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Molecular Analysis of Simpson-Golabi-Behmel Syndrome: An Overgrowth Condition Associated With an Increased Risk of Embryonal Tumour

A thesis submitted to the School of Graduated Studies at the University of Ottawa in partial fulfillment of the requirements for the degree of Master of Science, Department of Biochemistry, Faculty of Medicine.

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ABSTRACT

Simpson-Golabi-Behmel syndrome (SGBS) is a recently described rare X-linked condition associated with pre- and postnatal overgrowth and an increased risk of developing embryonal tumours. Features of the syndrome include a characteristic coarse face, broad nasal root, wide mouth, cleft palate, full lips with a midline groove of the lower lip, grooved tongue with tongue tie, prominent mandible, cardiac arrhythmias, supernumerary nipples, splenomegaly, large dysplastic kidneys, cryptorchidism, hypospadias, umbilical or diaphragmatic hernias, skeletal and limb abnormalities. Mild intellectual impairment has been reported in some families but many affected males are of normal intelligence.

Genetic linkage analysis of two SGBS families in our lab has been conducted. The closest linkage to SGBS was observed for the chromosome Xq26 locus HPRT (Z max = 7.45, θ max = 0.00). SGBS-Xq marker recombinations map the disease locus to the Xqcen-DXS425-SGBS-DXS1123-Xqter interval on Xq25-q27. Recently, linkage analysis conducted on two SGBS families in Europe have also showed cosegregation of SGBS with locus HPRT (Z max = 4.45 , θ max = 0.00). A young girl with a balanced translocation, 46XX, t (X;1) (q26;q12), was previously described as having Beckwith Wiedemann syndrome (BWS) but has been reassessed and now is considered to have SGBS (Punnett 1994). The close linkage between the disease locus and the HPRT locus strongly suggests that the causative gene for SGBS is located in the chromosome Xq26 region and is disrupted by the translocation.

SGBS shows significant clinical overlap with another overgrowth disorder

called Beckwith-Wiedemann syndrome (BWS). BWS is usually sporadic but in some families with more than one affected individual, a autosomal dominant inheritance has been suggested. Both disorders demonstrate an increased risk for developing embryonal tumours. Two genes from the BWS linked chromosome 11p15 region, IGF2 and H19, are known to normally undergo parental imprinting. Relaxation of this imprinting has recently been demonstrated in some BWS cases as well as in some instances of isolated Wilms' tumour (WT). Since SGBS and BWS have been postulated to result from distinct defects in a common pathway (Bird et al.,1993), we have studied the methylation pattern and transcriptional activity of IGF2 and H19 in isolated SGBS +/- WT tissue. No consistent methylation abnormalities were observed in genomic DNA isolated from SGBS leucocytes, placenta, skin fibroblasts or cleft lip tissues. Genotyping of H19 cDNA polymorphisms showed biallelic expression in both SGBS and normal placentas and monoallelic expression in one SGBS fibroblast. However, monoallelic expression of IGF2 was seen in control placenta and fibroblast but biallelic expression of IGF2 was shown in one SGBS placenta, and two SGBS fibroblasts tissues.

It would thus appear that loss of genomic imprinting may play a role in the pathogenesis of SGBS. This is the first example that we are aware of a *trans* imprinting function for a human gene.

Genotyping with microsatellite polymorphism did not show inheritance of a common chromosome 11p13-p15.5 region in the 3 children with WT, arguing against the presence of a predisposing mutation in the H19, IGF2, WT1 loci in these cases. Loss of maternal allele deletion was detected in the 11p13-p15.5

loci in the tumour tissues from two children with SGBS. A complete demethylation pattern of IGF2 has been observed in the WT tissue of one child with SGBS. This finding may result from the loss of maternal allele or loss of a methyl group in the maternal copy of the gene.

ACKNOWLEDGMENTS

I would like to thank my supervisor, Dr. Alexander E. MacKenzie, for his guidance and support during these studies. I especially thank Dr. Robert G. Korneluk and Dr. Rhia M. Hughes-Benzie for their great help in my research study. I am grateful to the SGBS patients and their families for their willing to provide blood samples and to Dr. Linda Surh and the diagnostic laboratory for the use of the extracted DNA for my research. I appreciated my many colleagues and friends in our laboratory for all the technical help and their encouragement and friendship. I am grateful to my husband Kwok Fai Chan and my family for their understanding and support.

DEDICATION

To my husband Fai, my grandmother, my family, and Dr. Alex MacKenzie especially .

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LIST OF ABBREVIATIONS

A	adenine base in a nucleotide sequence
bp	base pair
BRL	Bethesda Research Laboratories
^{o}C	degrees Celsius
cDNA	complementary DNA
cM	centiMorgan
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
kb	kilobase
lod	logarithm of the odds
LOH	loss of herterozygosity
LOI	loss of imprinting
Mb	megabase
min.	minute
mM	millimolar
mRNA	messenger ribonucleic acid
ng	nanogram
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism

CHAPTER 1 INTRODUCTION

1. Somatic Overgrowth: Sporadic and Syndromes

Somatic overgrowth, defined as having greater than normal weight and length, can be present at birth and usually persists into postnatal life. There is no generally accepted definition of prenatal somatic overgrowth, although many workers regard a birth weight of greater than 4.5 kg as a useful cut off in defining the condition. Several genetic syndromes have been classified as overgrowth disorders (see Table 1-1). The following examples illustrate the problems with the definition of this disorder: children with Beckwith-Wiedemann syndrome have excessive pre- and postnatal overgrowth in both weight and height, associated with macroglossia and skeletal abnormalities, but have normal brain and head size; Marshall-Smith syndrome is associated with a normal birth weight but with advanced bone maturation; Patterson-David syndrome is characterized by prenatal overgrowth in length and weight but postnatal weight deficiency is seen and localized overgrowth disorders represent overgrowth only in specific tissues or organs (Weaver 1994).

a. Clinical profile

Patterns of overgrowth usually can be separated into three general categories: normal variants, prenatal overgrowth, and postnatal overgrowth. Normal variants, such as familial tall stature, include individuals whose height and weight lies above the 98th centile of the normal postnatal growth curve. Prenatal overgrowth may be associated with other congenital anomalies and

occasionally mental deficiency. Prenatal overgrowth may persist postnatally and may be asymmetrical. Postnatal overgrowth may be associated with excesses of humorally mediated factors such as growth hormone (eg. acromegaly) or estrogens or androgens (eg. sexual precocity). Prader-Willi syndrome (PWS) is associated with marked postnatal obesity, short stature and mental retardation and is believed to involve abnormalities in genomic imprinting. Although overgrowth is usually observed in isolation, it can also occur as a part of a defined overgrowth syndrome. A list of overgrowth syndromes is shown in Table 1-1.

The incidence of overgrowth syndromes varies widely. For example, Klinefelter syndrome affects 1 in 1000 male births; Beckwith-Wiedemann syndrome is a relatively common overgrowth condition with over 500 cases and at least 27 families reported (Viljoen, et al., 1992); in contrast, Elejalde syndrome is very rare, occurring in less than 1/1,000,000 births (Weaver 1994).

The etiologies and modes of transmission of the overgrowth syndromes and disorders are also diverse. Marfan syndrome results from an autosomal dominant mutation in the fibrillin gene (FBN1), an important component of connective tissue. Perlman syndrome is an autosomal recessive disorder. (Weaver 1994). The BWS is usually sporadic, and can be associated with cytogenetic abnormalities such as duplication of 11p15. It can also be inherited as an autosomal dominant disorder with a locus mapped to 11p15.5 by linkage analysis (Koufos et al., 1989; and Ping et al., 1989). PWS is caused by the deletion of the paternal copy or the maternal uniparental disomy of chromosome 15q11-13 (Weaver 1994). Simpson-Golabi-Behmel syndrome is an X-linked

disorder which occurs both as familial and sporadic cases. In this thesis, I present the localization of SGBS to chromosome Xq26, the second classical overgrowth syndrome to be genetically mapped.

b. Cancer predisposition

Children with large birth weights are at increased for several types of cancer in the first four years of life, especially Wilms' tumour, neuroblastoma and leukemia (Irving 1970, Wertelecki and Mantel 1973, Daling et al., 1984). The precise mechanism of the cancer predisposition seen in somatic overgrowth is unknown. It has been postulated that an increased mitotic activity in overgrowth syndromes merely increases, in a non-specific manner, the odds of requisite oncogenic mutations occurring (Cohen, 1981). However, infants of diabetic mothers experience marked cellular hyperplasia and resultant somatic overgrowth, but have no change in their risk for malignancy. Also, the cancer risk associated with various overgrowth syndromes is not consistently determined by somatic size, indicating that additional, and as yet unknown, factors are involved.

Several overgrowth syndromes have been associated with a significant increased incidence of malignancy (Simpson-Golabi-Behmel, Beckwith-Wiedemann, Weavers, Sotos, Hemihypertrophy, Bannayan-Riley-Ruvalacaba, and Proteus). The mechanism of overgrowth and cancer disposition has been most intensely studied in BWS.

The main clinical features of the BWS include pre- and postnatal

overgrowth, accelerated osseous maturation, macroglossia, capillary nevus flammeus, ear lobe creases and pits, pancreatic hyperplasia, adrenocortical cytomegaly, large kidneys with renal medullary dysplasia, etc.. BWS is also characterized by variable clinical manifestations including visceromegaly, omphalocele, macroglossia and gigantism (Pettenati et al., 1986). About 85% of the BWS occur sporadically but occasionally BWS may be transmitted as an autosomal dominant disorder. The incidence of Wilms' tumour in sporadic BWS is 7-7.5% (Wiedemann 1983, Jones 1994), and in familial BWS the incidence of tumours is 1 in 61 individuals from 14 families (Grundy et al., 1994).

In two BWS families linkage between the disease phenotype and markers on chromosome 11p15.5 has been observed (Koufos et al., 1989; and Ping et al., 1989). Furthermore, sporadic cases of BWS have demonstrated chromosomal abnormalities involving 11p15.5 including duplications (Turleau 1984) and translocations (Mannens et al., 1991). Genes mapped to this region include WT1, WT2, IGF2, H19, and insulin and IGF2 receptor. Recently, an increased gene dosage of IGF2 associated with a disruption of normal genomic imprinting patterns for the IGF2 locus has been implicated as the mechanism underlying some cases of BWS (Weksberg et al,. 1993). In chapter 3 of this thesis, the effect of the SGBS mutation on 11p13-15.5 loci is reported. Specifically, changes of the methylation status or transcriptional activity of two genes from the 11p region was assayed and evidence for an LOI of IGF2 is presented.

Table 1-1. Overgrowth Syndromes:

1. Bannayan-Riley-Ruvalcaba syndrome
2. Elejalde syndrome
3. Familial rapid maturation
4. Familial tall stature
5. Fragile X syndrome
6. Gigantism/acromegaly
7. Hyperthyroidism, congenital
8. Hyperthyroidism, infancy and childhood
9. Klinefelter syndrome
10. Marfan syndrome
11. Nevo syndrome
12. Perlman syndrome
13. Precocious puberty
14. Prader-Willi syndrome (PWS)
15. Proteus syndrome
16. Simpson-Golabi-Behmel syndrome (SGBS)
17. Sotos syndrome
18. Teebi-type overgrowth syndrome
19. Trisomy 8 mosaicism (Warkany syndrome)
20. Beckwith-Wiedemann syndrome (BWS)
21. Marshall-Smith
22. Patterson-David
23. Hemihypertrophy
24. Weaver syndrome

(adapted from Cohen 1989; Weaver 1994).

Table 1-2. Congenital defects associated with Wilms' tumour.

SYNDROME	LOCUS	GENETIC ABNORMALITY WITH WT	FREQUENCE OF WT
WAGR*	11p13	Deletion in 11p13	>30%
Denys-Drash	11p13	WT1 point mutation	>90%
BWS	11p15.5	Duplication of paternal allele	<7.5%
SGBS	Xq26.1	Loss of maternal allele in 11p13-15.5	~7%
Sotos	----	----	3.9%
Hemihyperplasia	----	----	3.8%
Aniridia	11p13	Deletion in 11p13	0.84%

WAGR*- Wilms' tumour with aniridia, genitourinary malformations, and retardation syndrome. (adapted from Cohen et al.1989, Carol L 1993, Coppes 1994, Hughes-Benzie, 1994 pers. communication).

2. Simpson-Golabi-Behmel syndrome (SGBS)

Simpson-Golabi-Behmel syndrome (SGBS) is a relatively recently described rare X-linked condition associated with pre- and postnatal overgrowth and an increased risk of developing embyonal tumour. Other features included characteristic coarse facies, hypertelorism, broad nasal root, cleft palate, full lips with a midline groove of the lower lip, grooved tongue with tongue tie and prominent mandible; congenital heart defects and arrhythmias; supernumerary nipples; splenomegaly and large dysplastic kidneys; cryptorchidism and hypospadias; broad hands and feet, polydactyly, hypoplasia of the index finger nail and distal phalanx mild, cutaneous syndactyly of the hands and anomalies of the vertebrae, ribs and sacrum; umbilical and diaphragmatic hernias;. developmental delay and mental retardation have been reported in some families but many affected males are of normal intelligence (Hughes-Benzie et al., 1992 and Hughes-Benzie et al., 1994).

The first SGBS family was described by Simpson et al. in 1975. Two cousins with macrocephaly, abnormal face, high arched palates, broad hands with "dysplastic" fingernails, short and broad neck, and normal psychomotor development were described. In 1984, Golabi and Rosen reported on a "new" X-linked condition characterized by pre- and postnatal overgrowth. In the same year, Opitz reported what he considered to be three instances of another affected family and named the condition Golabi-Rosen syndrome. Shortly after that, Behmel et al., (1984) reported another affected family and called attention to the cases reported by Simpson et al., in 1975. Meanwhile, a Japanese boy diagnosed as Weaver-like syndrome (Tsukahara et al., 1984) was re-

diagnosed as Golabi-Rosen syndrome (Kajii and Tsukahara 1984). Because of the similarity between Golabi-Rosen syndrome and the syndrome described by Behmel et al., in 1984 and that by Simpson et al., in 1975, Neri et al., suggested that the condition be renamed Simpson-Golabi-Behmel syndrome (Neri, et al.,1988). Since then, there have been approximately 30 families or sporadic cases reported in the world (Golabi and Rosen, 1984; Opitz, 1984; Behmel et al., 1984; Tsukahara et al., 1984; Opitz et al., 1988; Neri et al., 1988; Behmel et al., 1988; Garganta et al., 1988; Konig et al., 1991; Kaariainen et al., 1991; Garganta CL. et al., 1992; Hughes-Benzie et al., 1992b; Di Rocco M. et al., 1993; Chen E. et al., 1993).

Genetic mapping of SGBS is complicated by the low prevalence of this disorder resulting in a paucity of patient material; recombination events which define the mapping region are therefore also rare. Furthermore, similarities of the overgrowth syndrome with others such as BWS has the potential of leading to misdiagnoses of SGBS. In 1992, Hughes-Benzie et al., first reported the linkage data of the Dutch-Canadian SGBS family. Twenty one DNA samples of the family members and the four DNA probes (404E2, 50M2, 72R1, and 329H2) obtained from Dr. D. Barker (Salt Lake City, Utah) were used in the genetic linkage analysis. With MLINK program analysis, a maximal lod score of 2.81 with a recombinant frequency of 0.00 for the SGB-DXYS68 pairing was obtained mapping the disorder causative gene to Xqcen-q22 region (Hughes-Benzie et al., 1992b). An extension of this linkage study is discussed in the second chapter of this thesis.

a. Possibility of genetic anticipation in SGBS

A noteworthy finding in our Dutch-Canadian SGBS kindreds is that the severity of the symptoms increases with each successive generation. Such an increase in the severity of the disease with the intergenerational passage is termed genetic anticipation. The female obligate carrier (idiv. II-1) in this family was 5'6"; whereas, all of her affected granddaughters are over 6'. The affected males show a similar trend. It is most evident when pictures of the affected male from different generations are compared (Figure 1-2). The coarseness of the faces which is mild and almost undetectable in the earliest generation (III-2) increases markedly in severity in the grandsons of the first generation (V-2, V-5, V-6) (MacKenzie 1994 unpublished) (Figure 1-1 and Figure 1-2). Recently, a biological basis for anticipation has been identified as the progressive expansion of trinucleotide CTG or CCG repeats. Stretches of the repeated trinucleotides can, after reaching a certain length, become unstable. Several human genetic disorders: myotonic dystrophy (DM), fragile X syndrome (FRAXA), Spinal and bulbar muscular atrophy (SBMA), Huntington disease, spinocerabellar ataxia type 1 (SCA1), and FRAXE mental retardation have been found to be involved in expansions of trinucleotide repeat (Wieringa B. 1994. Research reports showed that at least 50 human genes have been found containing expansion of five or more trinucleotide repeats (Morell V. 1993).

b. Cancer predisposition; statistical consideration.

