Paternally Inherited Submicroscopic Duplication at 11p15.5 Implicates Insulin-like Growth Factor II in Overgrowth and Wilms’ Tumorigenesis


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Abstract
Loss of imprinting at insulin-like growth factor II (IGFII), in association with H19 silencing, has been described previously in a subgroup of Beckwith-Wiedemann syndrome (BWS) patients who have an elevated risk for Wilms’ tumor. An equivalent somatic mutation occurs in sporadic Wilms’ tumor. We describe a family with overgrowth in three generations and Wilms’ tumor in two generations, with paternal inheritance of a cis-duplication at 11p15.5 spanning the BWS IC1 region and including H19, IGFII, INS, and TH. The duplicated region was below the limit of detection by high-resolution karyotyping and fluorescence in situ hybridization, has a predicted minimum size of 400 kb, and was confirmed by genotyping and gene-dosage analysis on a CytoChip comparative genomic hybridization bacterial artificial chromosome array. IGFII is the only known paternally expressed oncogene mapping within the duplicated region and our findings directly implicate IGFII in Wilms’ tumorigenesis and add to the mutation spectrum that increases the effective dose of IGFII. Furthermore, this study raises the possibility that sporadic cases of overgrowth and Wilms’ tumor, presenting with apparent gain of methylation at IC1, may be explained by submicroscopic paternal duplications. This finding has important implications for determining the transmission risk in these disorders. [Cancer Res 2007;67(5):2360–5]

Introduction
Beckwith-Wiedemann syndrome (BWS; OMIM 130650) is an overgrowth disorder involving imprinted genes on 11p15.5 and affects 1 in 15,000 births. It has a complex phenotype, typically including prenatal and postnatal overgrowth, macroglossia, exomphalos, umbilical hernia, hemihypertrophy, and hypoglycaemia in the neonatal period. Affected patients have a 1,000-fold increase in risk for developing tumors in childhood, including Wilms’ tumor, adrenal cortical carcinomas, and hepatoblastoma (1–3). Six BWS genetic subtypes have been described. These include patients with imprinting mutations affecting two separate imprinting centers in 11p15.5, IC1 and IC2, patients with mosaic uniparental paternal isodisomy for 11p15.5, patients with inactivating point mutations in CDKN1C, patients with translocations disrupting IC2 on maternal 11p, and patients trisomic for 11p with paternal duplication (4–15).

Recent studies on BWS have enabled correlations to be made between phenotype, imprinting mutations, and chromosomal abnormalities (8–10, 16). Uniparental disomy (UPD) for 11p and IC1 hypermethylation confers a high risk for Wilms’ tumor in BWS. Conversely, isolated IC2 hypomethylation has not, to date, been reported in any BWS case with Wilms’ tumor. IC1 hypermethylation is also linked to macrosomia with higher birth weights recorded compared with those with IC2 hypomethylation or CDKN1C mutation; however, IC1 BWS cases frequently display other features of BWS, including umbilical hernias and macroglossia. IC1 hypermethylation is relatively infrequent in BWS and affects only 2% to 5% of cases. IC1 is an intergenic region 5’ to the H19 promoter that maintains differential patterns of parental methylation during development. IC1, also referred to as the H19DMR1, is normally paternally methylated and maternally unmethylated. Methylation within IC1 is linked mechanistically to maintaining imprinting at H19 (OMIM 103280) and insulin-like growth factor II (IGFII; OMIM 147470). In BWS, IC1 acquires a methylation signature on the maternal chromosome, equivalent to that on the paternal chromosome, leading to silencing of maternally expressed H19 and loss of silencing at maternal fetal IGFII promoters, resulting in maternal IGFII transcription. IGFII encodes a fetal growth factor and its expression is confined to tissues affected in BWS. Gain of methylation at IC1 and associated loss of imprinting (LOI) at IGFII have also been described in sporadic Wilms’ tumor and lead to a 2-fold increase in IGFII expression (17). It has been hypothesized that increased IGFII dosage in the mature nephroblast leads to failure of proliferation arrest and the development of tumors at the periphery of the renal lobe. Thus, the presence of IC1 hypermethylation in BWS predisposes patients to developing Wilms’ tumor. Mice carrying deletions of the cis-acting sequences that control IGFII imprinting (h19A13 mouse model), raising the tissue levels of IGFII 2-fold, display some features of BWS, including overgrowth and placentomegaly; however, affected animals do not develop renal tumors (18, 19).

