Am. J. Hum. Genet. 61:304–309, 1997

Low Frequency of p57^{KIP2} Mutation in Beckwith-Wiedemann Syndrome

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Summary

Beckwith-Wiedemann syndrome (BWS) is an autosomal dominant disorder of increased prenatal growth and predisposition to embryonal cancers such as Wilms tumor. BWS is thought to involve one or more imprinted genes, since some patients show paternal uniparental disomy, and others show balanced germ-line chromosomal rearrangements involving the maternal chromosome. We previously mapped BWS, by genetic linkage analysis, to 11p15.5, which we and others also found to contain several imprinted genes; these include the gene for insulin-like growth factor II (IGF2) and H19, which show abnormal imprint-specific expression and/or methylation in 20% of BWS patients, and p57^{KIP2}, a cyclin-dependent kinase inhibitor, which we found showed biallelic expression in one of nine BWS patients studied. In addition, p57^{KIP2} was recently reported to show mutations in two of nine BWS patients. We have now analyzed the entire coding sequence and intron-exon boundaries of p57^{KIP2} in 40 unrelated BWS patients. Of these patients, only two (5%) showed mutations, both involving frameshifts in the second exon. In one case, the mutation was transmitted to the proband's mother, who was also affected, from the maternal grandfather, suggesting that p57^{KIP2} is not imprinted in at least some affected tissues at a critical stage of development and that haploinsufficiency due to mutation of either parental allele may cause at least some features of BWS. The low frequency of p57^{KIP2} mutations, as well as our recent discovery of disruption of the K_vLQT1 gene in patients with chromosomal rearrangements, suggest that BWS can involve disruption of multiple independent 11p15.5 genes.

Received January 10, 1997; accepted for publication May 20, 1997.
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0002-9297/97/6102-0008$02.00

Introduction

Beckwith-Wiedemann syndrome (BWS) is a disorder of prenatal overgrowth characterized by abdominal wall defects, macroglossia, ear creases and pits, hemihypertrophy, enlargement of the kidney and liver, and neonatal hypoglycemia. BWS patients also show a 1,000-fold-increased risk of embryonal tumors, including Wilms tumor, rhabdomyosarcoma, and hepatoblastoma (Wiedemann 1983). We and others previously had mapped BWS to 11p15.5 by genetic-linkage analysis (Koufos et al. 1989; Ping et al. 1989), in which some patients had shown balanced germ-line chromosomal rearrangements (Waziri et al. 1983; Turleau et al. 1984; Okano et al. 1986). These rearrangements specifically involve the maternal chromosome, and other BWS patients show paternal uniparental disomy (UPD) (Henry et al. 1991), suggesting that the BWS gene is imprinted—that is, is expressed from a specific parental allele. To date, we and others have identified four imprinted genes on 11p15.5: (1) IGF2 is a paternally expressed autocrine growth factor (Giannoukakis et al. 1993; Ogawa et al. 1993; Ohlsson et al. 1993; Rainier et al. 1993); (2) H19 is a maternally expressed, untranslated RNA (Moulton et al. 1994; Steenman et al. 1994) that may serve as a tumor-suppressor gene (Hao et al. 1993); (3) p57^{KIP2} is a cyclin-dependent kinase inhibitor proposed, on the basis of its map location (Matsuoka et al. 1995), as a BWS or tumor-suppressor gene, which we found to be expressed from the maternal allele (Matsuoka 1996); and (4) K_vLQT1 encodes a potassium channel expressed preferentially from the maternal allele (Lee et al. 1997).

Of these genes, all have shown alterations in BWS. First, we and others described abnormal imprint-specific expression and/or methylation affecting IGF2 and H19 in the embryonal tumors to which BWS patients are predisposed, as well as in normal tissues of BWS patients (Ogawa et al. 1993; Rainier et al. 1993; Weksberg et al. 1993; Steenman et al. 1994; Reik et al. 1995). We have recently also found biallelic expression of p57^{KIP2} in a BWS patient as well, which could be due to either activation of the paternal allele or silencing of the maternal allele to the low level of normal paternal expression (Thompson et al. 1996). Second, two of nine BWS pa-

tients were recently found to harbor apparent mutations in the coding sequence of p57^{KIP2}, one of which was also present in the proband's mother (Hatada et al. 1996). This was thought to play a causal role, since imprinting would lead to nonexpression of the gene, since the paternal allele is normally silent (Hatada et al. 1996). Third, we have recently found that five BWS chromosomal rearrangement breakpoints on 11p15 (the so-called BWSCR1 cluster) are spanned by the K_vLQT1 gene and that this gene is also normally imprinted and expressed from the maternal allele (Lee et al. 1997).