SGBS is relatively recently described overgrowth syndrome and has had a fairly low profile; consequently, the risk for WT in the syndrome is unknown. The estimation in some SGBS families, a rate of approximately 12 %

(6/52) has been obtained. This number is slightly greater than those seen in BWS (7.5%) although these may be an over representation of cases due to ascentainment bias. More, it is possible that there are a number of SGBS cases with WT mistakenly diagnosed with BWS or other overgrowth syndrome.

c. Cancer predisposition; a representative casehistory

Three children from the Dutch-Canadian SGBS family have been diagnosed with Wilms' tumour under the age of two. One of them died at the age 19 months with a embryonal tumour involving the right kidney and right adrenal 26 years ago (Hughes-Benzie et al., 1992). According to the clinical report, the 22 month old female (indi. V-1) sib of an SGBS male presented with haematuria and abdominal pain and swelling. Abdominal CT scan showed a 12 cm X 11.3 cm X 8.7 cm cystic mass in the upper pole of the right kidney. The tumour was a classical, non-anaplastic WT. Presence of peripheral intralobar nephrogenic rest tissue suggested an increased risk for tumour formation in the contra lateral kidney. Staging procedures were negative. She was treated as Stage II disease using the National Wilms' Tumour Study (NWTS) protocol; treatment which included Vincristine and Dactinomycin for 54 weeks. After six months of treatment, she was disease free (MacKenzie 1994 unpublished). As a result, Dr. Hughes-Benzie started a tumour screening program for the six children of two female SGBS heterozygotes (3 affected males and their sisters) in this family.

The tumour screening program included abdominal ultrasound, serum alpha-fetoprotein (AFP), serum beta-hCG, and urine VMA. Individual V-2 had

typical features of SGBS including pre- and postnatal overgrowth, coarse face, grooved tongue, supernumerary nipples, umbilical and inguinal hernias, hepatosplenomegaly and bilateral renal enlargement. Abdominal ultrasound screening showed large kidneys with dysplasia. His serum AFP ranged between 18-300 mg/l (normal < 10 mg/l) from one month of age. At 15 months, an abdominal CT scan showed normal configuration of the kidneys. At 19 months, abdominal ultrasound done for tumour screening showed a 3.4 cm X 3.2 cm mass in the upper pole of the right kidney. Over the next 14 days, the mass increased in size to 4.1 cm X 4.8 cm. The mass was removed by heminephrectomy for preservation of normal renal parenchyma and because of the potential for bilateral involvement. Pathological examination revealed a non-anaplastic triphasic WT with infiltration into the immediately adjacent renal parenchyma. Staging studies were negative and he was sucessfully treated with the NWTS protocol for Stage II tumour. A fourth WT male from an unrelated Portuguese SGBS family, died at two years of age from a tumour and a fifth affected child has been identified from the NWTS registry (MacKenzie 1994, unpublished). In addition, Hughes-Benzie et al., in 1993 reported the sixth case of WT in an unrelated SGBS male with persistent renal blastema forming subcapsular nephrogeneic rests who died unexpectedly at 4.5 months.

Figure 1-1. The pedigree of the Dutch-Canadian SGBS family.

Black solid squares represent affected males, White squares represent unaffected males, White circles with black spot represent carriers. White circles represent unaffected females. T - individual with Wilms' tumour. Numbers indicate the samples presented in the linkage data in chapter 2.

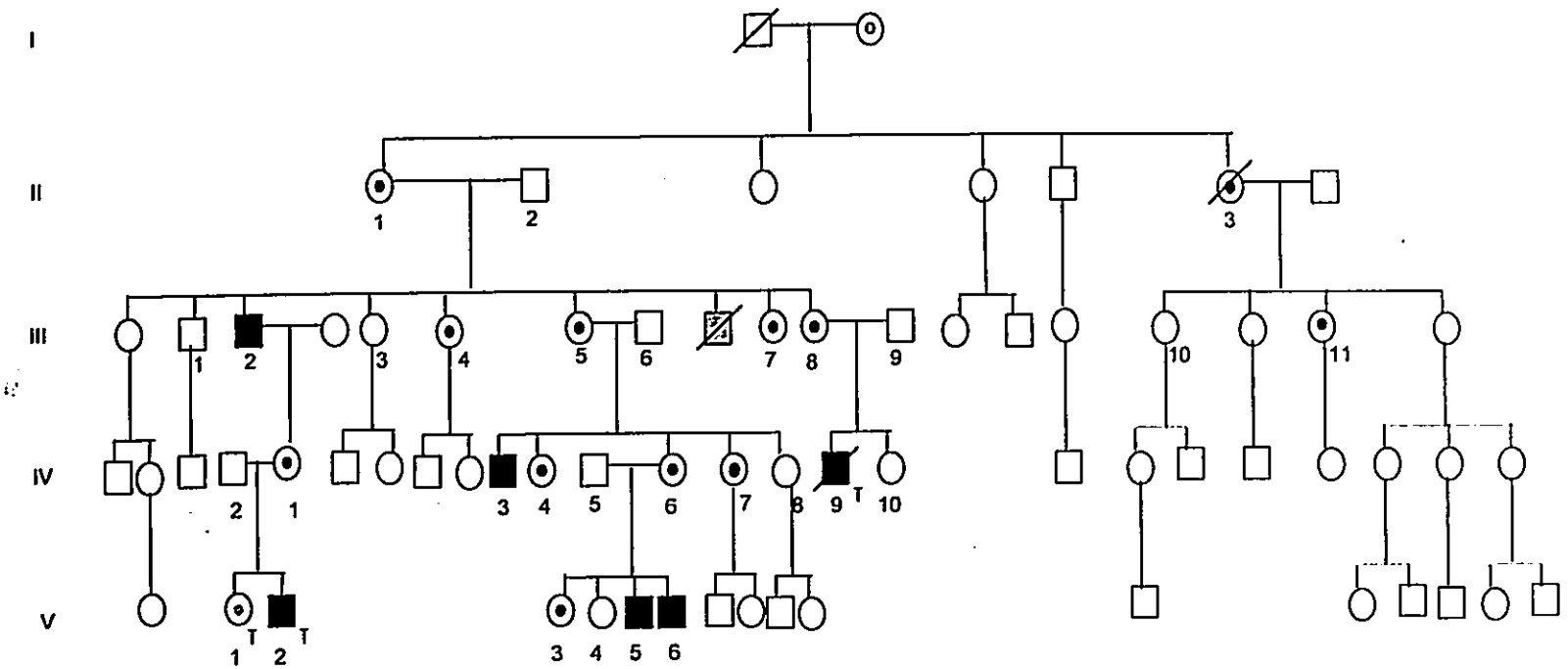
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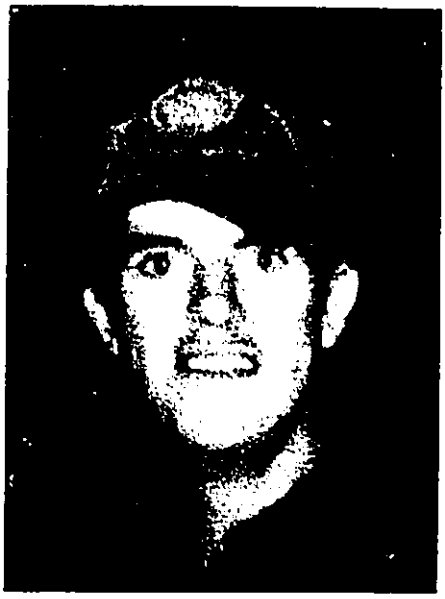
Figure 1-2. The pictures of some of the affected individual in the Dutch-Canadian SGBS family.

These pictures present the typical feature of SGBS coarse face and the anticipation of this feature from generation to generation. (A) III-2, a affected male, at age 59 years with wide mouth, long face, hypertelorism, large ears, prominent mandible. (B) VI-3, an affected male, at age 18 years with a long face, hypertelorism, wide mouth, large ears, and prominent mandible. (C) V-2, an affected male, at one month old with broad nasal base, depressed bridge and full lips. (D) V-6, the index patient in this family, at 14 months old with broad nasal base, upturned nose, and midline groove in tongue and lower lip.

(A) III-2 at age 59 years.



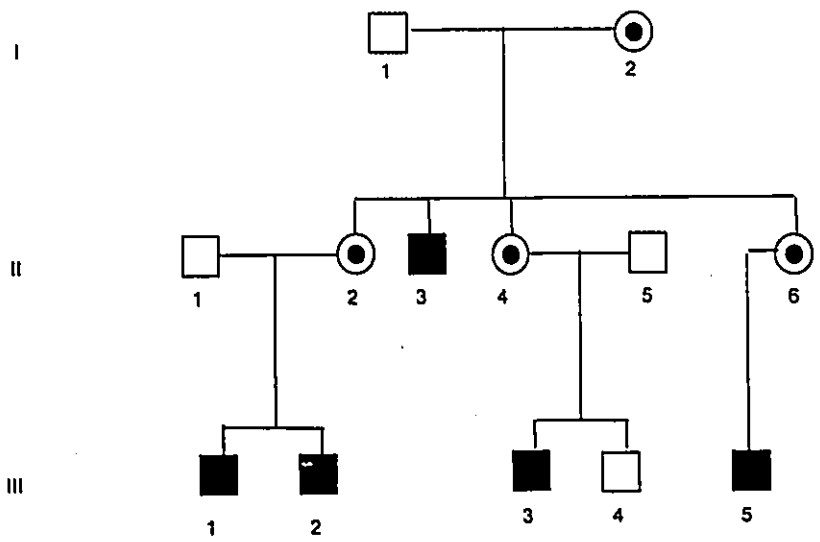
(B) VI-3 at age 18 years.

(C) V-2 at one month old.

(D) V-6 at 14 months old.

Figure 1-3. The pedigree of the English SGBS family.

Black solid squares represent affected males, White circles with black spot represent carriers. White circles represent unaffected females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3. Chromosome 11p loci methylation and genomic imprinting

Genomic imprinting is a process in which a gamete-specific modification leads to differential expression of the two alleles of a given gene. In such cases, the imprinted genes are normally expressed monoallelically with one copy, determined by the parent of origin, being transcriptionally quiescent. At least four genes in mouse showing such parentally determined differential expression have been identified: Insulin-like growth factor-II (IGF2), Small nuclear ribonucleoprotein-associated polypeptide (SmN, or Snrpn), IGF2 receptor, and H19. H19 and IGF2r are expressed from the maternal alleles (Bartolomei et al., 1991, Barlow et al.,1991), while IGF2 and Snrpn appear to be expressed from the paternal allele (DeChiara et al., 1991, Leff et al., 1992).

a. Chromosome 11p loci methylation in health

In keeping with the genomic imprinting observed in mice, recent studies have shown that in human the paternal H19 allele is inactive in fetal tissues (Kalscheuer et al., 1993) with exclusive expression from the maternal chromosome (Zhang et al., 1993). In contrast, the maternal allele of IGF2 is normally inactivated (Diaz de Bustamante, et al., 1990). Biallelic expression of IGF2 has been demonstrated in normal human liver showing that genomic imprinting can be tissue specific (Kalscheuer 1993, Davies 1994).

It is believed that imprinting is started in the germ line or shortly after fertilization with a heritable molecular marker which is both capable of regulating gene expression and is removed before the next germ cell development (Surani

1993). DNA methylation could be one such marker.

DNA methylation has been found to underlie gene expression, DNA restriction process, and DNA repair process (Mathews 1990; and, Laird and Jaenisch 1994). In animal DNA, about 3-5% of cytosine residues have been found to be methylated at the fifth carbon position of cytosine residues (5-methylcytosine), the cytosine residue is followed immediately by a guanine (G) residue to form a CpG sequence. The methylation at a particular site of eukaryotic DNA is heritable during DNA replication by the function of a maintenance methylase. A control role for DNA methylation in the tissue-specific inactivation of genes has been suggested by many studies.

Although distinct methylation patterns have been observed for the IGF2 and H19 loci, the precise role of DNA methylation in the expression of IGF2 and H19 is obscure. It has been observed that the CpG island in 3 kb upstream of IGF2 and in the promoter region of H19 are both methylated on the paternal chromosome but appear to have opposite effects in the activation of the genes (Surani 1993). The 5' portion of exon 9 of the maternal IGF2 allele is specifically hypomethylated (Schneid et al., 1993). Although differences in DNA methylation between maternal and paternal IGF2 gene were not consistently detected, it was suggested that DNA methylation may play a role in genomic imprinting with relaxation of this imprinting possibly resulting in the pathogenesis of different diseases and tumourigenesis (Surani 1993).

A physical map that includes H19 and IGF2 gene in mice has been established by pulsed-field gel electrophoresis and large-fragment DNA cloning.

IGF2 lies about 90 kb 5' to H19 with the same transcriptional orientation (Zemel et al., 1993). In humans, the physical proximity of the mapping of IGF2 and H19 are conserved. The distance between the two genes in humans is at most 200 kb, according to the results of pulsed-field gel electrophoresis of high molecular weight DNA from a human hepatoma cell line. The physical linkage of the H19 and IGF2 genes raise the possibility that they may constitute a functional imprinting domain, and may share transcriptional regulatory elements (Zemel, et al., 1993).

b. Chromosome 11p loci methylation in disease

Genomic imprinting appears to play a role in genetic disease and tumourigenesis in human. In this regard, the rather complex relationship between genomic imprinting and BWS and WT has been an area of intense research activity in recent years. Evidence for a loss of imprinting (LOI) in the pathogenesis of BWS and WT includes the observation of chromosome 11 aberrations such as uniparental disomy; 11p deletion or translocation; loss of heterozygosity (LOH); sex dependent transmission pattern; abnormal methylation patterns and changes in allelic expression of H19 and IGF2. A model of genomic imprinting is shown in Figure 1-4.

i. In Beckwith-Wiedemann syndrome

An incomplete sex-specific penetrance pattern has been shown in familial BWS which is an autosomal dominant trait was suggested (Koufos et al.,1989). In sporadic BWS, chromosome 11 uniparental disomies (Henry et al., 1991), trisomy 11p15 (Turleau et al.,1989, Lucas et al., 1985, Henry et al.,

1989) and isodisomy in 11p15 (Grundy et al., 1991, Henry et al., 1991) always involves duplication of the paternal (and not maternal) chromosome 11. This has led to the following model: normally the maternal alleles of the relevant 11p15.5 loci are inactivated, presumably by methylation based gene imprinting, leading to a functional hemizygosity for those genes. BWS is caused by the loss of this functional hemizygosity with the resulting increased 11p15.5 gene dosage giving rise to the observed phenotype.

The observation of increased methylation in H19 promoter of BWS patient with uniparental disomy suggests that H19 promoter undergoes parental -origin-specific methylation in human (Marjak 1994). The fetal growth factor IGF2 also shows imprinting with hypomethylation and inactivation of the maternal allele in humans (Schneid et al., 1993). Duplication of the paternal IGF2 allele and the resultant increased gene expression, has been associated with overgrowth in mice (Ferguson-Smith et al., 1991) and with tumourigenesis (Henry et al., 1989).

An imprinting mutation model in familial Prader-Willi syndrome (PWS) has been suggested (Figure 1-5). In somatic cells (S) at locus D15S63, the maternal allele is methylated and the paternal allele is unmethylated. The imprinting is observed in the germ line (G). Sperm cells carry only the unmethylated allele while the oocytes carry the methylated imprint. A *de novo* mutation is postulated to result in a failure to reset the methylation pattern and consequently disturb the expression of the imprinted gene (Reis A. et al., 1994).

Biallelic expression of IGF2 was observed in kidney, peripheral blood leukocytes and Wilms' tumour observed in one case of gigantism (Ogawa, 1993)

and in 4 out of 6 fibroblast strains and one tongue tissue in BWS cases (Weksberg et al., 1993). Loss of imprinting has been also observed in normal tissues of one-third of BWS (Marja et al., 1994). These findings strongly suggest that biallelic expression of IGF2 caused by a disruption of IGF2 imprinting and the loss of normal suppression of the maternal allele underlies at least some cases of BWS.

ii. In Wilms' tumour

Wilms' tumour is the most common intra-abdominal solid tumour of childhood, with a frequency of 1 in 10,000 children worldwide. Some of the congenital defects associated with WT are listed in Table 1-2. BWS and Aniridia have been linked to the 11p13-p15.5 loci which have been implicated in Wilms' tumourigenesis suggesting the disruption of the neighboring genes may affect to the Wilms' tumour-suppressor gene (WT1 and WT2) resulting in the increased tumour rate seen in these disorders.

The WT1 gene has been recently cloned from the 11p13 region and is comprised of 10 exons encoding a regulated transcriptional factor (Coppes 1994). Although deletions of WT1 are observed with some WT cases, many tumours do not have deletion or detectable abnormalities in 11p13. In this context, the BWS gene mapped to 11p15.5 has been shown to be associated with a high risk of WT. This suggests the existence of a second WT suppressor gene (WT2) in 11p15 region which may obviate a WT1 mutation. The evidence that Wilms' tumour locus have been mapped to 11p15.5 region in two Wilms' tumour families supported the existence of such a second WT gene (Koufos et al., 1989). Finally, a linkage study on additional Wilms' tumour families have excluded the

tumour-predisposing locus from both the 11p13 and the 11p15 region in these families suggesting the presence of a third Wilms' tumour locus (Grundy et al., 1988; and Francke 1990).