Morison et al. (20) described a syndrome they termed “IGFII overgrowth disorder,” following the identification of four patients with constitutional relaxation of IGFII imprinting and abnormal H19 methylation. This epimutation was in essence indistinguishable from what has been more referred recently in BWS cohort studies as an IC1 epimutation. All four children showed nephromegaly and two developed Wilms’ tumor; however, other than overgrowth, there were no other phenotypic characteristics of BWS. They proposed that BWS may be one extreme manifestation of “IGFII overgrowth disorder”; however, it is now apparent that...
IGFII overgrowth disorder is more likely an extreme manifestation of BWS. Sparago et al. (21) have identified familial cases of BWS with maternally inherited deletions within IC1 leading to LOI at the IGFII locus. More recently, Prawitt et al. (22) described a family in whom maternally inherited deletions in IC1 were shown to be insufficient to cause BWS. However, when these deletions occurred in individuals in association with cis-duplication of the maternal IGFII locus and flanking region, those affected displayed a BWS phenotype with Wilms’ tumor. Moreover, IGFII expression from maternal promoters was significantly elevated in the fibroblasts from these patients.

Here, we described a family of mixed Maori/Anglo-Saxon ancestry with macrosomia in three generations and Wilms’ tumor in two generations, in whom we have identified evidence for duplication of the distal 11p15.5-imprinted domain IC1 on the paternally inherited chromosome. The proximal IC2 domain is excluded from the duplicated region. Our studies on this unique family directly link a double dose of paternal IGFII to Wilms’ tumor and macrosomia, in the absence of any predicted effects on H19 expression, providing insight into the mechanisms associated with Wilms’ tumorigenesis and overgrowth.

Materials and Methods

DNA was extracted from peripheral blood mononuclear cells (PBMC) using the QIAamp DNA Blood mini kit. Alleles on 11p were examined by PCR amplification of 50 to 100 ng DNA using primers end labeled with Fam, Hex, or Tet across microsatellites listed in order from telomere to centromere D11S576, D11S922, TH, and 244.14 (KCNQ1). (all 11p15.5), HBB (11p15.4), WT1 (11p13), and D11S2020 (11p11.2). PCR products were electrophoresed on 4.5% of 0.2 mm denaturing polyacrylamide gels on an ABI 377 DNA sequencer using TAMRA 500 (Red) size standard. Sample lanes were tracked with Genescan software version 3.1.2 and analyzed using Genotyper version 2.1. Genotyping at H19 was done as described in Zhang and Tycko (23). Band quantitation following background subtraction was done using Quantity One software on the GelDoc 1000 (Bio-Rad, Hercules, CA).

Methylation was examined at KCNQ1OT1 (IC2; OMIM 604115) by Southern blotting using the differentially methylated region probe (DMRP; ref. 5). This method detects a methylated 4.2-kb band and an unmethylated 2.7-kb band in normal individuals. Five micrograms of genomic DNA were digested with 40 units each of EcoR1 and Not1 and run overnight on 1% agarose gels in 1 × TAE buffer. Transfer onto Zeta-Probe GT Genomic tested blotting membrane (Bio-Rad) was done overnight in 20 × SSC buffer and filters were fixed under UV light. Membranes were prehybridized at 60°C in ExpressHyb buffer (Clontech, Mountain View, CA) and hybridized with 32P-labeled DMRP for 18 h. Membranes were washed at room temperature with 2 × SSC/0.5% SDS for 40 min and then with 0.1 × SSC/0.1% SDS for a further 40 min at 55°C and exposed to phosphorimager screens for 24 to 48 h. Bands were visualized and quantified with ImageQuant TL version 2003.02 software following digital capture on a scanning PhosphorFlume (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). The methylation index was calculated as the peak volume of the methylated band divided by the sum of the volumes of the methylated and unmethylated bands after background subtraction.

