTGGGTGTA
				DMTSx3.2-R	GCATCTGTGAACCTGTTGG
	4	146 bp	321bp	DMTSx4-F	ATTTTCACTTTGTTTTAGTAGGT
				DMTSx4-R	ACAGAGGAGATAAGAGACTTGAGTA
	5	197 bp	a - 337 bp	DMTSx5.1-F	ATTGGTCTATTTTGGTTATGTA
				DMTSx5.1-R	GATGGTGAGGATGTAGGACT
			b - 331 bp	DMTSx5.2-F	GCGGGAGAAGTGGGAGAG
				DMTSx5.2-R	ATCCTGAATTTCCATCCATATT
	6	87 bp	282 bp	DMTSx6-F	CGCCAAACATTTCTGACCTA
				DMTSx6-R	AAGAACTCCCTGAAAAAAGA

* - Primer used for cycle sequencing only, and not for PCR

Gene	Exon	Exon Length	Amplimer Length	Primer Name	Primer Sequence (5' to 3')
ADAMTS12	7	125 bp	335 bp	DMTSx7-F	AAGAACTCCCCTGAAAAAAGA
				DMTSx7-R	TTACAAGAGCAGCAAAGAATAGT
	8	150 bp	347 bp	DMTSx8-F	GAGGTTTTTCCAGATGTTCC
				DMTSx8-R	AGGTTGAGGAGGGTGAGTC
	9	144 bp	308 bp	DMTSx9-F	TTATTACGCTCTTAGGAAACT
				DMTSx9-R	CTTCTCTGTGTAAAACTCAATGC
	10	144 bp	320 bp	DMTSx10-F	TGAAAGGCTGAAGGTAAAGG
				DMTSx10-R	GCTTCTATTTCTGTGTCTGGAT
	11	92 bp	322 bp	DMTSx11-F	TGGCATTATTGTGAGTGTGTG
				DMTSx11-R	TGTTTGACCTCCATCCACTG
	12	148 bp	316 bp	DMTSx12-F	TTTAGGGCTCTTGGCTCAG
				DMTSx12-R	GGAGGCTTGGGGTCACTG
	12	148 bp	312 bp	DMTSx12-F	TTTAGGGCTCTTGGCTCAG
				DMTSx12.1-R	CTTCTGTCTGTGTTTGTCTCTG
	13	170 bp	333 bp	DMTSx13.1-F	GAGGGCATAGAGAGACATCAT
				DMTSx13.1-R	TGCTTTGAGAATGGCTGTT
	14	134 bp	348 bp	DMTSx14-F	TTGCCAGAGTAAATGACACTTG
				DMTSx14-R	GATGAGGTGTAAATGCTGTGAA
	15	117 bp	337 bp	DMTSx15-F	CAGGCAAGAGTGGCATCC
				DMTSx15-R	CCCTTCCTCCTTTGTGGTA
	16	235 bp	327 bp	DMTSx16-F	GGAGGCTGGAAAGAGGATT
				DMTSX16-R	ACTCATTGGTGGGCCTGT
			280 bp	DMTSx16.1-F	GAGCAAGGGACATAAGAGTG
				DMTSx16.1-R	TTGGGGAAAAAGGAGAAGTAT
	17	139 bp	302 bp	DMTSx17.1-F	CTGACATTTTGTGGTGTTTTAA
				DMTSx17.1-R	AAGCAAATCACTGGTGTAAATCAC
	18	130 bp	283 bp	DMTSx1b-F	TGGGCTCAGGATTCAATACG
				DMTSx1b-R	CCAAATAACAAAACCACACAATA
	19	211 bp	378 bp	DMTSx2b.1-F	CCCTGCTTTCTCAGTGGTT
				DMTSx2b-R	TAGGGTCACTTTGGATTGGAT
	20	1106 bp	a - 346 bp	DMTSx3.1b-F	GCATAGTTTAGTTTCCATCAGG
				DMTSx3.1b-R	GTGGGTGTGGTCAGCATTC
			b - 353 bp	DMTSx3.2b-F	AAACCCACCAACACTAAAGC
				DMTSx3.2b-R	AAGATGACAAGCCAGAACCA
			c - 312 bp	DMTSx3.3b-F	CAGCCAAGTGAGGAAAATGT
				DMTSx3.3b-R	ATCTGGTGTAGTGGAGGTG
			d - 359 bp	DMTSx3.4b-F	CAGAGTACCTGGAAATGACG
				DMTSx3.4b-R	GCAAAAAGCCCTCAGTAATC
			e - 315 bp	DMTSx3.5b-F	AACCGTAACCACCTGAAACT
				DMTSx3.5b-R	ATGGGTCTGATTGCTTCCT
	21	152 bp	305 bp	DMTSx4b-F	GCAAACGGGACTGAGAACTGA
				DMTSx4b-R	CAACCCATCCCCACCTTCT
	22	187 bp	296 bp	DMTSx5b-F	ACGGCTGAGGGGAAGGAAG
				DMTSx5b-R	CTTGCCAGGTTGTGTGAGGA
	23	144 bp	364 bp	DMTSx6b-F	ATGTCTCCACCTGACTTTTACCT
				DMTSx6b-R	AATGATGAGTTGATGGGTGC
			144 bp	DMTSx6.1b-F	CACTGGGAACTGGGACCT
				DMTSx6b-R	AATGATGAGTTGATGGGTGC
	24	160 bp	364 bp	DMTSx7b-F	TGTTCTACTGGTGTTCCTTGG
				DMTSx7b-R	AGGGTTACTGGTTGACCTCTT