More than 20 years ago, it was proposed that some familial cancers might result from a somatic mutation resulting in a given cell becoming homozygous for the inactivated cancer causing mutation (De Mars 1970). Since then, a number of cancers have been reported to show a concurrent loss of alleles at different loci. For example, WT associated with LOH of 11p loci has been well established. One study of loss of heterozygosity (LOH) in WT demonstrated 11 of 44 tumours with LOH; 10 of these were limited to 11p; two of them observed in patients with BWS (Mannens et al., 1990) and in a second study, LOH in the 11p15.5 loci was observed in three WT cases associated with BWS (Koufos et al., 1989).

In addition to LOH, relaxation of imprinting alone has been observed. The maternal H19 allele which normally expressed in kidney was lost in Wilms' tumour (Zhang et al., 1993). A study of the role of H19 and IGF2 in genomic imprinting showed that approximate 2/3 of the WTs undergo biallelic expression of one or both genes (Rainier et al., 1993). A similar observation was made with BWS, where relaxation of imprinting of the maternal IGF2 allele was found in 69% of Wilms' tumours not undergoing LOH at 11p, representing a second mechanism for gain of function associated with increased gene dosage (Ogawa et al., 1993 and Rainier et al., 1993). In this regard, an increased level of IGF2 expression has been detected in Wilms' tumour (Scott et al., 1985; Reeve et al., 1985; Tricoli

et al., 1986).

Mechanisms possibly involved in these genetic changes include unequal mitotic recombination , deletion, unbalanced translocation and aneuploid loss with endoreduplication (Mannens, et al.,1988,). It is noteworthy that these mechanisms are observed in the significant majority of WT, irrespective of the presence of BWS. It thus appears that the condition which underlies some cases of BWS is a necessary step in the pathogenesis of many WT's.

An increased risk of embryonal tumours is an important feature shared in between BWS and SGBS and the importance of differentiating SGBS and BWS has been underlined (Hughes-Benzie et al., 1992a and 1992b). Clinical similarities between SGBS and BWS raise the question of whether SGBS and BWS share a common pathway which causes overgrowth and an increased risk for embryonal tumours. A hypothesis made by Bird et al., in 1993 was that BWS and SGBS may represent as an modified and modifier pair. In other words, if BWS is a result of resistance to the imprinting by 11p15.5 loci (e.g. IGF2), SGBS may represent failure of a gene which although mapping to the X chromosome is responsible for the imprinting of the same chromosome 11p15.5 loci. A hypothesis model for the relationship between SGBS locus and 11p loci is illustrated in Figure 1-6. Assessment and discussion of the role of 11p loci in SGBS pathogenesis is given in the Chapter 3 of this thesis.

Figure 1-4. A Model of imprinting.

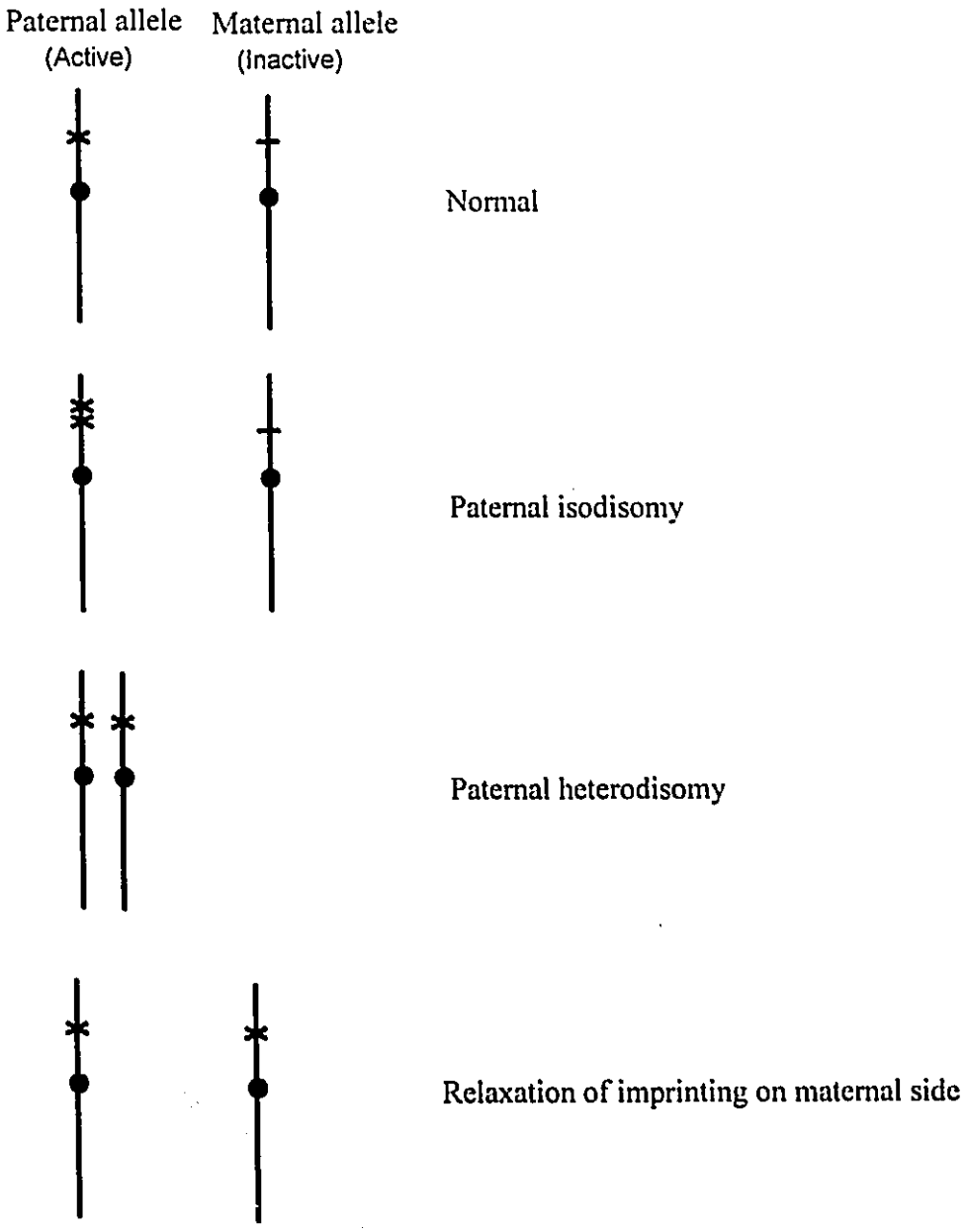

Paternal allele
(Active)
Maternal allele
(Inactive)
Normal
Paternal isodisomy
Paternal heterodisomy
Relaxation of imprinting on maternal side

Figure 1-5 Imprinting mutation model in familial Prader-Willi syndrome (PWS).

In somatic cells (S) at locus D15S63, the maternal allele is methylated and the paternal allele is unmethylated. The imprinting is observed in the germ line (G). Sperm cells carry only the unmethylated allele while the oocytes carry the methylated imprint. A *de novo* mutation results in a failure to reset the methylation pattern (adapted from Reis A. et al., 1994).

Familial PWS

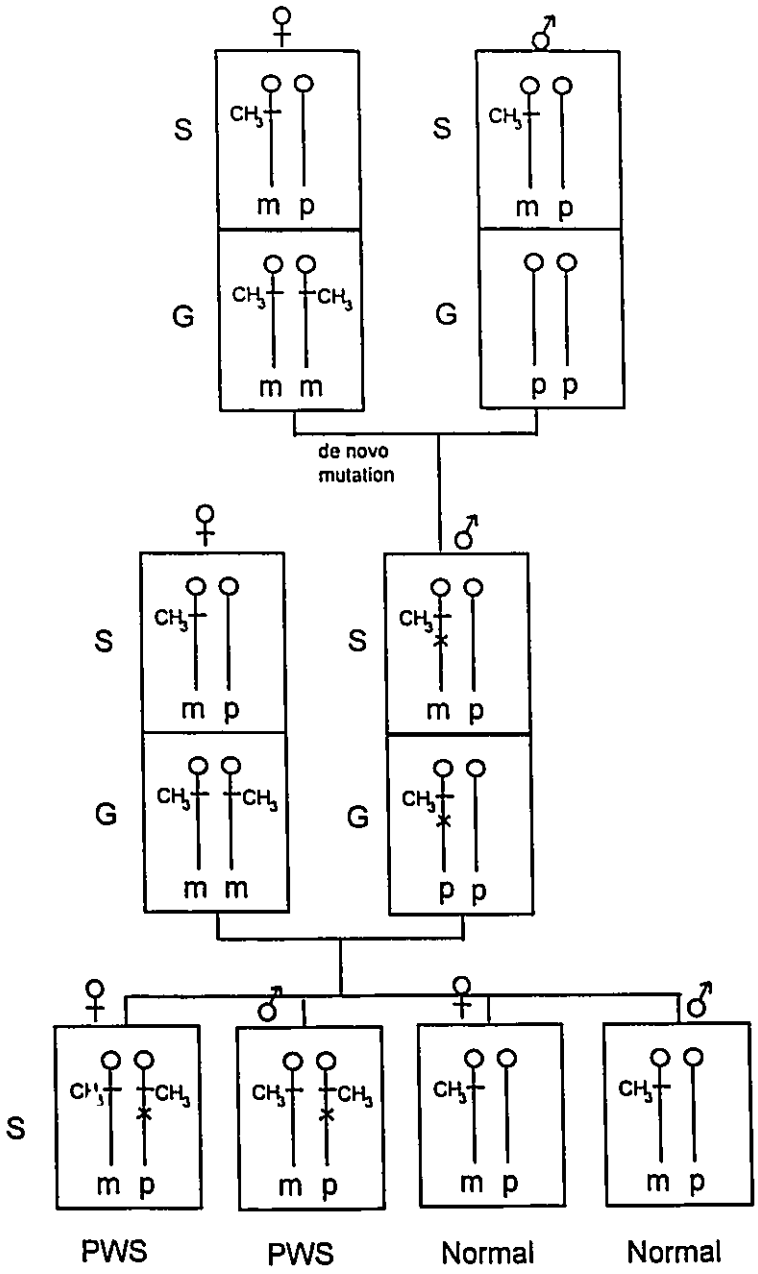


Figure 1-6. The hypothetical model of the relationship between SGBS locus and 11p loci.

This model represents the possibility that two overgrowth syndromes, BWS and SGBS, may share a common pathway prior to somatic overgrowth and tumorigenesis.

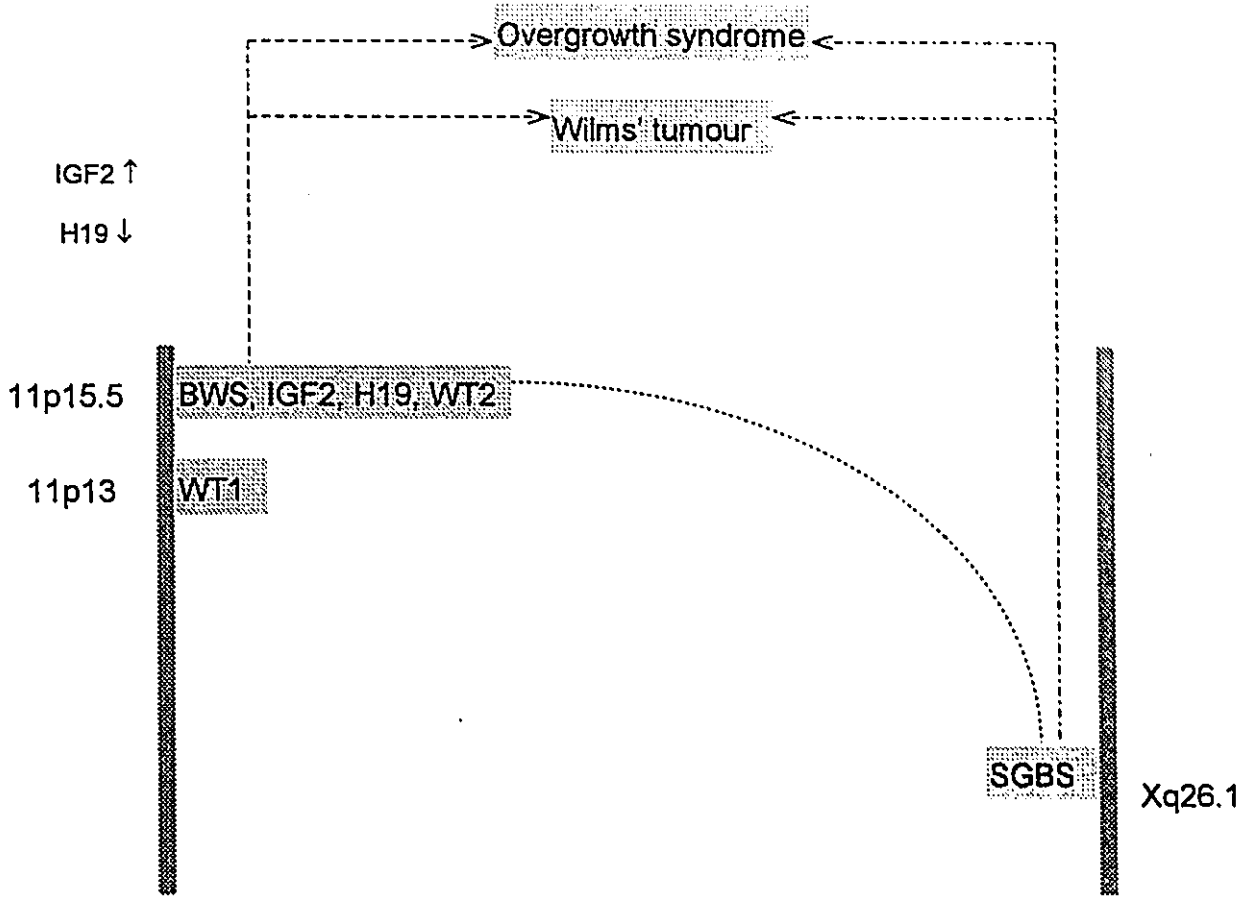

Overgrowth syndrome
Wilms' tumour
IGF2 ↑
H19 ↓
11p15.5
BWS, IGF2, H19, WT2
11p13
WT1
SGBS
Xq26.1

Table 1-3. Allelic expression of IGF2 in normal and overgrowth tissues and Wilms' tumour.

Diagnosis	Tissue(#)	Allele expression	Reference
Normal	tongue (1)	M	Weksberg, et al. (1993)
"	kidney (4)	M	Rainier (1993)
"	kidney (9)	M	Ogawa (1993b)
"	placenta (2)	M	Weksberg, et al. (1993)
"	placenta (6)	M	Giannoukakis et al., (1993)
"	liver	B	Davies (1994)
"	liver	B	Kalscheuer (1993)
"	fibroblasts (5)	M	Weksberg, et al. (1993)
Fetal	leukocytes	B	Giannoukakis (1994)
Postnatal	leukocytes (68)	B	Giannoukakis (1994)
"	leukocytes (9)	M	Giannoukakis (1994)
BWS	kidney (1)	B	Steenman (1994)
"	kidney	M	Ohlsson (1993)
"	placenta	M	Ohlsson (1993)
"	fibroblast (4)	B	Weksberg, et al. (1993)
"	tongue (1)	B	Weksberg, et al. (1993)
"	WT(1)	B	Steenman (1994)
Gigantism	leukocyte (1)	B	Ogawa (1993b)
"	kidney (1)	B	Ogawa (1993b)
"	WT (1)	B	Ogawa (1993b)
Wilms' tumour	WT (11)	B	Rainier (1993)
"	WT (5)	M	Rainier (1993)
"	WT (4)	B	Ogawa (1993a)
"	WT (2)	M	Ogawa (1993a)
"	WT(9)	B	Steenman (1994)

M--monoallelic expression, B--biallelic expression. The WTs are not LOH.

Table 1-4 Allelic expression of H19 gene in normal and overgrowth tissues and Wilms' tumour.

Diagnosis	Tissue	Allele expression	Reference
Normal fetal	kidney	M	Rainier (1993)
"	heart	M	"
"	lung	M	"
Normal fetal	liver	M	Zhang (1993)
"	brain	M	"
"	leptomeninges	M	"
"	placenta	B	"
"	spleen	M	"
"	heart	M	"
"	lung	M	"
"	kidney	M	"
"	adrenal	M	"
"	thymus	M	"
BWS	fibroblasts(8)	M	Weksberg (1994)
Wilms' tumour	WT(12)	M	Rainier (1993)
"	WT(2)	B	"

M--monoallelic expression, B--biallelic expression. The WTs are not LOH.

CHAPTER 2 MOLECULAR GENETIC LINKAGE STUDY OF SIMPSON-GOLABI-BEHMEL SYNDROME

1. Introduction

As outlined previously, SGBS is an X-linked condition associated with pre and postnatal overgrowth. Since the first family was described by Simpson et al., in 1975, approximately 30 families with similar clinical profiles have been recognized and the phenotypic variability of the syndrome has been delineated. In 1992, Hughes-Benzie et al., reported an association of renal dysplasia and two cases of embryonal tumour with SGBS in a Dutch-Canadian family. In the same report, preliminary data from our laboratory showed a maximal lod score of 2.81 with a recombination frequency of 0.00 for the SGB-DXYS68 pairing mapping the SGBS gene to Xqcen-q22 region (Hughes-Benzie et al., 1992a). To further refine the SGBS region, additional linkage analysis with more DNA samples from the Dutch-Canadian family and X chromosome markers were performed.

2. Results

The genotypings of the partial pedigree of the Dutch-Canadian and the British kindred are shown in Figures 2-1 and 2-2.