For fluorescence in situ hybridization (FISH) studies, IGFII cosmide clone RPC11-11-46C8 (IGFII genomic sequence Genbank accession no. AC132217.15) was labeled by nick translation according to the manufacturer’s instructions (Vysis kit using fluorophore Spectrum Orange, Vysis, Downers Grove, IL). Slides were prepared after harvest from a standard phytohemagglutinin-stimulated culture of peripheral blood lymphocytes. They were then aged overnight in a desiccator. The following day, the slides were pretreated with 0.1 mg/mL RNase at 37°C for 30 min, washed 3 × 5 min in 2 × SSC, dehydrated through an ethanol series (70%, 90%, and 100%) at room temperature, and allowed to air dry. The slides were then denatured in 70% formamide/2 × SSC at 73°C for 5 min and then immediately dehydrated through a cold ethanol series (70%, 90%, and 100%) stored at −20°C for 3 min each wash. The IGFII probe and the chromosome 11 centromere (pBR11; M. Rocchi, University of Bari, Bari, Italy) was denatured at 75°C for 5 min and then placed directly into ice. The probes were then dispensed onto the slide, cover slipped and sealed with art cement, and placed into a 37°C humidified chamber overnight. Posthybridization washes were done in 0.4 × SSC/0.3% NP-40 at 73°C for 2 min, rinsed in 2 × SSC/0.1% NP-40 at room temperature for 1 min, air dried, and counterstained with 4,6-diamidino-2-phenylindole. Once dry, the slides were cover slipped using antifade mount (2.3% 1,4-diazabicyclo[2.2.2]octane in 40% glycerol/0.02 mol/L Tris-HCl). The slides were viewed with a Zeiss Axioskop 20 microscope (Carl Zeiss Inc., Thornwood, NY) powered by a 100 W mercury burner. Vysis filter sets and CytoVision version 3.6 were used for capturing and evaluation.

Array comparative genomic hybridization (CGH) was done on CytoChips (BlueGene Ltd., Cambridge, United Kingdom), spotted with a bacterial artificial chromosome (BAC) library of over 3,300 clones, and spaced at ~1 Mb intervals to provide even coverage of all chromosomes, including telomeres. Clones on the array are derived from the validated Roswell Park Cancer Institute BAC library. Each cytochip contains duplicate subarrays so that two independent labelings and hybridizations can be completed in a single dye-swap experiment. These chips contain extra tiling in regions of clinical interest, including 11p15.5. Four hundred nanograms of proband DNA from III-3 were hybridized against 400 ng control DNA in duplicate. Reciprocal labeling of DNA samples was done with Cy3 and Cy5 according to a standard BlueGene protocol. Blocking, prehybridization, and hybridization were done as per the BlueGene standard protocol in a Hybaid hybridization oven. Scanning was done on an Agilent 4000 B series scanner (Agilent Technologies, Santa Clara, CA) and data analysis was carried out using GenePix Proverison 6.0 software. Array data were subject to block-wise normalization. The positions of the duplicated BACs within 11p have been confirmed through reference to the Sanger Institute (Cambridge, United Kingdom) Web site.5

Further details on the scanning procedure, and the GenePix data file itself, are available from Elizabeth M. Algar on request.