Gene	Exon	Exon Length	Amplimer Length	Primer Name	Primer Sequence (5' to 3')
ADAMTS12	25	186 bp	385 bp	DMTSx8b-F	TGTTATGGCTCCTTTCTTGGA
				DMTSx8b-R	GCAAAACCTCAACGAAGCA
SALPR	1	1825 bp	a - 684 bp	SALPR-1F	CAGAGGAAACGGATAAAGGTA
				SALPR-1R	TACACCACGCTGATGAGAAT
			b - 718 bp	SALPR-2F	ATGAATAAGGCAGCAGGC
				SALPR-2R	AACGCACCAGGCACAGC
			c - 618 bp	SALPR-3F	TGTTCTTCTCACTGCCAT
				SALPR-3R	ACACGCTCACAGGGAACG
			d - 334 bp	SALPR-2F	ATGAATAAGGCAGCAGGC
				SALPR-5R	TGGTGACGAAGAGGTTGATA
			e - 372 bp	SALPR-4F	TCCTGTCCTTCTTCTGTGT
				SALPR-6R	TGTAGACCACGACGCCAG
			f - 292 bp	SALPR-7F	CCTTCACCGCCACTACCA
				SALPR-4R	TGCTCTGCCCATCCTCCT
MATP	1	409 bp	a - 350 bp	AIM1x1.1-F	ATCTCTGTTGGTTGCTCGG
				AIM1x1.1-R	CCAGGATGGGGCTGAGGAAC
			b - 262 bp	AIM1x1.3-F	CAGCGTATGTGACCCCACT
				AIM1x1.3-R	TATCCCTGTCTGCCCACCTGT
	2	177 bp	287 bp	AIMx2-F	GCGGATGATTCTAAAACAGGAT
				AIMx2-R	GTGGAAGTGCCTCATTGTCTGG
	3	326 bp	a - 315 bp	AIM1x3.1-F	GGTTGCCTCTGCTGTCTTCA
				AIM1x3.1-R	AGGGGTTTGCTGTGGGGGAA
			b - 261 bp	AIM1x3.2-F	AGCCCCACTTACAGAGGTTGC
				AIM1x3.2-R	TCAAACAGACAAAACAAACAAT
	4	144 bp	300 bp	AIM1x4-F	TGTGTGTTCTGGCTCCCTTA
				AIM1x4-R	TACCCTTTCTTACATTCTTACTG
	5	124 bp	348 bp	AIM1x5-F	CAGAGGTGGAGAAGCAGAGTG
				AIM1x5-R	GGTGATAGTTTTTCTGACG
	6	212 bp	377 bp	AIM1x6-F	AAAGGATGTCTAAAAAGGTGAAT
				AIM1x6-R	CTCCCCAGCCTTCAGATG
	7	260 bp	a - 262 bp	AIM1x7-F	CTGACCTGTGCCCTAAATG
				AIM1x7.1-R	GACAAAGCAACAGCCTATCA
			b - 293 bp	AIM1x7.2-F	CCAGACAACAGCGTGAGAG
				AIM1x7-R	GCAGACCCTAAGATTTCAGAGC
AMACR	1	336 bp	597 bp	AMACRx1-F	GCACAAAAAGGAAAAGCAACC
				AMACRx1.1-R	GGCAACAGGGCAAAGGTG
				AMACRx1-1F*	GATTGGGAGGGCTTCTTG
				AMACRx1-2F*	TGCTATGGTCCCTGGCTGA
	2	144 bp	356 bp	AMACRx2-F	GGTGCTATTTTATGTTTGTCTCA
				AMACRx2-R	ATGCTCTCTTGATTGATTCTGAT
	3	161 bp	385 bp	AMACRx3-F	CTGCTGGAGAGATTGATTATTC
				AMACRx3-R	GGTAACCTGGCTTGAACTTC
	4	187 bp	294 bp	AMACRx4-F	ATGCTTTGACACTTGAGTTATCT
				AMACRx4-R	AAAGAACATCCACAGCAAAAG