The tentative mapping of the SGBS gene to Xqcen-q22 in our lab

prompted a search for candidate genes mapping to this region. The functional pseudogene CR-3 which has been localized to Xq21.3-q22 encodes the growth hormone CRIPTO (Plowman et al., 1990) and was identified as a potential candidate gene. An XbaI CR3 RFLP was characterized (Besner et al.,1993) and genotyped in the SGBS kindred revealing 2 instances of non-cosegregation for CR3-SGBS ruling the CR3 gene out as the causative gene for SGBS and mapping it to the DXS345-DXS87 interval (Figure 2-3).

Further expansion of the Dutch-Canadian SGBS family revealed that an obligate SGBS carrier (individual III-8) showed recombination with markers in Xqcen-q22. Consequently, genotyping with a variety of additional Xq markers was undertaken. This revealed that the recombination event observed in individual IV-3 which had originally defined the distal boundary of the SGBS region to be DXS458 was, in fact, a double recombination resulting in a remapping of the locus distally on Xq. The more telomeric recombination of the double recombinants in this individual is depicted in Figure 2-1. Two point SGBS-Xq marker LOD scores are shown in Table 2-2; the highest LOD score, 7.45, is observed for the HPRT-SGBS pairing with no recombinants localizing SGBS to the Xq26 region. The SGBS region is now defined centromerically by DXS425 (individual IV-3) and telomerically by DXS1123 (individual V-6) in the Dutch-Canadian family (Figure 2-4). This mapping somewhat surprisingly appears to exclude a 3 1/2 year old female (individual V-1) with Wilms tumour (WT) as an SGBS carrier. The only other feature in this girl compatible with SGBS was the presence of skeletal abnormalities in the form of hemivertebrae. As a result of this potential inconsistency in mapping, the remainder of the X

chromosome was assayed for SGBS linkage, and none was found (Table 2-2).

3. Discussion

The SGBS locus has been mapped to the DXS425-DXS1123 interval on Xq25-q27 by virtue of a proximal recombinant event between DXS425 and (SGBS - DXS1114) (individual IV-3) and a distal recombination between (SGBS -HPRT) and DXS1123 (individual V-6).

The only potential inconsistency generated by this mapping is the exclusion of individual V-1 as an SGBS carrier. This 3 1/2 year old girl was diagnosed with a right-sided Wilms tumour at 22 months of age. Pathological examination at nephrectomy identified nephrogenic rests. She had multiple thoracic hemivertebrae and a Sprengel's deformity of her right shoulder but no other features of SGBS. Vertebral anomalies have been previously reported in males with SGBS by Opitz et al., and Neri et al., in 1984. Genotyping of individual V-1 appears to map the SGBS locus proximal to DXS425. However, the recombination observed in individual IV-3 who is unequivocally affected with SGBS (demonstrating both overgrowth and characteristic coarsened facial features, see Figure 3 in Hughes-Benzie,R.M. 1992b) maps the disease locus distal to DXS425 outside the SGBS X chromosome region inherited by individual V-1. Despite the clinical findings of V-1 which are compatible with SGBS, she is of normal height and without coarsened facial features both which are characteristic of affected and carrier individuals in this SGBS family. Consequently, we believe it is possible that despite some suggestive findings,

individual V-1 is not an SGBS carrier. Her mother is an obligate carrier; a possible explanation for the clinical anomalies found in this female is that the SGBS gene product exerts a intrauterine effect either directly or by regulation of other maternal genes, the products of which may be transferred across the placenta to affect the fetus. In this regard, the fact that the other 2 individuals with WT in this kindred were offspring of SGBS mothers and not SGBS fathers may acquire new significance. Recently, Schneid et al. (1993) reported an ostensibly unaffected sibling of a Beckwith-Wiedemann Syndrome (BWS) individual who developed a neuroblastoma.

There also exists the possibility that individual V-1 has undergone a double recombination and has actually inherited the mutated SGBS allele. A relatively long region of homozygosity between DXS1114 and DXS52 is seen in Xq26-28 in the mother of individual V-1 (individual IV-2, figure 2-1, DXS52 data not shown). Consequently, a double recombination could have occurred in this approximately 30 cM (Dr. P. Fain, personal communication) interval and not have been detected. Informative markers within this region are currently being sought in order to exclude such a double recombination.

In order to rule out an atypical somatic mosaicism possibly underlying this result, DXS425 genotyping of individual V-1's renal tumour was conducted and compared with that of her leukocyte DNA; no inconsistencies were detected.

The clinical status of individual V-1 does not alter our mapping of the SGBS locus to the Xq25-q27 region; the HPRT-SGBS LOD score is unchanged and scores of over 4 for DXS425 and DXS1001 are attained regardless of her

affection status.

In addition to individual V-1, 2 affected members of the Dutch-Canadian kindred developed WT. The observed rate of 2/27 or 7.5% SGBS positive individuals developing WT is similar to that cited for Beckwith-Wiedemann syndrome (BWS) of 7-7.5 % (Wiedemann 1983, Jones 1994) and somewhat greater than Sotos syndrome of 2% (Hersh et al., 1992).

The only other classic overgrowth syndrome which has been genetically mapped is BWS; two studies have shown linkage of this syndrome to 11p15 region markers with a combined LOD score of approximately to 7 (Koufos et al., 1989 and Ping et al., 1989). The deletions of the 3p21 region which have been described in all major types of lung cancer (Kok et al., 1987) have been suggested to play a role in the etiology of a Sotos overgrowth syndrome case with small cell lung carcinoma (Cole et al., 1992). In addition, a child with Sotos syndrome and a balanced reciprocal *de novo* 3p /6p translocation has also led to the suggestion that this syndrome may be related to a chromosomal deletion at 3p21 or 6p21 (Schrander-Stumpel et al., 1990). However, no clear evidence of linkage for Sotos syndrome has been reported to date.

The relative rarity of SGBS makes the eventual cloning of the causative gene by positional cloning alone relatively unlikely. Therefore an individual with an Xq26 translocation and SGBS would clearly be an important resource. A recently reported translocation in the Xp11 region in a child with BWS (Diaz de Bustamante et al., 1990) is unlikely to be related to SGBS as large negative LOD scores were obtained for SGBS linkage in this region (Table 2-2). In contrast, a

1974 report of a female who was originally diagnosed as BWS with a balanced reciprocal (X;1) (q26;q12) translocation has recently been rediagnosed as SGBS (Punnett et al., 1974 and Punnett 1994). Moreover, linkage analysis performed in two European families have showed inkage with LOD score of 4.45 for SGBS with Xq26 markers.

The complete linkage between the disease locus and HPRT combined with the presence of a cytogenetic breakpoint in this region in an affected female strongly suggests that the gene which underlies this syndrome is located in Xq26 region. The identification of the translocation breakpoint gene may therefore shed light into the pathogenesis of this disorder. Given that this syndrome is associated with an increased risk of embryonal renal tumours, its eventual cloning may also help in the elucidation of the molecular pathogenesis of these malignancies.

4. Methodology

a. Family members:

The study was conducted upon a five generation Canadian-Dutch kindred comprised of 6 affected males and 12 carrier females (Figure 1-1 and 2-1) and a British kindred comprised of 5 affected males and 4 carrier females (Figure 1-3 and 2-2). A total of 48 DNA samples were analyzed. The clinical diagnosis of the Canadian-Dutch family has been previously reported (Hughes-Benzie, R.M. et al. 1992b). Family members were said to be affected if they were obligate

carriers or had at least two of the following diagnostic criteria:

- Overgrowth,
- Coarse facies,
- Tongue groove/tie,
- Renal dysplasia,
- Extra nipple,
- Skeletal abnormalities.

The sole exception to this diagnostic scheme is individual V-1 in the Dutch-Canadian family (see results and discussion in this chapter).

b. DNA studies:

For RFLP analysis, genomic DNA was extracted from peripheral white blood cells, digested with the appropriate enzymes, fractionated on 0.8 % agarose gel, and then transferred by Southern blotting to Hybond N (Amersham). DNA probes (Table 2-1) were labeled with ^{32}P using primer extension, and hybridized to the blots at 65^{o}C for ~16 hours. The blots were then washed with 2 X SSC, 0.1% SDS. The stringency of washing was increased as required. Specific amplification of the 5'portion of CR3 was achieved by using primers CR3-1 (**5' AAG CTT GCG CGC CAT GTA AGG 3'**), and CR3-2 (**5' AGA CCC ACA GTT CTC TTT GCG 3'**). PCR condition: a total of 25µl reaction containing 100ng genomic DNA, 5 pMoles of each primer, 10X PCR Buffer, 200 µM dNTPs, 1 unit of Taq polymerase. 30 cycles: 94^{o} C (90s), 50^{o} C (90s), 72^{o} C (90s). The 1.2kb PCR product was used to probe to Xba I blots generating two

alleles A_1=8.5kb (freq.= 0.38) and A_2=6.4kb (freq.= 0.62) (data not shown).

For microsatellite analysis, PCR reactions were performed in a total volume of 25 µl using 30 different primer pairs (Table 2-1). The coding strand primer was end-labeled for 1hr at 37°C in a 23.3 µl reaction containing 10µl [γ ^{32}P] ATP, 10µl primer (100ng), 1µl T_4 polynucleotide kinase, 2.3µl T_4 kinase buffer. 0.5µl of the labeled primer was added to a total volume of a 25µl PCR reaction containing 1µl of genomic DNA template, 2.5µl each oligonucleotide primer (20 ng/µl), 4µl dNTP mix, 0.2µl Taq polymerase (8 u/µl), 2.5µl 10xPCR buffer (Pharmacia). PCRs were performed as previously described (Table 2-1).

For linkage analysis both X-linked dominant inheritance and 100% penetrance were assumed. Two point LOD scores were calculated using LINKAGE 5.1 computer program (Lathrop et al., 1985).

Figure 2-1. Genotyping of chromosome Xq loci in a partial pedigree of SGBS the Dutch-Canadian family.

The haplotypes depicted are the most likely alignments based on phasing of genotype data. Arrows indicate key recombinant chromosomes in the kindred. The recombination event between DXS425-DXS1114 in individual IV-3 defines the proximal boundary of the SGBS region. The recombination between HPRT-DXS1123 in individual V-6 defines the distal boundary of the SGBS region. T - individuals with renal embryonal tumours.

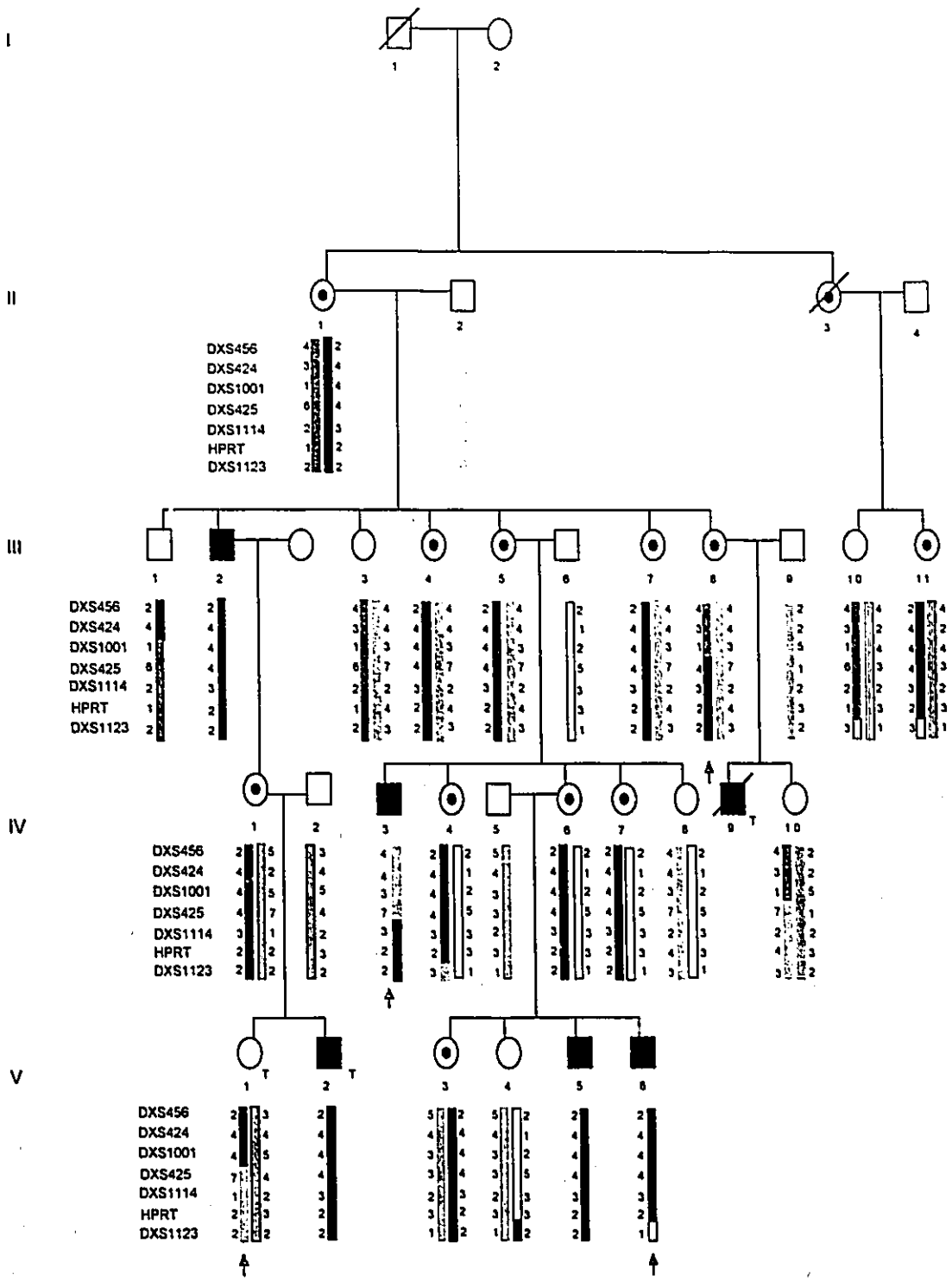

I
II
III
IV
V
DXS456
DXS424
DXS1001
DXS425
DXS1114
HPRT
DXS1123

Figure 2-2. Genotyping of chromosome Xq loci in the British SGBS family.

The data demonstrate cosegregation of SGBS with Xq24-q26 markers.

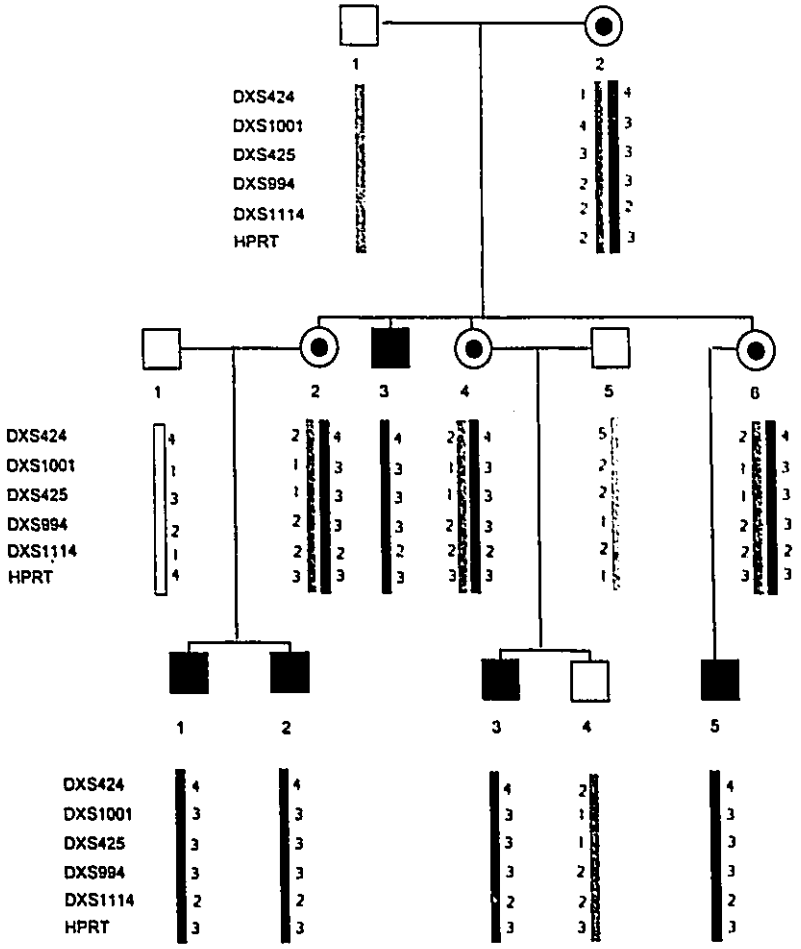

1
2
DXS424
DXS1001
DXS425
DXS994
DXS1114
HPRT
1
2
3
4
5
6
DXS424
DXS1001
DXS425
DXS994
DXS1114
HPRT
1
2
3
4
5
DXS424
DXS1001
DXS425
DXS994
DXS1114
HPRT

Figure 2-3. Genotyping of CRIPTO gene (CR-3) in the Dutch-Canadian SGBS family.