Results

This study was conducted in compliance with institutional guidelines for research studies in human genetics (approval no. RCH EHRC 21121C) and informed consent was obtained from participating individuals. The relevant pedigree is shown in Fig. 1A. Patient III-2 and III-3 were born with high birth weight (5,860 and 5,060 g respectively). Patient III-2 developed bilateral Wilms’ tumor with pulmonary metastases at 18 months of age, was macrosomic, but otherwise developmentally normal. He had no history of hypoglycaemia, macroglossia, or abdominal wall defects and no ear lobe creases or pits. His sister, patient III-3, had enlarged kidneys and prominent pelvicalyceal collecting system on midtrimester ultrasound in pregnancy. She was macrosomic from birth and had hypertelorism and a broad face but no macroglossia, hypoglycaemia, abdominal wall defects, or ear creases. Postnatal ultrasound confirmed nephromegaly with diffuse areas suggestive of bilateral nephroblastomatosis. Unilateral Wilms’ tumor was diagnosed at 5 months. III-2 and III-3 had similar facial features, including full cheeks and an upturned nose. Their brother III-1 was born with high birth weight (5,568 g) and developed unilateral Wilms’ tumor at 2.5 years of age. He was otherwise developmentally normal. The paternal grandfather I-1 had tall

5 https://enigma.sanger.ac.uk/perl/PostGenomics/decipher/manager?action= arraytypes&array_id=1&chr=11
stature, had birth weight of ~ 5 kg, and was of mixed Maori/Anglo-Saxon background (paternal/maternal). The paternal grandmother I-2 was phenotypically normal. II-1 was the only child of I-1 and I-2 who were subfertile. There were no chromosomal abnormalities detected on high-resolution GTG banding in the affected family members.

To investigate the genetic basis for the overgrowth/Wilms' tumor phenotype in the family, DNA isolated from PBMCs was genotyped using microsatellite markers mapping to 11p. Allele analysis revealed coinheritance of an identical paternal haplotype in the region extending from HBB in 11p15.4 to D11S922 within 11p15.5 in the affected individuals and not in the unaffected family members (Fig. 1A). Furthermore, allelic imbalance approximating a 2:1 ratio (paternal/maternal), confined to TH, was identified in the affected family members and not in the unaffected individuals (Fig. 1B). This suggested the possibility of cis-duplication within 11p15.5 involving TH and excluding D11S922 distally and HBB at 11p15.4 proximally. Methylation analysis at KCNQ1OT1 on DNA isolated from PBMC in III-2 revealed maintenance of normal methylation patterns consistent with exclusion of the proximal 11p15.5 IC2 region from the duplication (data not shown). This was also shown by normal allelic inheritance for the microsatellite marker D11S4088 located within this region.

The segregation of paternal 11p15 haplotype with overgrowth and Wilms' tumor together with evidence for paternal/maternal allelic imbalance approximating a 2:1 ratio implied the existence of cis-duplication within 11p15.5, including IC1 but excluding IC2. Based on the distance between D11S922 and D11S4088, we estimated the maximum size of the duplicated region to be 1,148 kb [Homosapiens build 36.2 National Center for Biotechnology Information (NCBI) Map Viewer]. The 11p15.5 genes TH, INS, and IGFII are clustered in an interval of <50 kb within this region. H19 is located 142 kb distally relative to IGFII. To ascertain the direct involvement of H19 within the region of duplication, informative family members were genotyped at a previously described polymorphic Rsa1 site within H19 exon 5. III-1, III-2, and III-3 were informative. II-1 was homozygous a/a (829 bp) and II-2 was homozygous b/b (693 bp). Normal allelic ratios were identified in the unaffected child III-1, whereas III-2 and III-3 had allelic ratios skewed to the paternal allele (Fig. 2).

FISH using an IGFII cosmid (22) was done on lymphoblasts from II-1 to ascertain the mechanism of duplication. Analysis of 100 interphase lymphoblasts showed skewing to three and four copies of IGFII in II-1 compared with normal lymphoblasts. However, it was not possible to determine unequivocally whether the increased copy number was attributable to separation of signals from sister
chromatids in G2 interphases or to IGFII duplication. Metaphase FISH for IGFII excluded trisomy caused by unbalanced translocation affecting 11p15.5 providing indirect evidence for cis-duplication of the affected region.