* - Primer used for cycle sequencing only, and not for PCR

Gene	Exon	Exon Length	Amplimer Length	Primer Name	Primer Sequence (5' to 3')
AMACR	5	1213 bp	a - 749 bp	AMACRx5a-F	TGATTTTACATTTTTTCTCTTGAC
				AMACRx5a-R	CAAAAGCCCTAATGATAACCA
				AMACRx5a-1F*	ACTCCCAATCAGATGAGCA
				AMACRx5a-2F*	TGACTTTTGAGGAGGTTGTTC
				AMACRx5a-3F*	ATAGGAGAACACACTGAGGAGA
				AMACRx5b-F	GGAGGAACAGTATTACAGTGTCC
			b - 801 bp	AMACRx5b-R	AATCCCGTCTTCTTTGTCA
				AMACRx5b-1F*	ATGGTAGTTATTCTGCCTTCC
				AMACRx5b-2F*	CGTGAAACAGAGTGATTGGT
				AMACRx5b-3F*	AAAATAATGAGGAAATGTGTTGG
			5c	AMACRx5c-F	AAGAGGGACGGGGGTGGT
				AMACRx5c.1-R	CAACAGGGCAAAGGTGTGG
				AMACRx5c-1F	GCCCGTTCTGTGCTATGG
C1QTNF3 (isoform a)	1	153 bp	322 bp	CTRP3x1-F	TGCCTACAGCCTTATTTATTACA
				CTRP3x1-R	CTCTCACTTTCTCCCTCCTG
	2	113 bp	315 bp	CTRP3x2-F	CCCATCCCATCTTTTGTGA
				CTRP3x2-R	TTGCCCTTTGCTCCTACTTT
	3	155 bp	263 bp	CTRP3x3-F	AAATGGATGGTAAGTATGAGTGA
				CTRP3x3-R	GTCAGGCAGGTTGGAAG
	4	130 bp	314 bp	CTRP3x4-F	TTCCCAATCTTGCTGTTATGTA
				CTRP3x4-R	CTCTTCTTTCTTCCTTCCTTA
	5	100 bp	240 bp	CTRP3x5-F	TGAAGGCAGTTGGCAAAT
				CTRP3x5-R	GAACAAACAATGGCAGCA
	6	1046 bp	a - 333 bp	CTRP3x6-F	GGGGAGCACAGACTTTGG
				CTRP3x6-R	CAGCAATGTAAAACCCTCAA
			b - 319 bp	CTRP3x6.1-F	CTTTGGGGAAGACTTGTAGC
				CTRP3x6.1-R	TAAAAGCAGAGCAGTCAAAAT
			c - 364 bp	CTRP3x6.2-F	TGACGCAGGTTGGAAATA
				CTRP3x6.2-R	TGGGGGAACATACACAAAA
			d - 309 bp	CTRP3x6.3-F	TGATCCACAAAATACATTAAAC
				CTRP3x6.3-R	TATCTAAAGCCAGGTCAAAATC
			e - 315 bp	CTRP3x6.4-F	ATGTTGACCAGAGGGATTTTAT
				CTRP3x6.4-R	TTAACCATAGTGATTTACAATTCT
C1QTNF (isoform b)	1	3003 bp	a - 1653 bp	C1Qc-F	AGAAAGAGGAGCCAGAGACA
				C1QTNFa-R	ACAGGCTGAACAGAGAGGCTTA
			b - 1713 bp	C1QTNFb-F	ATCGGTCAGTGTAGACAATCAT
				C1qh-R	CTTCAGCCACAGCCAACTC
				C1Qg-F*	GAGGGGGCAAGAAGAGTTAC
				C1Qh-F*	AGAGGAAGCAGAAGGAAGTTA
RAI14	1b	258 bp	674 bp	RAIx1b2b-F	ATTATGGTGTGTGTCGTTTCC
				RAIx1b2b-R	CCTCTCCCTCCCTAAAAGC
				RAIx1b-R*	CACATTCCCCACATCCTA
	2b	60 bp	674 bp	RAIx1b2b-F	ATTATGGTGTGTGTCGTTTCC
				RAIx1b2b-R	CCTCTCCCTCCCTAAAAGC
				RAIx2b-F*	GGTCACCTGTTCTCACTTGC
	2	84 bp	339 bp	RAIx2-F	CCCAAGTTGTAAATCCAATCAG
				RAIx2-R	GCACACAAACAGCAAGGAA