Genotyping of CR-3 in the partial pedigree of the Dutch-Canadian SGBS family demonstrates the absence of SGBS-CR-3 cosegregation. The CR-3 interval is defined centromerically by DXS345 (individual IV-7) and telomerically by DXS87 (individual IV-12).

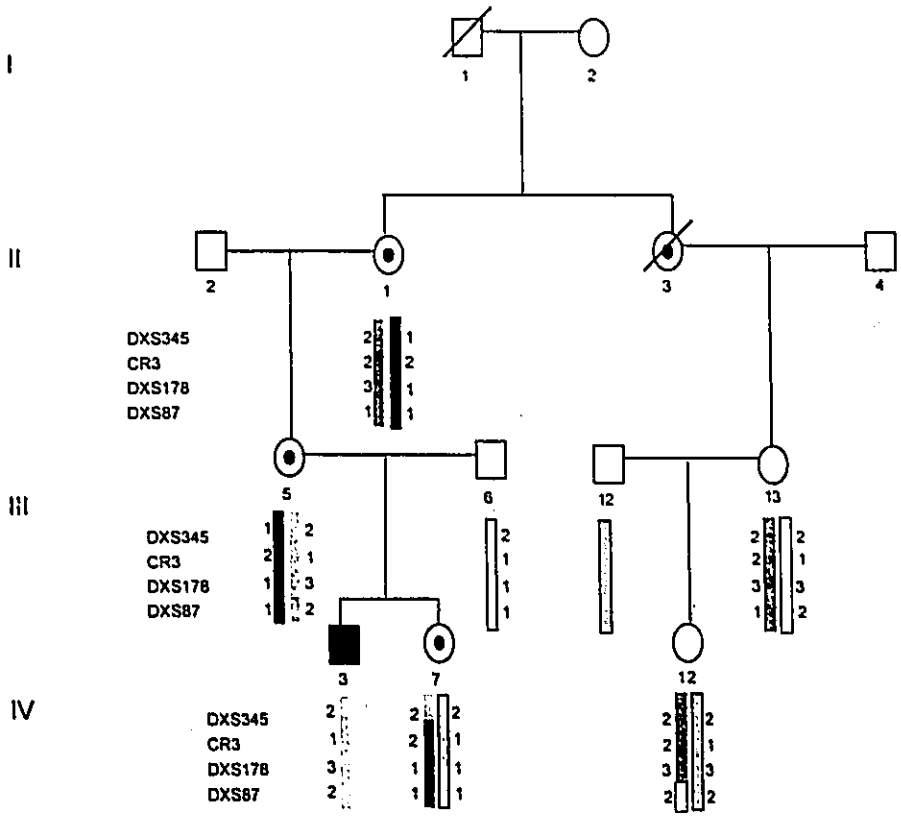

I
II
III
IV
1
2
2
1
3
4
DXS345
CR3
DXS178
DXS87
5
6
12
13
3
7
12

Figure 2-4. Map of SGBS locus and X chromosome.

The SGBS locus has been mapped to the DXS425-DXS1123 interval on Xq25-q27. The highest two point LOD scores of 7.45 are observed for the HPRT-SGBS pairing with no recombinants localizing SGBS to the Xq26 region.

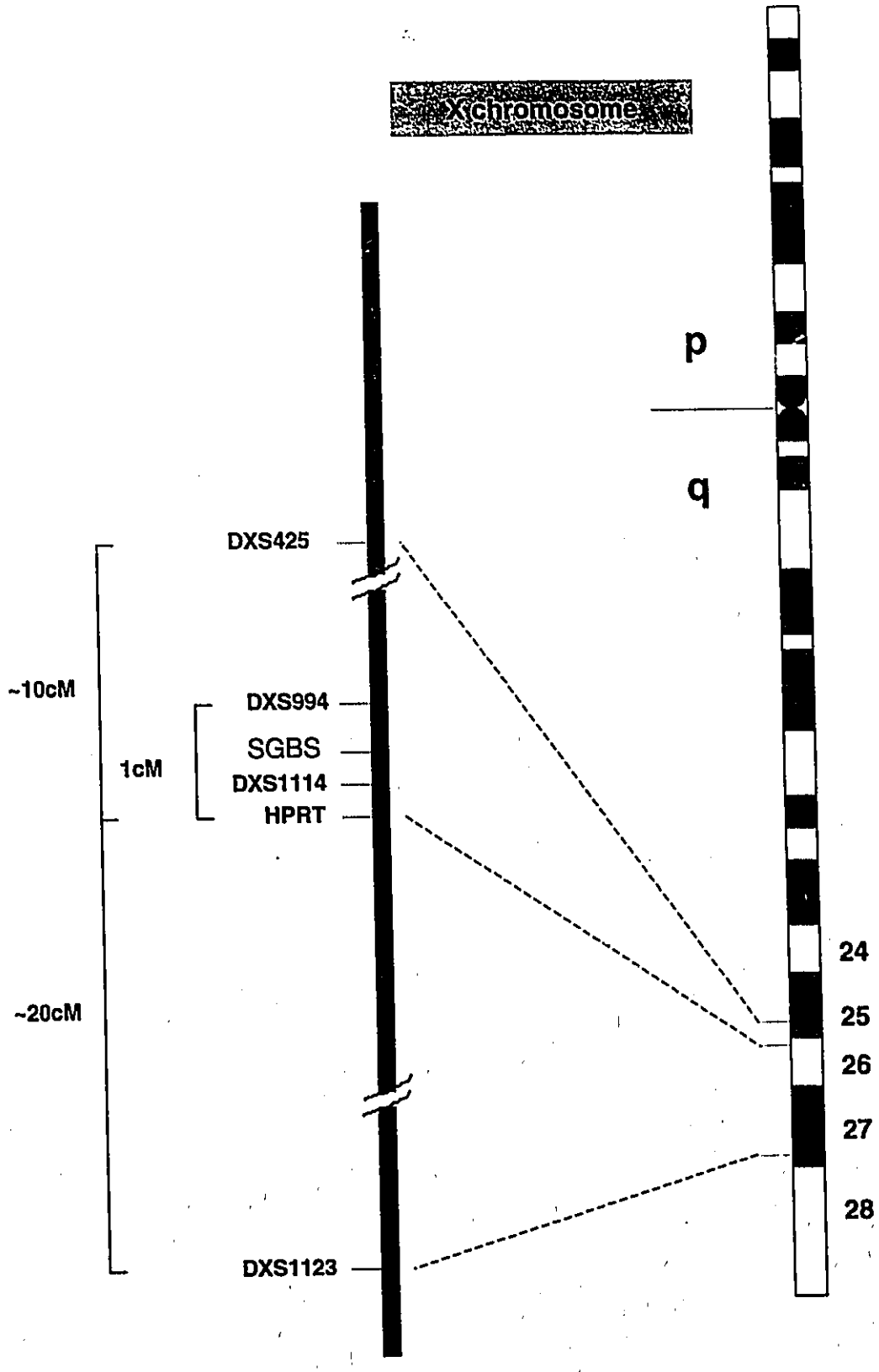

X chromosome
p
q
DXS425
~10cM
DXS994
SGBS
1cM
DXS1114
HPRT
~20cM
DXS1123
24
25
26
27
28
38

Figure 2-5. The genotyping of HPRT locus in the Dutch-Canadian SGBS family.

The PCR was conducted using HPRT primer. Four alleles were observed. The SGBS carriers and affected males inherit allele 2 showing complete linkage for this locus. Arrow indicates the allele 2.

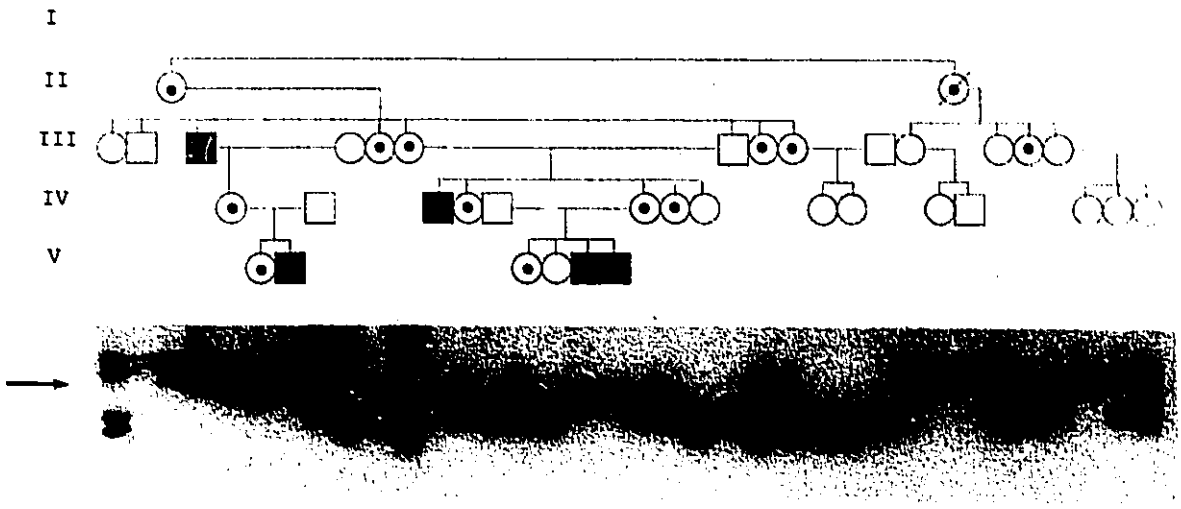

I
II
III
IV
V

Table 2-1. DNA Markers Used for Linkage Analysis.

Marker	Locus	Location	Enzyme	Reference
DXS237 PCR1.1/1.2*	DXS237	Xp22.32	.....	"GDB"@
3'CA F/R*	DMD3'	Xp21.2	.....	"GDB"
DXS228 PCR1.1/1.2*	DXS228	Xp11.4	.....	"GDB"
AR PCR1.1/1.2*	AR	Xq12	.....	"GDB"
MFD66*	DXS453	Xq11.23-q21.1	.....	Weber et al., [1990]
DXS441.PCR 2*	DXS441	Xq13.2-q13.3	.....	"GDB"
404E2	DXS447	Xq13-q21.1	Bgl II	Barker [1991]
50M2MA	DXYS68	Xq21.3	Rsa I	Barker et al., [1989]
DXYS1X1/4-1*	DXYS1X	Xq21.3	.....	Browne et al., [1991], "GDB"
AFM136yc7*	DXS990	Xq21.	.....	Weissenbach et al.,[1992], "GDB"
AFM116xg1*	DXS986	Xq21.	.....	Weissenbach et al.,[1992], "GDB"
DXS3-1*	DXS3	Xq21.3	.....	Stanier et al., [1991], "GDB"
MFD79*	DXS458	Xq21.1-q23	.....	Weber et al., [1990]
MFD72*	DXS454	Xq21.1-q23	.....	Weber et al., [1990]
329H2	DXS366	Xq21.1-q24	Taq I	Barker [1989]
72RI	DXS345	Xq21.2-q22.1	Bgl II	Barker et al., [1989]
CR1-2	CRIPTO-3	Xq21-q22	Xba I	"GDB"
E03167CA*	DXS178	Xq22	.....	Weers et al., [1992], "GDB"

Table 2-1. DNA Markers Used for Linkage Analysis (conti.).

Marker	Locus	Location	Enzyme	Reference
DXS87PCR1	DXS87	Xq21.33-q22	Bgl II	"GDB"
XG30B*	DXS456	Xq21-q22	.....	Luty [1990]
849/850*	DXS424	Xq24-26	.....	"GDB"
AFM248we5a/5m	DXS1001	X	.....	"GDB"
860/862A	DXS425	Xq25-26	.....	"GDB"
AFM205wd2a/2m*	DXS994	Xq26	.....	"GDB"
HPRT PCR2.1/2.2*	HPRT	Xq26.1	.....	"GDB"
ACAG/TGTC*	DXS1114	Xq26.1	.....	"GDB"
DXS102 PCR1.1/1.2*	DXS102	Xq26	.....	"GDB"
AFM105xc5a/5m*	DXS984	Xq27	.....	"GDB"
41ADF/41ACR*	DXS1123	Xq27	.....	"GDB"
St14	DXS52	Xq28	VNTR	"GDB"

* Microsatellites. @ "GDB"- Genome Data Base.

Table 2-2. LOD scores at different recombinant fractions (θ) between X chromosome markers and SGBS.

		X CHROMOSOME MARKERS											
	LOCI	DXS237	DMD3'	DXS228	AR	DXS424	DXS1001	DXS425	DXS994	DXS1114	HPRT	DXS102	DXS1123
	MAP	Xp22.32	Xp21.2	Xp11.4	XQ12	Xq24-26	Xq	Xq25-26	Xq26	Xq26.1	Xq26.1	Xq26	Xq27
	Z max	0.08	0.04	1.87	0.60	4.09	5.13	6.73	3.37	4.18	**7.45**	2.00	0.50
	Θ max	0.50	0.44	0.23	0.30	0.10	0.09	0.03	0.09	0.05	0.00	0.00	0.31
	0.001	-14.21	-20.72	-11.91	-0.96	-0.66	0.53	5.63	0.33	2.93	7.43	0.19	-10.16
	0.01	-8.52	-11.48	-4.78	-4.81	2.34	3.52	6.54	2.30	3.88	7.31	0.19	-5.03
	0.02	-6.79	-8.97	-2.99	-3.45	3.06	4.22	6.70	2.77	4.07	7.19	0.18	-3.67
	0.03	-5.64	-7.59	-1.83	-2.66	3.44	4.59	6.73	3.01	4.15	7.06	0.17	-2.87
	0.04	-4.83	-6.43	-1.14	-2.11	3.68	4.81	6.72	3.16	4.18	6.94	0.17	-2.32
	0.05	-4.22	-5.69	-0.52	-1.69	3.84	4.95	6.68	3.25	4.16	6.82	0.16	-1.90
Θ	0.06	-3.71	-5.02	-0.13	-1.35	3.95	5.04	6.62	3.31	4.16	6.69	0.16	-1.56
	0.07	-3.36	-4.44	0.27	-1.08	4.02	5.09	6.55	3.35	4.13	6.56	0.15	-1.28
	0.08	-3.05	-4.06	0.55	-0.85	4.06	5.12	6.47	3.36	4.09	6.43	0.14	-1.04
	0.09	-2.74	-3.61	0.75	-0.65	4.08	5.12	6.38	3.36	4.05	6.30	0.14	-0.84
	0.10	-2.43	-3.35	0.92	-0.48	4.09	5.11	6.30	3.36	4.00	6.18	0.13	-0.69
	0.20	-0.98	-1.23	1.81	0.40	3.60	4.42	5.10	2.89	3.27	4.79	0.08	0.26
	0.30	-0.34	-0.35	1.72	0.60	2.59	3.17	3.61	2.04	2.29	3.25	0.04	0.49
	0.40	-0.07	0.05	1.04	0.44	1.27	1.57	1.83	0.98	1.12	1.54	0.01	0.39

CHAPTER 3 ASSESSMENT OF CHROMOSOME 11P LOCI IN SIMPSON-GOLABI-BEHMEL SYNDROME (SGBS).

1. Introduction

As previously outlined, Simpson-Golabi-Behmel syndrome (SGBS) is an X -linked condition characterized by pre- and postnatal overgrowth, coarse facial features and an increased risk for embryonal tumours (Hughes-Benzie et al., 1992a and 1992b). We and others have mapped the SGBS gene to the Xq26 region with complete linkage to HPRT (Zmax = 7.5, θmax = 0.00) (Xuan et al., 1994, and Orth et al., 1994).

SGBS shares many clinical features with the more common Beckwith-Wiedemann syndrome (BWS) including macrosomia, macroglossia, cleft palate, visceromegaly, umbilical hernia, neonatal hypoglycemia, and an increased risk of embryonal tumors (Hughes-Benzie et al., 1992a, and Hughes-Benzie et al., 1992b). Linkage analysis on BWS families has localized the causative gene for a subgroup of BWS to 11p15.5 (Ping et al., 1989 and Koufos et al., 1989), a region containing the IGF2 and H19 genes. As well, derangement in the normal parental imprinting patterns of IGF2 has been observed in at least some BWS cases suggesting a possible underlying pathogenic mechanism involving upregulation of this autocrine growth factor (Weksberg, et al., 1993, Rainier, et al., Reik, et al., and Hedborg, et al., 1994).

Given the significant clinical overlap between SGBS and BWS, and the

apparent resistance of IGF2 to imprinting in some cases of BWS, the possibility that SGBS represents a mutation in a gene normally involved in the *trans* imprinting of 11p loci has been suggested (Bird, LM 1993).

To elucidate the role of chromosome 11p loci in the pathogenesis of SGBS, the methylation pattern and allelic expression of the IGF2 and H19 genes in SGBS patients was studied. The possibility of inheritance of a common chromosome 11p in the three individuals with WT in the Dutch-Canadian SGBS family was also explored none was seen. Finally, loss of heterozygosity in the 11p13-p15.5 loci was assayed in WTs.

2. Results

a. Allele specific methylation of H19 and IGF2 in control and SGBS tissues

i. Methylation analysis of H19 in normal and SGBS tissues.