Our initial studies of a smaller number of BWS patients revealed no mutations in p57^{KIP2}. The purpose of the experiments described here was to determine the frequency of p57^{KIP2} mutations in a much larger number of patients than that examined by us or by Hatada et al. (1996), as well as its pattern of transmission.

Subjects and Methods

BWS Patients

All patients in this study were part of a self-referred BWS registry. A subset of patients was examined by one of us if they reported at least two of the following features: macroglossia, birth weight and length above the 90th percentile, hypoglycemia in the 1st mo of life, ear creases or pits, and abdominal wall defects (omphalocele, diastasis recti, and umbilical hernia). Only samples from patients both having a diagnosis of BWS confirmed by physical examination by board-certified geneticists and for whom other syndromes were excluded were subjected to molecular analysis.

Isolation of DNA

Cells were suspended in TE9 (0.5 M Tris.HCl, pH 9.0, 20 mM EDTA, 10 mM NaCl), digested with proteinase K (0.2 mg/ml) in the presence of 1% SDS at 50°C overnight, purified by phenol/chloroform extraction, and precipitated with sodium acetate/ethanol.

PCR Amplification and Sequencing Analysis

The following primers were used: primer set 1, forward primer GGGCCATGTCCGACGCGTCCCTCCGCA and reverse primer GCGGTTCTGGTCCTCGGCGTTCAGCTC; primer set 2, forward primer GGAGCTGAGCCGCGAGCTGCAGGCCCG and reverse primer CAGCAGGCGGCAGCGCCCCACCTGCAC; primer set 3, forward primer CTTCCAGCAGGACATGCCGCTG and reverse primer TGGAGCCAGGACCGGGACT; primer set 4, forward primer ACTGCCTAGTGTCCCGGTC and reverse primer GTCAGCGAGAGGCTCCTGG; primer set 5, forward primer GGCGCCTCAAGAGAGCGCCGAGCAGGG and reverse primer GGGACCGGCCGGCCGGACAAAGCGGGG; primer set 6, forward primer CGACGTAAACAAAGCTGACC and reverse primer CCCCAGGTGCGCTGTA; and H19, forward primer CAATGAGGTGTCCCAGTTCCA and reverse primer CACATAAGTAGGCGTGACTTGA. PCR reactions contained 0.5 μM primers, 0.2 mM dNTP, 50 ng DNA, 1 × PCR buffer A (InVitrogen), 12.5% dimethyl sulfoxide, 1.6 mg BSA/ml, and 0.6 units *Pfu* DNA polymerase (Stratagene) in 25 μl and were performed with a Robocycler (Stratagene), as follows: initial denaturation at 95°C for 3 min, followed by 30 cycles each of 95°C for 1 min, 60°C for 1 min, and 74°C for 1.5 min, and a final primer extension at 74°C for another 7 min. PCR products were resolved on 2% agarose gels, were purified by use of a Qiaex gel extraction kit (Qiagen), and were either sequenced with an ABI automatic sequencer or digested with the indicated restriction enzymes. Sequence analysis was performed by use of the Sequencher program (Gene Codes) and visual inspection of the chromatogram. To confirm the presence of mutations, we subcloned PCR products in the PCR2.1 vector and sequenced several independent clones from both normal and mutant alleles.

Results

p57^{KIP2} is a general inhibitor of G1 cyclin-dependent kinases, and it is closely related to p27^{KIP1} (Lee et al. 1995; Matsuoka et al. 1995). p57^{KIP2} contains three domains—a Cdk-inhibitory domain shared among p21^{CIP1}, p27^{KIP1}, and p57^{KIP2}; a highly polymorphic hexanucleotide repeat encoding proline-alanine (PAPA repeat): and a domain containing glutamine and threonine (QT domain), conserved between p27^{KIP1} and p57^{KIP2}. The exon-intron genomic structure, along with the protein domains, are illustrated in figure 1. We designed six sets of PCR primers (fig. 1), in order to amplify the entire coding region and exon-intron boundaries. These six sets of PCR primers ensured that there was enough

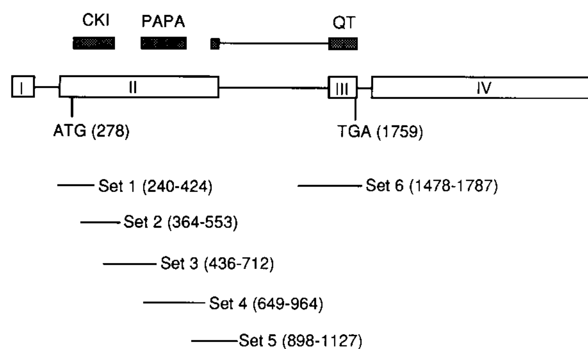

Figure 1 Structure of the human p57^{KIP2} gene. The exon-intron structure is based on the genomic sequence of p57^{KIP2} (Tokino et al. 1996). Shaded boxes denote the predicted protein domains. Sets 1–6 indicate the PCR fragments used for sequencing. The sequences of the primers are given in the Subjects and Methods.