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Gene	Exon	Exon Length	Amplimer Length	Primer Name	Primer Sequence (5' to 3')
RAI14	2c	117 bp	277 bp	RAI2c-F	ACCTTGAGACTCCTGTATGTAGC
				RAI2c.1-R	TGATGAAACCCTGTCTCTACA
	2d	164 bp	843 bp	RAI2d3b-F	TGGATTGGAGGAAGCAGTTAT
				RAI2d3b-R	TCCCTTGGAATAGAAAAAGACA
				RAI2d-R*	TTCACGGCAAAGCAATAAAAT
	3b	181 bp	843 bp	RAI2d3b-F	TGGATTGGAGGAAGCAGTTAT
				RAI2d3b-R	TCCCTTGGAATAGAAAAAGACA
				RAI3b-F*	AGATGCTTATGGGATGATGGTA
	3	131 bp	320 bp	RAI3-F	CACACTCATTTCGGGAGCAG
				RAI3-R	CCAGAGAACACCCCAAAAA
Cadherin-18	3	119 bp	250 bp	cdh18x3-F	CATAACCATTTTCTGTGTTC
				cdh18x3-R	GCAACTTACTGTAGCAAACGC
	4	168 bp	314 bp	cdh18x4-F	TATGTATGTGTATGTTTGTG
				cdh18x4-R	CTGTTCAAGTATTTTCATTTA
	7	137 bp	263 bp	cdh18x7-F	TTTCCTTGGTTCAGTTGT
				cdh18x7-R	ATTTCAGTATGTCTTCTTGTA
	8	122 bp	297 bp	cdh18x8-F	TATGGAACCAGTGACAAG
				cdh18x8-R	ACCTAAGAATAATAAAAAATAAG
	9	118 bp	306 bp	cdh18x9-F	TCAAGAGACAAGCAAAAT
				cdh18x9-R	CATAACACAGTAGCATCAA

* - Primer used for cycle sequencing only, and not for PCR

SNP Name	Amplimer Length	Primer Name	Primer Sequence (5' to 3')
rs840388	377 bp	CTRP3snp-F	CTGGGTTCAGTGACTCTCCTA
		CTRP3snp-R	TCAATATAATCTCTTTGTGTCCAA
rs840345 rs840346	364 bp	CTRP3x6.2-F	TGACGCAGGTTGGAATA
		CTRP3x6.2-R	TGGGGGAACATACACAAAA
rs1366648	245 bp	snp136-F	TTAGGTGATGAGGTTTACTTGTT
		snp136-R	TGAAGTTATTTGAAGGGCACA
rs1440429	317 bp	snp144-F	CCTACTTTGAGAATGCTGCTTTA
		snp144-R	ATCAAGGCTTCTTACAGACTCAG
rs2388676 rs2892568 rs2892569 rs2388677 rs2388678 rs2388679 rs2388680	331 bp	LOC215i3-F	ATGGCATCTGCTTGTGAAAT
		LOC215i3-R	TAGATTCTTTTGACGCTTCATT
rs2099761	550 bp	snp209-F	AACATTTGAACAGCCATCCA
		snp209-R	CAGTCAGACCACAAGACACCT
rs903844	307 bp	snp903-F	GTGGTGGGTAGGGGAAGA
		snp903-R	CACTCTGTTTCTAAGCACTTTTC
rs1992714	730 bp	snp199-F	CTCTGGAACAATCAGGTTTG
		snp199-R	GGGGCAGGAGTGTAATAA
rs466348	694 bp	RAIsnp2B-F	AGCAGCCATCCCAAGCAA
		RAIsnp2B-R	GACTCCTTTTCCCTATCACCAT