Southern blot analysis was used to investigate the H19 methylation pattern in SGBS. Southern blots containing DNA from SGBS and control individuals digested with Rsa I or Rsa I / Hpa II were hybridized with the genomic probe generated by the primers P1 and P2 spanning H19 exons 3, 4 and 5 (Zhang Y. and Tycko B. 1992). DNA from SGBS heart, foreskin, fibroblasts, and Wilms' tumour samples (WT_1 and WT_2), all heterozygous at the Rsa I site, showed monoallelic H19 methylation upon Rsa I/ Hpa II double digestion (Figure 3-1 and Table 3-1). A general reduction in methylation of the H19 locus was

observed for the remaining tissues which were all homozygous for the RsaI polymorphism (Figure 3-1 and Table 3-1).

ii. Methylation analysis of IGF2 in normal and SGBS tissues.

The methylation patterns of IGF2 in leukocyte genomic DNA from normal and SGBS individuals were investigated by hybridizing Southern blots of Ava II or Ava II/Hpa II digested DNA with a 1.4kb probe generated by IGF2 primers P1 and P3 (Ogawa O. et al., 1993) . Ten samples (three normal individuals, four carriers, and three severely affected males) from our Dutch-Canadian SGBS family were tested. Five individuals were heterozygous for the Ava II polymophism demonstrating 1.1 and 0.9 kb bands with the remainder being homozygous for the 1.1 kb band. No methylation abnormality was consistently detected in SGBS WBC with complete demethylation of one allele and partial demethylation of the second allele observed in SGBS and control individuals alike (Figure 3-2).

Similar analysis of DNA from SGBS cleft lip, fibroblast and foreskin tissues demonstrated monoallelic methylation while either partial methylation of both alleles (SGBS placenta and control lip and placenta) or a general reduction of methylation in IGF2 AvaII homozygotes (control foreskin and fibroblast) was observed (Figure 3-3 and Table 3-1).

b. H19 and IGF2 transcriptional activities in control and SGBS

PCR amplification of the H19 region spanning exons 3-5 which contains a polymorphic RsaI site was used to study the expression of H19 alleles. RNA's from normal placenta, SGBS placenta, and SGBS fibroblast FB_1 which

were all heterozygous for the Rsa I polymorphism, were employed. The 829 bp genomic DNA fragment generated as outlined in the methodology is cleaved into 693 bp and 136 bp bands by RsaI digestion and the 539 bp H19 cDNA fragment is cleaved into 403 bp and 136 bp fragments. Rsa I digestion of RT-PCR products from SGBS and control placenta generated two alleles at 539 bp and 403 bp reflecting biallelic expression. In contrast, the Rsa I digestion of RT-PCR products from SGBS fibroblast FB_2 gave only a single band at 593bp suggesting monoallelic expression of the H19 gene (Figure 3-4 and Table 3-1).

An Apa I polymorphism in exon 9 was employed to investigate the allelic expression of the IGF2 gene. Six tissue samples including one control placenta, and skin fibroblast, one SGBS placenta, and three SGBS skin fibroblasts (FB_1, FB_2 and FB_3), all heterozygous for the Apa I site were studied. RNA samples were subjected to PCR to test for DNA contamination, only those not generating PCR products were used for reverse transcription. The 236bp PCR product of genomic DNA was cleaved by Apa I digestion into 63bp and 173bp fragments. Apa I digestion of RT-PCR products showed only the lower band (173bp) for the normal placenta and normal fibroblast, only the upper band (236bp) for SGBS fibroblast (FB_2), but both lower and upper band for the SGBS placenta and the SGBS fibroblasts FB_1 and FB_3 (Figure 3-5 and Table 3-1). The cDNA samples were digested three times with Apa I to ensure that the observed pattern was not a result of partial digestion. Thus the IGF2 gene is monoallelically expressed in normal placental tissues, and normal fibroblast as has been previously shown (Weksberg R. et al., 1993), but biallelically expressed in SGBS placenta, and two of three SGBS skin fibroblast tissues.

c. Linkage analysis of chromosome 11p11-p15.5 loci in WTs

Given the role of 11p loci in the genesis of WT, the possibility that three WT individuals from the Dutch-Canadian SGBS kindred inherited the same 11p chromosome which contained WT predisposing mutations was tested. Genotyping with four microsatellite repeats (D11S554, D11S956, D11S921, and D11S922) mapping to 11p11-15.5 was conducted to assay for such cosegregation (Table 3-2). Siblings 010 and 011 inherited the same D11S922 allele from their SGBS mother and the same paternal chromosome 11 was also inherited. However, as can be seen, inheritance of the same chromosome 11 pattern in the three children was not observed (Figure 3-8).

d. Chromosome 11p loci LOH and methylation analysis in WTs

A loss of imprinting by means of a loss of heterozygosity has been previously documented in a significant proportion of WT. To assay for the presence of LOH in the SGBS associated WT, four 11p13-15.5 region microsatellite repeats (D11S922, D11S569, D11S921 and D11S935) and the RLFP of IGF2 locus were genotyped (Figure 3-9 and Table 3-3). In 11p15.5, loss of maternal alleles in locus D11S922 was demonstrated in both WT_1 and WT_2; in 11p13, a loss of the maternal allele in locus D11S935 was detected only in WT_2 since WT_1 was homozygous (Table 3-3). A loss of maternal allele was also demonstrated for WT_2 using the markers D11S569 and D11S921 (data not shown).

In contrast, Southern blot analysis of IGF2 showed that WT1 DNA was homozygous for the Ava II site with a 1.1kb band observed and that the

parental alleles were digested completely by Hpa II (data not shown). The demethylation pattern suggested that either loss of the maternal copy of IGF2 or demethylation in the maternal copy of the IGF2 in the WT_1 tissue. Furthermore, the analysis of H19 showed that WT_1 and WT_2 retain both parental alleles (Figure 3-1). Consequently, LOH were not observed in H19 locus. These results are consistent with a role for LOH in the11p15.5 region in WT tumourigenesis in this family.

3. Discussion

Since its original description in 1963, a number of observations have implicated a derangement of genetic imprinting in the genesis of BWS. These include a predominantly maternal transmission of the disorder in familial cases, an over-representation of paternal chromosomes 11 in 'gain of function' cytogenetic abnormalities (e.g. uniparental chromosome 11 disomy and 11p duplication) and virtually exclusive maternal inheritance of putative 'loss of function' chromosomal 11 abnormalities(e.g. translocations and inversions) (Viljoen D. and Ramesar R. 1992, and Mannens M. et al., 1994).

In recent years, a role for genetic imprinting has been borne out at the molecular level by the documentation, of the biallelic transcriptional activity of IGF2 in some cases of BWS, in contrast to normal monoallelic IGF2 expression (Weksberg R. et al., 1993). The attendant upregulation of the autocrine growth factor is proposed to result in or contribute to the BWS phenotype including the

increased risk of malignancy (Hedborg F. et al., 1994, Christofori G. et al., 1994).

While no aberrant H19 expression was observed in SGBS fibroblast, we have shown that a loss of IGF2 imprinting is also a feature of the BWS-like SGBS. It thus appears that mutational inactivation of the SGBS gene may lead directly or indirectly, to a loss of imprinting in IGF2 resulting in or contributing to somatic overgrowth and an increased risk of embryonal tumorigenesis.

In keeping with this model is the recent description of the *Tme* mouse as an IGF2 receptor deletion mutant resulting in a de facto overexpression of IGF2. This mouse shows significant parallels with SGBS including somatic overgrowth, skeletal and cardiovascular abnormalities, and facial dysmorphism (Wang Z.Q. et al., 1994). As well, the oncogenic potential recently demonstrated for IGF2 in transgenic mice (Christofori et al., 1994) fits well with the increased risk of malignancy in SGBS.

The basis of differential IGF2 allelic activity which has been proposed resides in a site specific methylation region mapping to exon 9 of the gene (Schnied H., et al., 1993). Methylation of the IGF2 exon 9 region of the paternal allele imparts, in most tissues, transcriptional activity and absence of methylation in the maternal allele is associated with transcriptional quiescence. Consequently, should the methylation be the main agent for genetic imprinting as is as suggested by knockout mouse data (Li E. et al., 1993) then, the predicted abnormality in SGBS would be illegitimate methylation and consequent activation of normally unmethylated inactive maternal IGF2 allele. However, despite the loss of IGF2 imprinting on transcriptional assay, no consistent difference in IGF2

methylation between SGBS and control tissues was detected. This loss of the normally observed relationship between methylation and IGF2 imprinting is in keeping with a previous study on BWS which, although demonstrating biallelic transcriptional activity, has shown abnormalities of methylation only in one case and not the expected methylation of the maternal allele but, instead, hypomethylation of the paternal IGF2 (Weksberg et al., 1993).

In conclusion, our data suggests that the Xq26 SGBS locus plays an important role in the genetic imprinting of at least one autosomal locus. Since a chromosomal translocation causing SGBS has been identified (Xuan et al., 1994, and Punnette HH 1994), we believe that it is an inactivation of the SGBS gene product which underlies the loss of imprinting. Somatically acquired mutations inactivating the SGBS locus may underlie some of the spontaneous IGF2 imprinting relaxation observed in isolated WT. The comparatively frequent loss of the X chromosome observed in the karyotyping of WT tissue is consistent with this model (Mitelman F. 1991). Recently, the translocation breakpoint from an SGBS associated der (X;1) (q26;q12) chromosome has been cloned in an effort to isolate the SGBS gene (Pilia G. et al., 1994). The identification and characterization of the causative gene will permit the assessment of its role both in the spontaneous loss of imprinting in sporadic WT as well as embryonal tumourigenesis in general.

4. Methodology

a. Samples.

The clinical and genetic details of the Dutch-Canadian SGBS kindred which was the source of Wilms' tumour tissues (WT_1 and WT_2), SGBS foreskin, heart, cleft lip, and the fibroblast cell line 1 (FB_1), have been described previously (Hughes-Benzie et al., 1992b, and Xuan et al., 1994). The SGBS fibroblast cell line 2 and 3 (FB_2 and FB_3) and SGBS placenta used in the allelic methylation and expression analysis were from individuals diagnosed as SGBS following the clinical criteria given in Hughes-Benzie et al., (1992). The translocation *t* (X;1) (q26;q12) fibroblast cell line (Fbt) from an SGBS female (Punnett H.H 1994) was purchased from Coriell Cell Repositories, New Jersey. SGBS and control tissue samples were collected during surgical procedures and frozen at -80°C. Total DNA was extracted from tissues by the proteinase K method and total RNA by the method of Chomczynski et al., (1987).

b. Probes.

The IGF2 probe generated by PCR using primers P1 and P3 as described (Zhang Y and Tycko B. 1992). The H19 probe was generated using primers p1 and p2 as described (Ogawa O. et al., 1993). PCR products were cloned into pCR vector (Invitrogen, San Diego, CA) and transfected into *E. Coli.* The DNA probes were extracted from the cells by the alkaline rapid preparation method and labeled with α [^{32}P] dCTP.

c. Southern blots analysis.

To generate optimal Southern blots the following method (Carmen Sapienza, personal communication) was used; 10µg of DNA were digested with 50 units of restriction enzyme in 300 µl, then precipitated by 7.5M

NH_4OAc / 100% isopropanol, resuspended in 1x loading buffer in TE at 65 °C for 2-3 h, and run on agarose gels. For IGF2 analysis, genomic DNA was digested with Ava II or Ava II / Hpa II, fractionated on 0.8% agarose gel, and then Southern blotted to Hybond N (Amersham). For H19 analysis, genomic DNA was digested with Rsa I or Rsa I / Hpa II. Blots were hybridized at 65° C for ~ 16 hrs and then washed with 2 X SSC, 0.1% SDS.

d. H19 and IGF2 cDNA synthesis and RT-PCR amplifications.

Approximately 20 μg of total placental or fibroblast RNA treated with RNase-free DNase I, extracted with phenol/chloroform and assayed for genomic DNA contamination by PCR; only those showing no PCR product were used for the reverse transcription using 100ng of 3' primer and 200U of MLV reverse transcriptase (GIBCO BRL). The cDNAs were extracted with phenol/chloroform and precipitated in ethanol prior to PCR. IGF2 expression was analysed using an Apa I RFLP in exon 9. IGF2 cDNA containing this polymorphism was amplified with PCR primers IGF2 I1 and I3 as described (Rainier S. et al., 1993). The RT-PCR products were digested with ApaI and run on 3% Nu Sieve gel. H19 expression was analysed using a exon 5 Rsa I polymorphism amplified by PCR using the primer P5 (5' **AGG AAT CGG CTC TGG AAG GTG** 3') and P4 as described (Ogawa O. et al., 1993). Conditions for all PCR reactions were: ~1μg of DNA, 100ng of each primer, 0.2mM of each dNTP, and 2-2.5 U of Taq DNA polymerase in a total of 50μl reaction. Conditions for H19 cDNA PCR amplification were denaturation:94^{0}C, 4 min., amplification 30 cycles: 94^{0}C,90s; 60^{0}C, 60s; 72^{0}C, 120s; extension: 72^{0}C, 7.5min. The PCR generated a 600bp band from genomic DNA and a 539 bp fragment from cDNA. The PCR and RT-

PCR products were then digested with Rsa I and electrophoresed in 1% agarose gels.

e. Microsatellities

The microsatellite repeat polymorphisms in the 11p region were employed as described (Table 3-2). The 3' primers were end-labeled for 1 hour at 37 ^{o}C with γ [^{32}P] ATP. 0.3 μl of the labeled primer was added to the 15 μl of PCR reaction containing 1 μl of genomic DNA template, 1.5 μl each oligonucleotide primer (20 ng/μl), 200 μM of each dNTP, 0.12 μl Taq polymerase (8u/μl), 1.5 μl 10 X PCR buffer (Pharmacia). The PCR products were run on 8% acrylamide gel and the fragments were visualized by autoradiography.

Table 3-1. Methylation pattern and transcriptional activity in normal and SGBS tissues.

Locus/Map	Methylation											Allelic Expression *					
	Control tissue				SGBS tissue							Control tissue		SGBS tissue			
	Li	Pla	Fo	Fb	WT1	WT2	Li	Fbt	Pla	Fo	He	Pla	Fb	Pla	Fb1	Fb2	Fb3
IGF2/11p15.5	▦	▦	▬	▬	□	ND	◨	◨	▦	▦	ND	-/b	-/b	a/b	a/b	a/-	a/b
H19/11p15.5	▬	◨	ND	ND	◨	◨	▬	▦	▦	◨	◨	a/b	ND	a/b	ND	a/-	ND

He - Heart, Li - Cleft Lip, Pla- Placenta, Fb - Fibroblast, Fbt - X;1 (q26;q12) translocation skin fibroblasts, Fo - Foreskin. Black rectangles represent methylated alleles, white represent unmethylated, and gray represent partially methylated. ◨ - heterozygous, one allele methylated. □ - homozygous, demethylated. ▬ - homozygous, hemimethylated. ▦ - one allele unmethylated, but the other partial methylated. ▦ - heterozygous, both alleles partial methylated. ND - not done. "*" - all samples tested are heterozygous.

Table 3-2. Polymorphism markers used in chromosome 11p linkage studies.

Marker	Locus	Location	Reference
388II-A/388II-B	D11S554	11p12-p11.2	Phromchotikul et al, GDB
AFM254zb9	D11S935	11p13	Weisenbach,J et al., GDB
AFM212xa11	D11S921	11p13	Weisenbach,J et al., GDB
434-A/434-B	D11S569	11p15.2-p15.1	Phromchotikul et al, GDB
AFM217yb10	D11S922	11p15.5	Weisenbach,J et al., GDB

GDB-Genome Data Base.

Table 3-3. Assessment of LOH in chromosome 11p13-15.5 loci in two SGBS with WTs.

Locus/map	WT_1	WT_2
HRAS/11p15.5*		
D11S922/11p15.5		
IGF2/11p15.5		
H19/11p15.5		
D11S956/11p15.2		
D11S921/11p15.2		
D11S935/11p13		
FSH/11p13*		

Blank box-not imformative; Light gray-Loss of heterozygosity (LOH); Dark gray-remain heterozygosity. "*" - data supported by Paul Grundy (personal communication).

Figure 3-1. Southern blot analysis of H19 methylation.

Genomic DNA was extracted from normal and SGBS tissues and digested with (A) RsaI or (B) RsaI / HpaII. The RsaI polymorphism consists of 1.7 and 1.3 kb bands. N.pl-normal placenta, S.pl-SGBS placenta, WT_1-Wilms' tumour case 1, WT_2-Wilms' tumour case 2, S.Li-SGBS cleft lip, FBt-SGBS t (X;1) (q26;q12) cell line fibroblasts.