DNA from proband III-3 was hybridized to a CytoChip CGH array. The two BAC probes with origins located at 2,061,361 and 2,261,639 bp from 11pter showed clear evidence of duplication (Fig. 3A). In a pair of dye-swap hybridizations, reciprocal increases and decreases in the dye ratios of these two contiguous probes consistently indicated a duplication in the proband (compared with a normal control). A normal copy number was indicated for flanking probes at 1,687,193 and 2,627,884 bp, consistent with genotyping analyses and with maintenance of normal methylation at the proximal imprinting center, IC2, located within the proximal half of the BAC probe at 2,627,884 bp. IGFII is at 2.117 Mb and is flanked by the duplicated probes, placing it directly within the duplicated region Fig 3B. We predict, based on allelotyping and methylation analyses and the location of H19 at 1.975 Mb on the array, that the duplication has a minimum size of 400 kb and a maximum of 800 kb. These size estimates are necessarily approximate because the size of the BAC probes themselves (>100 kb each) must be taken into account. To give a positive signal on the array, the duplication must include most, but not all, of the sequence within the two BACs implicated. The BAC addresses above give the locations of the first base; hence, the minimum duplication size must be considerably larger. The true ends of the duplication could also lie within the nearest flanking BACs and so the maximum size estimate is also approximate.

![Figure 2. Genotyping at a polymorphic Rsa1 site in H19 exon 5. Numbers show ratios of allele a to b in informative individuals. II-1 and II-2 were homozygous for alleles a and b, respectively. III-2 shows significant allele skewing when compared with III-1. A maternal allele was detected in III-3 but was extremely faint and was not visible following digital capture of the gel image.](image)

![Figure 3. A, chromosome 11 gene dosage analysis on Cytochip CGH array. DNA from the proband III-3 was hybridized against control DNA in duplicate. Y axis, log of the Cy3/Cy5 ratio (base 2). Black triangles, the ratio when proband DNA was labeled with Cy3 and the control with Cy5; gray triangles, reciprocal labeling. The two BAC probes RP3-416J11 and RP11-113A6, located at 2,061,361 and 2,261,636, showed significant and reciprocal deviations above and below the mean in dye-swap experiments. IGFII is located at 2,117,000. Bottom, an expanded view of the duplicated region. The two probes flanking the duplication are RP11-2B17 and RP11-774H09 at 1,687,193 and 2,627,884, respectively. B, representation of the 11p15.5 region, showing the location and predicted approximate minimum (solid line) and maximum size (broken line) of the tandem duplication on the paternal chromosome. Numbers on the chromosome axis denote megabase distances from 11pter (Homosapiens build 36.2 NCBI Map Viewer). Stars, microsatellite markers; □, BAC clones; ▲, genes. The chromosome axis is not represented to scale.](image)
Discussion

Paternally inherited cases of overgrowth have been described previously; however, in these cases, unbalanced translocations leading to trisomy for 11p with paternal duplication have been responsible (15). Recently, Russo et al. (24) described a patient with features of BWS who had a de novo paternal tandem duplication at 11p15.5 spanning 1.8 Mb and involving IC1 and excluding IC2. This was proposed to result from an unequal recombination at paternal meiosis I. However, the present study is the first to report an inherited submicroscopic cis-duplication affecting the distal BWS imprinting center, IC1, and the first to report a paternal tandem duplication in association with familial Wilms’ tumor and overgrowth.

The findings in this study add to the mutation spectrum increasing effective IGFII dosage in overgrowth and Wilms’ tumor. These mutations now include the following: imprinting mutations leading to LOI at IGFII (i.e., maternal IC1 deletion and hypermethylation at IC1), paternal duplication of the IC1 region as a result of trisomy 11p15.5, and as we have now shown, small regions of cis-duplication on the paternal chromosome encompassing H19/IGFII. High-resolution genotyping and array CGH should therefore be considered as part of molecular screening in familial cases of overgrowth/Wilms’ tumor in particular those with paternal inheritance and an abnormal mosaic IC1 hypermethylation.