Curriculum Vitae

- Assisted in preparation of the FDA submission for the *TRUGENE HIV™* Genotyping Kit by coordinating and documenting studies related to development of the kit's negative control and writing documents describing standard operating procedures used within the lab.

Research Technician (June 1996-August 1999)

Visible Genetics Inc., Toronto, Ontario

Assisted in the research and development of gene analysis kits for use on the company's Open Gene Automated DNA Sequencing System™.

- Assisted in the optimization and testing of gene analysis kits including kits for analysis of the p53 tumor suppressor gene, HLA Class I and II genes, and antibiotic resistance-associated *M. tuberculosis* genes.
- Provided demonstrations of the Open Gene System™ to potential customers and investors as requested.

TEACHING EXPERIENCE

Let's Talk Science Volunteer (September 2001 – June 2002)

University of Ottawa, Ottawa, Ontario

Provided demonstrations to highschool students regarding various science topics, as requested by highschool teachers.

- Acted as a liason between one of the local highschools and the Let's Talk Science program, to arrange demonstrations of various science topics by university graduate students
- Assisted in demonstrations of DNA, forensic science, sexuality and sexually transmitted diseases at several local highschools.

Laboratory Demonstrator (September 2001 – December 2001)

University of Ottawa, Ottawa, Ontario

Provided laboratory demonstrations and teaching assistance for the third year undergraduate biochemistry lab course, BCH3356 – Molecular Biology Laboratory.

- Provided weekly laboratory demonstrations for 11 students, including a brief lecture about background information related to the laboratory procedures. Topics included DNA and RNA isolation, restriction endonucleases, vectors, recombinant DNA and protein analysis.
- Evaluated weekly lab reports and assignments.
- Provided extra tutorial to students as required.

Swimming Instructor and Lifeguard (September 1991 – June 1992)

Oakville Parks and Recreation – Aquatics Division, Oakville, Ontario

Delivered swimming instruction through the Red Cross Aquatics Program and safe guarded the lives of swimmers.

- Instructed children in swimming skills and water safety.
- Prepared daily lessons which stressed creativity and skill development.
- Delivered nine classes per week to some 60 students.

PEER REVIEWED PUBLICATIONS

Kirkpatrick, T.J., Au, K.S., Mastrobattista, J.M., **McCready, M.E.**, Bulman, D.E., Northrup, H. (2003). Identification of a mutation in the Indian Hedgehog (IHH) gene causing brachydactyly type A1 and evidence for a third locus. *Journal of Medical Genetics*, 40(1):42-4.

McCready, M.E., Sweeney, E., Fryer, A.E., Donnai, D., Baig, A., Racacho, L., Warman, M.L., Hunter, A.G., Bulman, D.E. (2002). A novel mutation in the IHH gene causes brachydactyly type A1: a ninety-five-year old mystery resolved. *Human Genetics*, 11(4-5):368-75.

Armour, C.M.*, **McCready, M.E.***, Baig, A., Hunter, A.G.W., Bulman, D.E. (2002). A novel locus for Brachydactyly Type A1 on chromosome 5p13.3-13.2. *Journal of Medical Genetics*, 39(3):186-8.

Saunders, M.E., Mackenzie, R., Shipman, R., Fransen, E., Gilbert, R., Jordan, R.C.K. (1999). Patterns of p53 gene mutations in head and neck cancer: Full-length gene sequencing and results of primary radiotherapy. *Clinical Cancer Research*, 5:2455-2468.