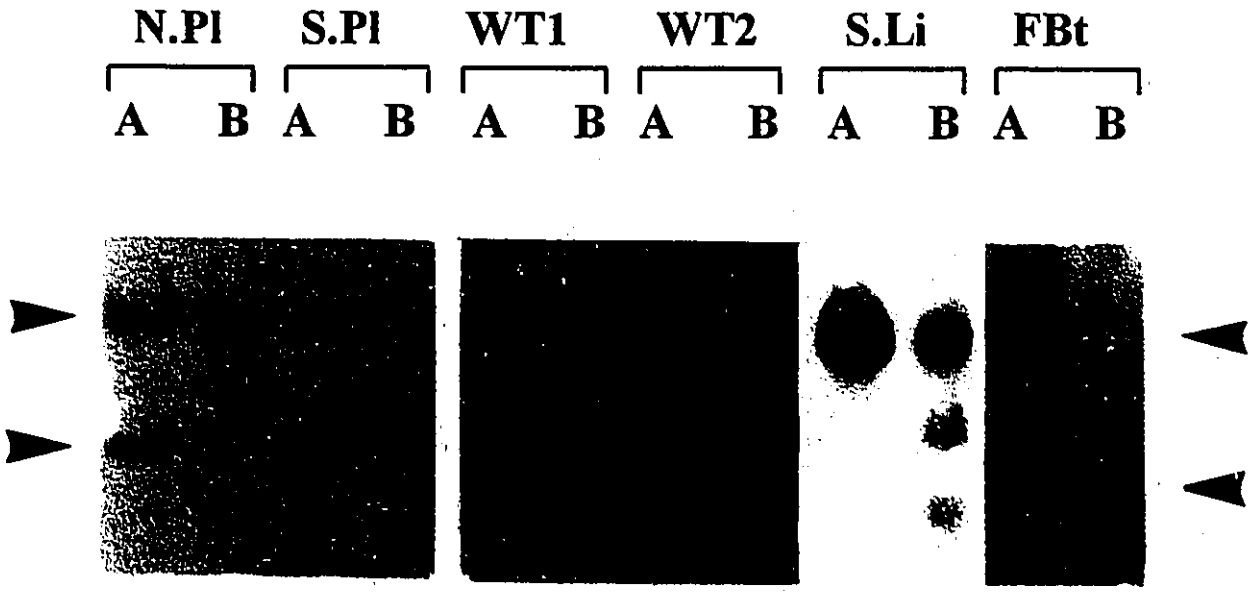

N.Pl
S.Pl
WT1
WT2
S.Li
FBt
A B A B A B A B A B A B

Figure 3-2. Southern blot analysis of IGF2 methylation in SGBS and control leucocyte DNA.

SGBS and control leucocyte DNA digested with (A) AvaII, or (B) AvaII/HpaII, and probed with IGF2 cDNA. Lanes 1-3, 6, and 8-10 contain SGBS DNA and lanes 4, 5 and 7 contain control DNA. The Ava II RFLP consists of two bands of 1.1 and 0.9 kb. In both SGBS and control DNA, one IGF2 allele is hypomethylated as can be seen with the HpaII digestion.

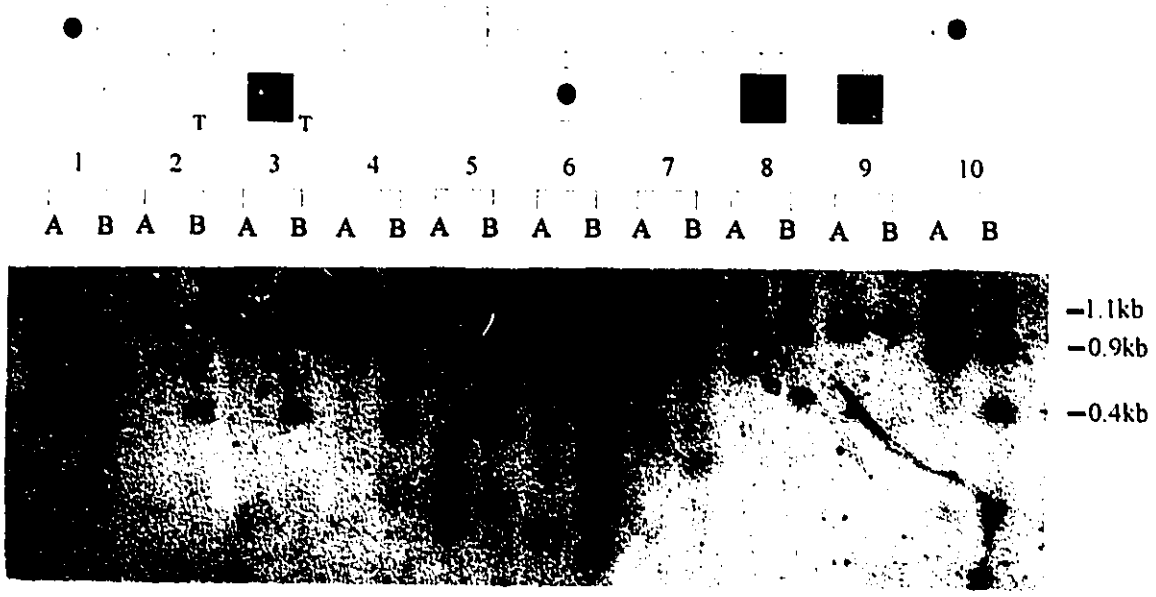

T
T
1 2 3 4 5 6 7 8 9 10
A B A B A B A B A B A B A B A B A B A B
1.1kb
0.9kb
0.4kb

Figure 3-3. Southern blot analysis of IGF2 methylation in SGBS and control tissues.

Genomic DNA from SGBS and control tissues digested with (A) AvaII (A) or (B) AvaII/HpaII. Blots were hybridized with the 1.4kb genomic DNA probe spanning the last two exons of IGF2. N.PI - normal placenta; S.PI - SGBS placenta; N.Fo - normal foreskin; S.Fo - SGBS foreskin; N.Li - normal cleft lip; S.Li - SGBS cleft lip; N.Fb - control fibroblast; Fbt - X;1(q26,q12) translocation cell line fibroblast.

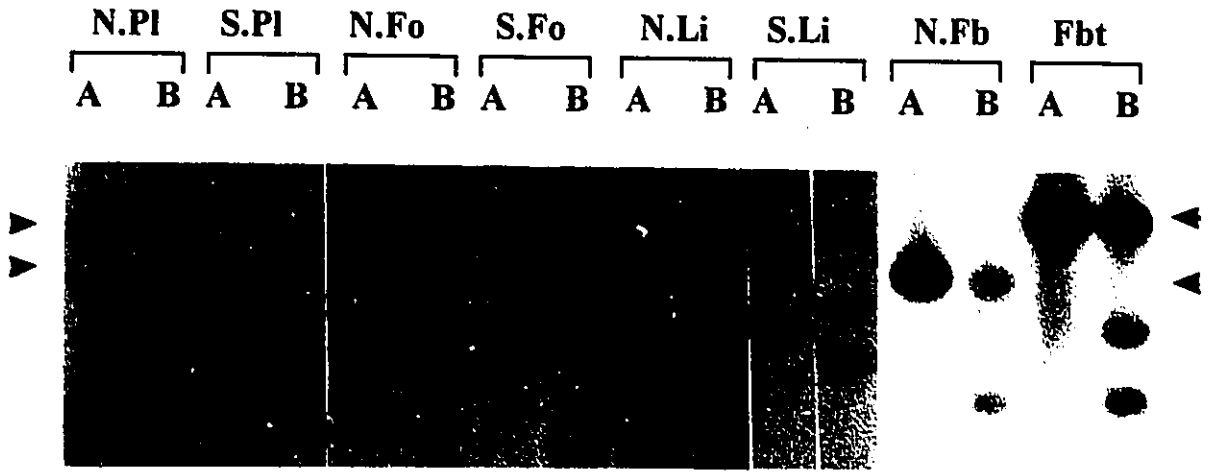

N.Pl
S.Pl
N.Fo
S.Fo
N.Li
S.Li
N.Fb
Fbt
A B A B A B A B A B A B A B A B

Figure 3-4. Allelic expression of H19 gene in normal and SGBS tissues.

Placenta and fibroblast RNA's were subjected to RT-PCR as described. A. PCR of genomic DNA digested with RsaI. B. RT-PCR of RNA digested with RsaI to show allelic expression pattern. In both A and B, lane 1, 2, and 3 contain product from normal placenta, SGBS placenta, and SGBS fibroblast cell line 2. The disroportionately intense lower band in land 2 (A), is a result of the greater concentration of the RsaI positive H19 alleles as can be seen in the Southern blot (Figure 3-1,lane S.Pl/B), possibly a result of 11p somatic recombination or an interstitial deletion. Nonetheless biallelic expression of H19 is seen for the SGBS placenta as well as the control, in contrast to the monoallelic expression of the SGBS fibroblast.

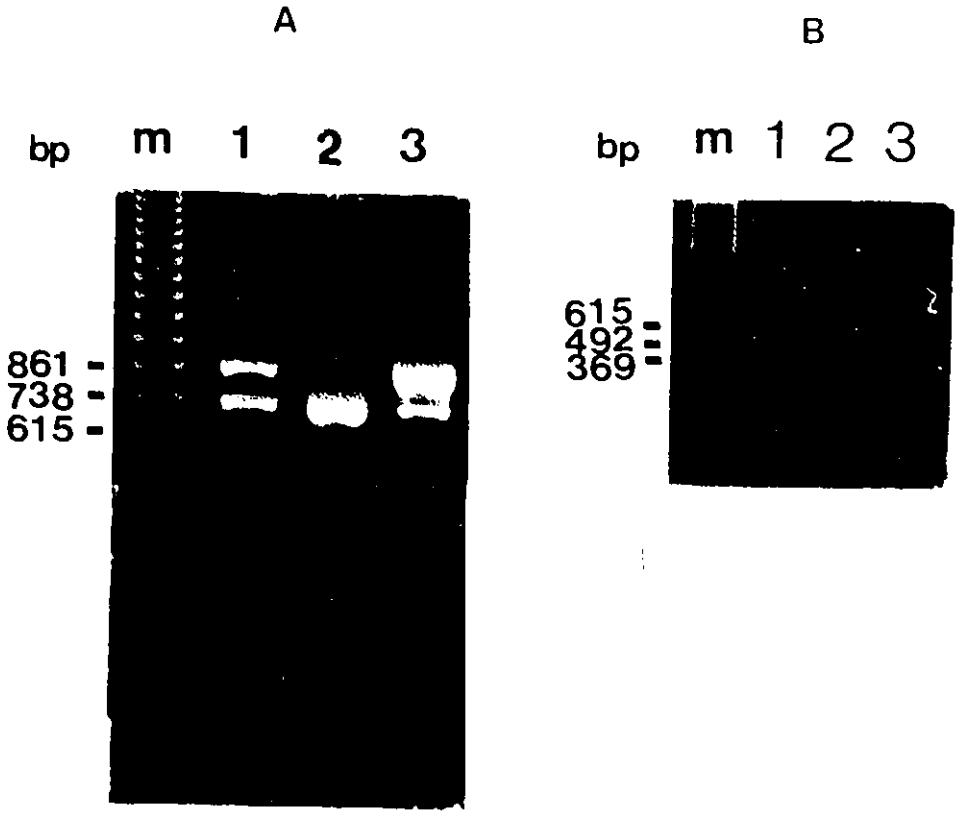

A
B
bp
m
1
2
3
861
738
615
bp
m
1
2
3
615
492
369

Figure 3-5. Allelic expression of IGF2 in control and SGBS tissues from placenta and fibroblast.

The 236bp RT-PCR products were either resistant to ApaI digestion or cleaved into 173bp and 63bp fragments. N.Pl - control placenta; S.Pl - SGBS placenta; N.FB - control fibroblast; $S.FB_1$, $S.FB_2$ and $S.FB_3$ - SGBS fibroblast cell line 1,2 and 3. "+" - RNA sample with reverse transcriptase added; "-" RNA sample without reverse transcriptase added.

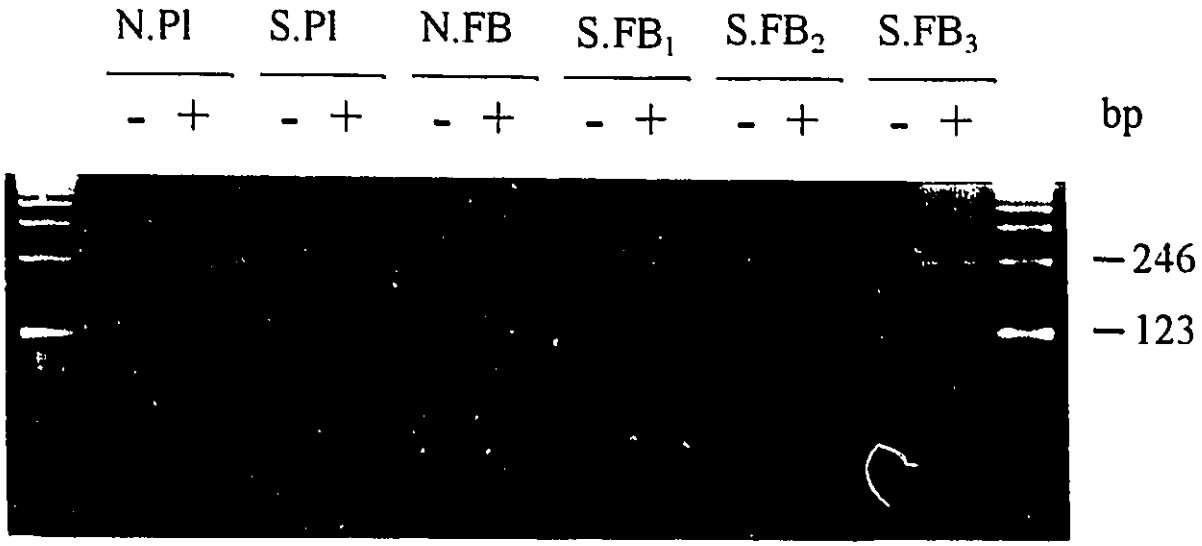

N.PI
S.PI
N.FB
S.FB1
S.FB2
S.FB3
- + - + - + - + - + - +
bp
246
123

Figure 3-6. Restriction map of 3' half of the H19 gene.

The rectangles represent exons and Rsa I site is indicated in exon 5. The PCR primers P1, P2, P3 and P4 have been described previously (Zhang et al., 1992). The sequence of P5 is given in the methodology section. Using primer P5 and P4, the PCR products generated from genomic DNA was 600bp and that from cDNA was 539bp.

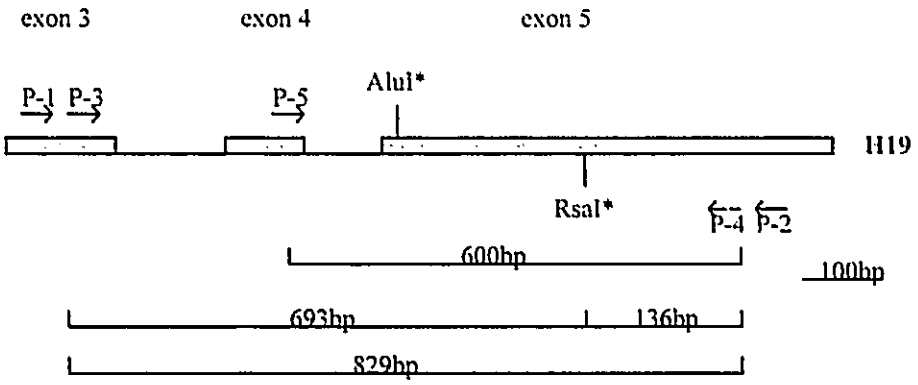

exon 3
exon 4
exon 5
P-1
P-3
P-5
AluI*
H19
RsaI*
P-4
P-2
600bp
100bp
693bp
136bp
829bp

Figure 3-7. Restriction Map of the the 3' exons in IGF2 gene.

The relationship between AvaII and HpaII sites on the IGF2 gene around exons 7, 8, and 9. A=AvaII site. H=HpaII site. H*=specific HpaII site, sensitive to methylation. (CA)n = CA rich area.

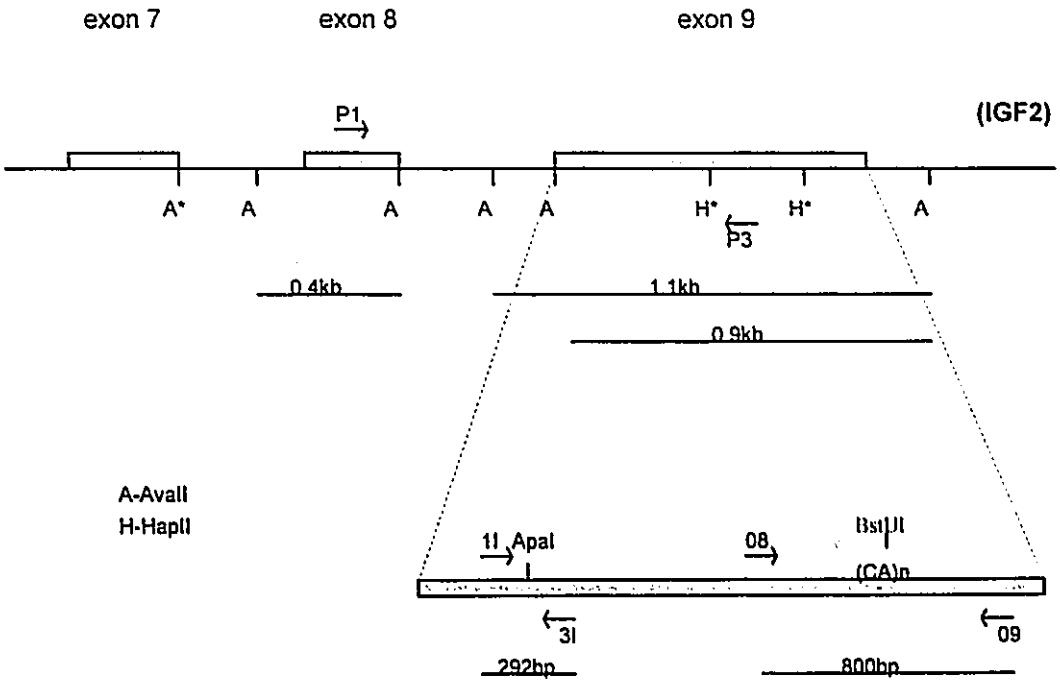

exon 7
exon 8
exon 9
(IGF2)
P1
A*
A
A
A
A
H*
H*
A
P3
0.4kb
1.1kb
0.9kb
A-AvaII
H-HapII
1l
ApaI
08
BstUI
(CA)n
3l
09
292bp
800bp

Figure 3-8. The Genotyping of the Dutch-Canadian SGBS Family on chromosome 11p11-15.5 loci.