The identified duplication is predicted to disrupt the dosage and expression of paternally expressed as well as nonimprinted genes. However, effective dosage of maternally expressed genes, including H19 and ASCL2 (25), is predicted to remain unchanged. IGFII and TRPM5 (26) are the only paternally expressed genes mapping within the duplicated region and IGFII is the only known candidate oncogene. Increased effective IGFII dosage has been implicated previously in overgrowth in mouse models and in familial BWS cases with Wilms’ tumor (22). However, it has not previously been possible to separate potential synergistic effects between H19 silencing and increased IGFII expression in predisposing to Wilms’ tumors occurring in association with BWS. In this study, we present evidence that duplication of the expressed paternal IGFII allele is the critical event in Wilms’ tumor predisposition in the absence of any altered H19 dosage. Furthermore, our findings suggest that increased effective IGFII dosage underpins high birth weight and macrosomia. Interestingly, inactivation of the maternal H19 allele (by aberrant hypermethylation or deletion within IC1), leading to H19 silencing, has been implicated in BWS in conjunction with loss of IGFII imprinting. Affected individuals show typical BWS features, including overgrowth, macroGLOSSIA, umbilical hernia, ear creases, and neonatal hypoglycaemia. However, none of these features (with the exception of overgrowth) was present in the family investigated, suggesting that they may be caused by disrupted H19 expression in conjunction with elevated IGFII or caused by disruption to other genes inactivated by the same mechanisms causing aberrant hypermethylation at the H19 IC1.

Studies on this family directly implicate IGFII dosage in Wilms’ tumor; however, they also suggest that a second hit may have been necessary for Wilms’ tumor to develop as the grandfather I-1 did not develop a tumor. This was despite evidence from H19 methylation analyses, showing mosaic hypermethylation (data not shown), that he also carried a paternally inherited cis-duplication increasing effective IGFII dosage. This is consistent with a requirement for a second genetic hit to facilitate complete malignant transformation in Wilms’ tumor and with IGFII LOI in nephrogenic rests, pockets of undifferentiated renal tissue representing precursor lesions to Wilms’ tumor found in normal fetal kidney tissue (27).

Patients with overgrowth who do not have hemihyper trophy may be candidates for germ-line cis-duplication of paternal 11p15.5. Hemihyper trophy is strongly linked to mosaic UPD at 11p15.5 and 23% of individuals with hypermethylation affecting IC1 also have hemihyper trophy (16). Gain of methylation at IC1 exhibits mosaicism, possibly explaining the co-occurrence of IC1 hypermethylation with hemihyper trophy in these cases. As germ-line duplication of IC1 is not predicted to be mosaic, IC1 hypermethylation in the absence of hemihyper trophy might imply the existence of a structural, rather than an imprinting abnormality. Further investigation of these cases using array CGH may reveal novel mutations linked to overgrowth and Wilms’ tumor. Interestingly, the 11p15.5 paternally duplicated region included the INS locus; however, none of those affected had experienced hypoglycaemia neonatally, although dosage is increased to 3:2. This suggests that mechanisms in addition to increases in insulin dosage may be required in order for hypoglycaemia to develop and is supported by the fact that hypoglycaemia occurs with all BWS mutations.

In summary, genetic analysis on this interesting family has provided a unique opportunity to further our understanding of the genetic origins of overgrowth and Wilms’ tumor. In addition, we anticipate that the novel mutation we have identified in this family may be found in sporadic cases of overgrowth and Wilms’ tumor. These studies will benefit professionals engaged in counseling families affected by Wilms’ tumor.

Acknowledgments

Received 9/12/2006; revised 12/14/2006; accepted 1/2/2007.

Grant support: Children’s Cancer Centre Foundation (E.M. Algar and V. Dagar), Cancer in Kids Auxiliary (V. Dagar), and the Murdoch Children’s Research Institute (E.M. Algar).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank the family who is the subject of this study, Dr. Geoff McCowage, Pediatric Oncologist, who initially referred the proband; and Sanger Institute (Cambridge, United Kingdom) for providing the statistical software for interpretation and representation of the array data.

References
Cis-Duplication Affecting Paternal H19/IGF2


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