*co-first authorship

CHAPTERS AND OTHER NON-PEER REVIEWED PUBLICATIONS

McCready, M.E., D.E. Bulman (in press). Brachydactyly Type A. In: Lange, R., Wilbertz, H. (eds.). *Encyclopedic Reference of Molecular Mechanisms of Disease*. Springer-Verlag:Germany.

POSTER PRESENTATIONS AND ABSTRACTS

McCready, M.E., Sweeney, E., Fryer, A.E., Donnai, D., Baig, A., Racacho, L., Warman, M.L., Hunter, A.G., Bulman, D.E. (2003). A novel mutation in the IHH gene causes brachydactyly type A1: a ninety-five-year old mystery resolved. Canadian Genetic Disease Network, Annual Meeting, Kanaskis, Alberta. May 15-19.

McCready, M.E., Armour, C.M., Hunter, A.G. W., Bulman, D.E. (2001). Evidence for genetic heterogeneity in Brachydactyly Type A1. American Society of Human Genetics. 51st Annual Meeting, San Diego, California, USA. October 12-16.

McCready, M.E., Armour, C.M., Hunter, A.G. W., Bulman, D.E. (2001). A novel locus for Brachydactyly Type A1 on chromosome 5p13.3-13.2. Canadian Federation of Biological Studies. 44th Annual Meeting, Ottawa, Ontario. June 20-23.

Saunders, M.E., Mackenzie, R., Shipman, R., Gilbert, R., Fransen, E., Jordan, R.C.K. (1999). Complete p53 Mutational Analysis in Laryngeal Squamous Cell Carcinoma. Canadian Association of Pathology, 50th Annual Meeting, Calgary, Alberta. June 19-23.

Shipman, R., **Saunders, M.E.** (1999). Complete p53 gene analysis, allelic loss and microsatellite instability analysis in primary non-small cell lung cancer. Canadian Association of Pathology, 50th Annual Meeting, Calgary, Alberta. June 19-23.

Saunders, M.E., Mackenzie, R., Shipman, R., Gilbert, R., Jordan, R.C.K. (1999). p53 mutational analysis in laryngeal squamous cell carcinoma: Results of full-length gene sequencing and primary radiation therapy. American Association of Cancer Research, 90th Annual Meeting, Philadelphia, USA. April 10-14.

Saunders, M.E., Mackenzie, R., Shipman, R., Gilbert, R., Fransen, E., Jordan, R.C.K. (1998). Full-length p53 gene sequencing in head and neck cancers and outcome radiation therapy. Eastern Great Lakes Head and Neck Association, 22nd Annual Meeting, Toronto, Ontario. November 7.

Saunders, M.E., Mackenzie, R., Shipman, R., Jordan, R.C.K. (1998). Complete p53 gene analysis in head and neck cancer: Mutation detection in genomic DNA from archival sources. American Association of Cancer Research, 89th Annual Meeting, New Orleans, USA. March 28-April 1.

Saunders, E., Shipman, R. (1997). Complete p53 gene analysis using the Open Gene™ System: Mutation Detection in genomic DNA from archival and fresh clinical specimens. Association for Molecular Pathology 1997, San Diego, USA. November 12-15.

INVITED ORAL PRESENTATIONS

The Genetics of Brachydactyly: Short Fingers, Lengthy History. Ottawa Health Research Institute Research Day, Ottawa, Ontario. January 24, 2003.

Characterization of the Gene(s) that Cause Brachydactyly Type A1. Children's Hospital of Eastern Ontario Genetics Rounds, Ottawa, Ontario. June 18, 2002.

Complete p53 gene analysis using the Open Gene System™: Mutation detection in genomic DNA from archival and fresh clinical samples. Dana Farber Cancer Institute, Chicago, USA. July 12, 1999

Complete p53 gene analysis using the Open Gene System™: Mutation detection in genomic DNA from archival and fresh clinical samples. Upstate New York Section of the American Association of Clinical Chemistry – Annual Spring Meeting, Niagra Falls, Ontario. May 20, 1999.

ACADEMIC AWARDS

2003-2006 – CIHR Doctoral Research Award

2003-2006 – University of Ottawa National Excellence Award

2001-2002 – Government of Ontario – Ottawa-Brennan Bookstore Graduate Scholarship in Science and Technology

1998-1999 – University of Toronto Open Fellowship

1997-1999 – Visible Genetics Inc. Award

1992 – Ontario Scholar