The chromosome pattern demonstrated no common chromosome inheritance in the three WTs. T - WT patient.

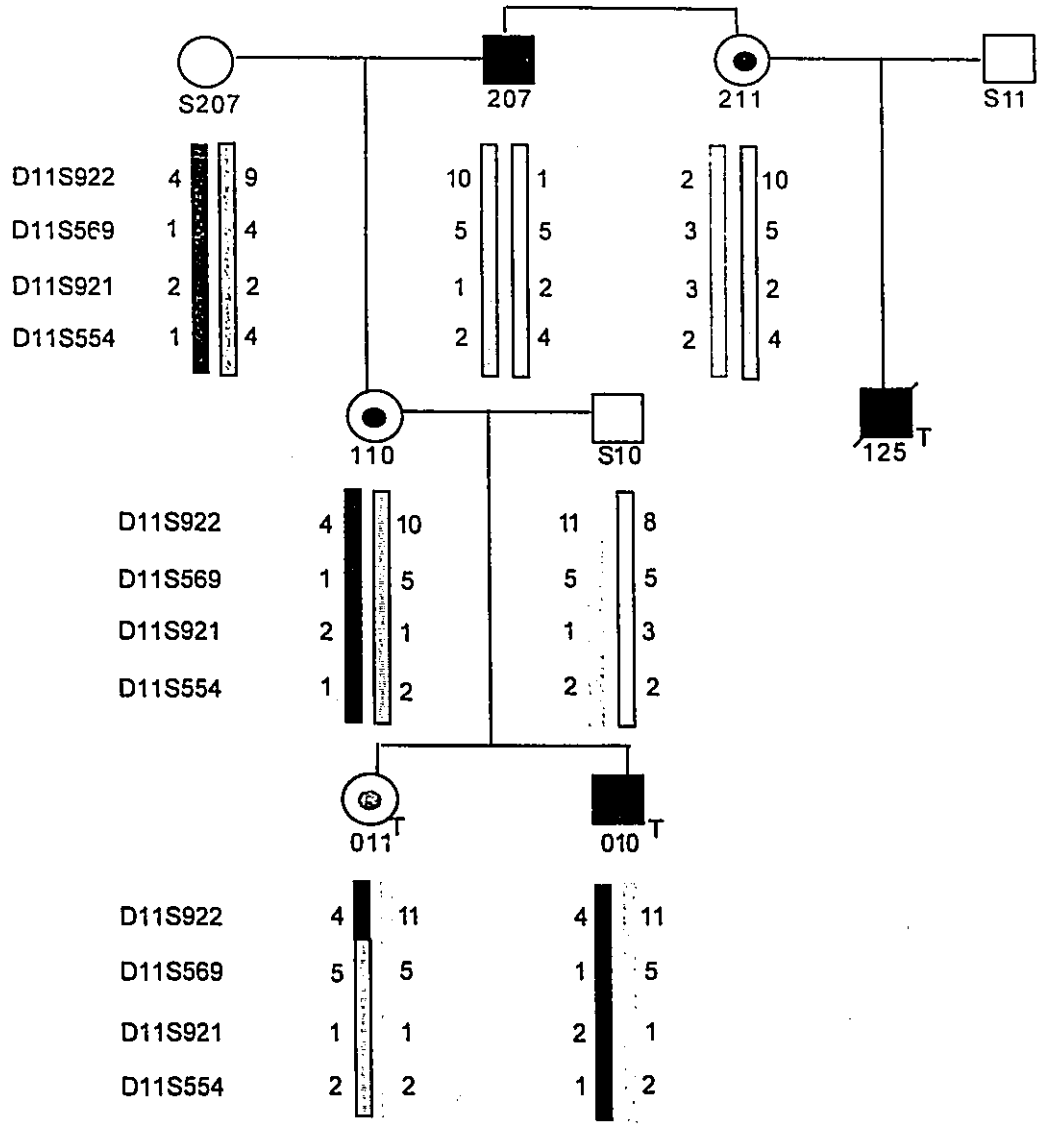
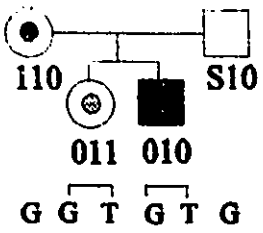

S207
207
211
S11
D11S922
D11S569
D11S921
D11S554
4 9
1 4
2 2
1 4
10 1
5 5
1 2
2 4
2 10
3 5
3 2
2 4
110
S10
125 T
D11S922
D11S569
D11S921
D11S554
4 10
1 5
2 1
1 2
11 8
5 5
1 3
2 2
011 T
010 T
D11S922
D11S569
D11S921
D11S554
4 11
5 5
1 1
2 2
4 11
1 5
2 1
1 2

Figure 3-9. LOH of 11p loci detected by microsatellite polymorphisms in two Wilms Tumour samples.

A. PCR was performed on white blood cell DNA (C) of SGBS. B. PCR was performed on Wilms tumour DNA (T) of SGBS patients. Loss of maternal allele (indicated by arrow) in locus D11S922 and D11S569 were observed in two cases of SGBS associated with WT.



D11S922

D11S569

CHAPTER 4 SUMMARY

Simpson-Golabi-Behmel syndrome (SGBS) is a rare X-linked condition associated with pre- and postnatal overgrowth and an increased risk of developing Wilms' tumours. Other features included characteristic coarse face, congenital heart defects, arrhythmias, supernumerary nipples, splenomegaly, large dysplastic kidneys, cryptorchidism, hypospadias, skeletal abnormalities, umbilical hernias. Most SGBS individuals are of normal intelligence. A noteworthy finding in a Dutch-Canadian SGBS kindreds is the severity of the symptoms increases with each successive generation - a event termed genetic anticipation. Since the first case of SGBS was described 20 years ago, more than 30 families or cases with similar clinical profiles have been recognized. The SGBS gene was initially mapped to the Xqcen-q22 (Hughes-Benzie et al., 1992), a region contains a functional pseudogene CRIPTO-3 (CR-3). CR-3 is localized in the Xq21.3-q22 region and encodes the growth factor CRIPTO which was identified as a potential candidate gene for SGBS.

The first objective of this thesis was to refine the region containing the SGBS gene. An XbaI CR-3 RFLP was genotyped in the Dutch-Canadian and British SGBS kindreds revealing 2 instances of CR3-SGBS non-cosegregation ruling CR-3 out as the causative gene for SGBS. Moreover, 30 polymorphic markers spanning the X-chromosome were genotyped in a total of 48 DNA samples from two large SGBS families. Using the LINKAGE 5.1 computer program, complete linkage with a maximum lod score of 7.5 for SGBS-HPRT pairing was observed, remapping the SGBS gene to the Xq25-q27 region (Xuan

et al.,1994).

This gene localization has been confirmed in two large European SGBS families which showed complete linkage with Lod score of 4.45 at the HPRT locus (Orth et al., 1994) and reassessed a SGBS female who has a translocation t (X;1) (q26;q12) and was previously diagnosed as BWS (Punnett 1974 and Punnett 1994). The close linkage between the disease locus and HPRT as well as the cytogenetic breakpoint in the SGBS critical region strongly supports the localization of the SGBS gene to the Xq26 region suggesting that the SGBS locus is disrupted by the chromosomal translocation.

According to the linkage data showed in chapter 2, individual V-1, a young girl with Wilms' tumour in the Ductch-Canadian SGBS family does not carry the mutant chromosome in the Xq26 critical region. It was suspected that her mild SGBS syndrome as well as the Wilms' tumour were an effect of the maternal SGBS gene product and that she may not be a SGBS carrier. To clarify the situation, genotyping of an AT repeat polymorphism derived from a phage clone (1B5) within the intron of GPC3 revealed that individual V-1 does not carries the mutant chromosome (data not shown) supporting the linkage data showed in chapter 2.

SGBS shares many clinical features with the more common Bechweth-Wiedemann syndrome (BWS) - an overgrowth syndrome mapped to the chromosome 11p15.5 region. Given the significant clinical overlap between SGBS and BWS, and the observation of loss of imprinting in some BWS cases, it was postulated that SGBS may represent failure of an X-chromosome gene

which normally imprints 11p loci. Based on this hypothesis, the second objective of this thesis was to elucidate the role of chromosome 11p loci in the pathogenesis of SGBS as well as in the tumourigenesis of WT in SGBS patient. The methylation pattern and allelic expression of the IGF2 and H19 genes in SGBS patients were studied. Genotyping of H19 cDNA polymorphisms showed biallelic expression in an SGBS and a normal placenta and monoallelic expression in one SGBS fibroblast cell line. IGF2 showed monoallelic expression in control placenta and fibroblast but biallelic expression in one SGBS placenta, and two of three SGBS fibroblast tissues. These results are similar to the observation of monoallelic IGF2 expression in control placentas and fibroblasts by others (Weksberg et al., 1993 and Giannnoukakis et al., 1994) and biallelic expression of IGF2 in BWS fibroblasts (Weksberg et al., 1993). It appears that tissue-specific loss of IGF2 imprinting may play a role in the pathogenesis of SGBS as well as other overgrowth syndromes.

As previously reported, the SGBS family is associated with high risk of Wilms' tumour. A Wilms' tumour gene (WT1) has been cloned in chromosome 11p13 where deletion or detectable abnormalities were found in 10-15 % of Wilms' tumour. In addition, Wilms' tumour locus have also been mapped to 11p15.5 region in two Wilms' tumour families indicated the existence of a second WT gene (Koufos et al., 1989). Finally, linkage analysis of additional Wilms' tumour families have excluded the tumour-predisposing locus from both 11p13 and 11p15 region suggesting the presence of a third Wilms' tumour locus (Grundy et al., 1988; and Francke 1990). To assay for the possibility that the three WT individuals from the Dutch-Canadian SGBS kindred inherited the same chromosome 11p containing WT predisposing mutations, genotyping with four

microsatellite repeats D11S554, D11S956, D11S921, and D11S922 (Table 1) mapping to 11p11-15.5 was conducted. We did not observe a common chromosome inheritance in either the 11p13 or 11p15.5 region for the three WT cases in this kindred supporting the possibility of a third WT locus exists. It is possible that LOH in chromosome 11p loci disrupt a tumour suppressor gene resulting in tumourigenesis in this kindred.

Recently, the SGBS candidate gene (GPC3) has been identified by positional cloning based on the SGBS female with X;1 (Xq26; 1q12) translocation in the University of Washington (Pillia G. et al., 1995). GPC3, a 2,130 bp cDNA, spans more than 500 kb in Xq26 and is interrupted with the X;1 (q26;q12) and an X;16 (q26;q24) translocation breakpoints. This cDNA encodes a putative protein chain of 580 amino acids and shows very high homology (94%) with OCI-5, a rat cDNA which is associated with the family of cell surface heparan sulfate proteoglycans.

Primer pairs derived from the GPC3 exons have been used to assay for the mutation in SGBS families. Deletion in exon 2 was found in all affected males in the Ductch-Canadian family (Pedigee 17207 in Figure 4-1). This deletion results in the loss of 40 amino acids in the putative protein from those patients. Deletion in exon 7 and exon 8 were also showed in another two small SGBS kindreds (Pedigee 23353 and Pedigee 26372 in Figure 4-1) confirming the GPC3 as a candidate gene for SGBS (Pillia G. et al., 1995). Moreover, Southern blot analysis using GPC3 exons as probe demonstrated deletions in 8 out of 11 SGBS families (Hughes-Benzie unpublished data). To prove that deletions are involve in pathogenesis of the disorder, PCR assays were conducted on 60 unrelated

control males using the GPC3 exon primers. No deletions were detected in control samples (data not shown).

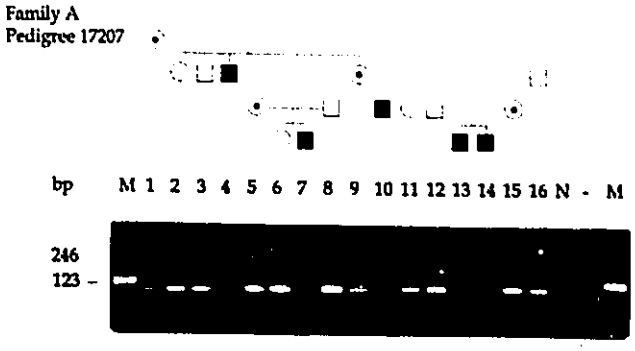
Given the nature of the GPC3, a member of the heparan sulfate proteoglycan family, its functions are more likely linked to the cell surface, modulate specific growth factor-receptor interactions, mediate interactions of the cell surface with enzymes and extracellular matrix proteins, and accelerate the formation of proteinase-proteinase inhibitor complexes (David 1993). Recently, a RT-PCR assay for the expression of GPC3 in different human tissues failed to amplify the band from skin fibroblast suggesting the gene may not be expressed in this tissuses (Pillia G. personal communi 1995).

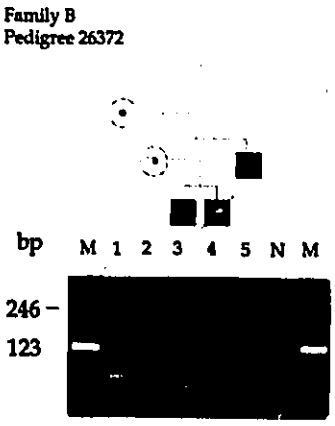
The predicted functions of the GPC3 as well as the assay of the gene expression in skin fibroblast are somewhat difficult to explain in view of the hypothesis that the mutant of SGBS gene may result in loss of IGF2 imprinting. However, the loss of IGF2 imprinting observed in some cases of BWS and the significant clinical overlap observed between BWS and SGBS still suggest that SGBS may be characterized by a relaxation of the genomic imprinting of IGF2. Moreover, the *Tme* mouse, an IGF2R deletion mutant leads to a *de facto* overexpression of IGF2, showed significant parallels to SGBS including somatic overgrowth, skeletal and cardiovascular abnormalities, and facial dysmorphism (Wang, Z.Q. et al., 1994). As well, the oncogenic potential recently demonstrated for IGF2 in transgenic mice (Christofori, G.et al., 1994) fits well with the increased risk of malignancy observed in SGBS. Cell membrane haparan sulfate proteoglycan associated with cell nuclei have been studied in the pass 20 years. By sulfate labeling, glycoproteins were demonstrated in the nuclei of rat brain

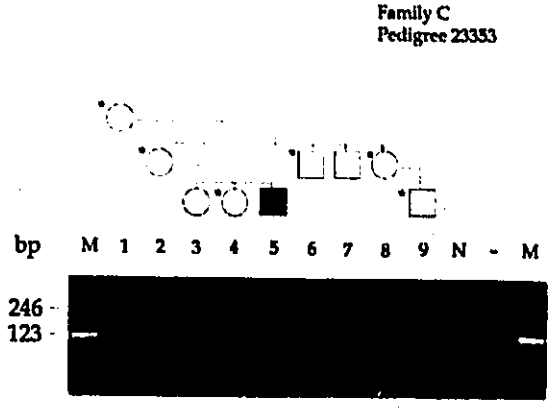
(Margolis et al., 1976). Nuclear heparan sulfate is also found in rat hepatocyte cell line (Ishihara et al 1986). A study of the effects of cell surface heparan sultfate proteoglycan on hepatoma cell growth showed that when adding HSPG to log phase cells, free heparan sulfate chains appeared in the nucleus and the growth of the treated cells was inhibited; following the decrease of nuclear HS level, the cells resumed logarithmic growth implying that the nuclear heparan sulfate proteoglycan level influences the rate of cell growth (Fedarko et al., 1989). Based on the above evidence, the SGBS gene product may be a constituent in a heparan sulfate proteoglycan pathway and could be released and transfered into the nucleus, regulate the IGF2 expression, lead to loss of IGF2 imprinting and eventully resulting in or contributing to somatic overgrowth.

Figure 4-1. The mutation analysis of the SGBS candidate gene GPC3 in three SGBS families.

Primer pairs derived from the GPC3 exons were used to assay for the mutation in SGBS families. Deletion in exon 2 was found in all affected males in the Ductch-Canadian family (Pedigee 17207). Deletion in exon 7 and exon 8 were also showed in two small SGBS kindreds (Pedigee 23353 and Pedigee 26372) confirming the GPC3 as a candidate gene for SGBS.


Family A
Pedigree 17207
bp M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 N - M
246
123


Family B
Pedigree 26372
bp M 1 2 3 4 5 N M
246
123


Family C
Pedigree 23353
bp M 1 2 3 4 5 6 7 8 9 N - M
246
123

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