overlapping sequence between adjacent PCR fragment, since sequencing toward the ends of PCR fragments is less reliable. Genomic DNAs were isolated from either fibroblast or lymphoblastoid cells from 40 BWS patients and were used to amplify the p57^{KIP2} gene. PCR fragments were sequenced directly by use of an ABI automatic sequencer. Because of the extensive overlap of these PCR fragments, many regions of the p57^{KIP2} gene were sequenced twice. If there was any ambiguity, we also sequenced the complementary strand.

In this manner, we analyzed each nucleotide of the entire coding region and exon-intron boundaries of the p57^{KIP2} gene, for the presence of mutations in 40 BWS patients. Of these patients, only two patients showed mutations in the p57^{KIP2} gene. Both were frameshift mutations due to deletion of a single nucleotide. Patient 1 was the proband of a BWS family in our earlier genetic-linkage study (family B in Ping et al. 1989). She had all the common features of BWS, including hypoglycemia, ear creases, macroglossia, craniofacial anomalies, and omphalocele. She also developed neuroblastoma. Both her mother and her maternal grandfather were also affected, although to a lesser degree. They both had ear creases and craniofacial anomalies. The mother also had macroglossia, and the maternal grandfather had cryptorchidism. The sequencing chromatogram of patient 1 by use of primer set 1 revealed deletion of a single nucleotide at position 347 (data not shown; the nucleotide coordinates are given in fig. 1). To confirm the apparent mutation, we then subcloned the PCR fragment and sequenced several individual subclones. The sequence analysis confirmed deletion of a single nucleotide G_{347} (fig. 2, *left*). As further confirmation of the mutation, the deletion of G_{347} in patient 1 also generates a predicted *Eag*I site, CGGCCG (fig. 2, *left*). Segment 1 is 185 bp, and digestion of the mutant allele with *Eag*I generates two fragments, 109 bp and 75 bp (fig. 3*a*). Indeed, digestion of the PCR fragment from patient 1 showed three bands—185 bp, 109 bp, and 75 bp (fig. 3*b,* lane 1)—corresponding to the wild-type and mutant alleles. The mutant allele was also present in the mother (fig. 3*b,* lane 2) and maternal grandfather (fig. 3*b,* lane 4) of patient 1, but it was not present in the maternal grandmother, a maternal aunt, and two unrelated individuals (fig. 3*b,* lanes 3 and 5–7). The mutation in the mother and the grandfather, as well as the absence of mutation in the grandmother and the maternal aunt, were also confirmed by direct sequencing (data not shown). The *Eag*I site was also not present in 50 unrelated individuals (data not shown). Therefore, the frameshift mutation in patient 1 cosegregated with the disease, and it was not seen in any unaffected individuals. The frameshift mutation would create a truncated protein retaining the first 24 amino acids and followed by six new amino acids (fig. 2). The truncated protein thus contains neither the Cdk-inhibitor domain nor the QT domain and is a true null mutant.

The mutation in patient 2 was also a frameshift mutation, in this case in PCR fragment 4, initially detected by direct sequencing of PCR products and then confirmed by subcloning and sequencing of multiple subclones (fig. 2, *right*). This frameshift mutation, like that in patient 1, was also caused by deletion of a single nucleotide, in this case a T at nucleotide 705. The mutation, like that in patient 1, creates a truncated protein lacking the PAPA and QT domains. Patient 2 also showed typical features of BWS, including macroglossia requiring surgery, as well as ear pits, nevus flammeus, and omphalocele. We do not know whether other family members carry the mutation, since the patient was adopted and her biological parents therefore are unavailable for study. We observed no additional mutations in the remaining 38 BWS patients. Thus, germ-line mutations of p57^{KIP2} were found in only 5% of the large number of BWS patients analyzed here.

We and others previously had shown that BWS in some patients involves alterations of imprint-specific methylation of a CpG island in the H19 gene (Steenman et al. 1994; Reik et al. 1995). In order to determine whether p57^{KIP2} mutations might exert an indirect effect on the genomic imprinting of other 11p15 genes, we used a sensitive and specific PCR assay (Jinno et al. 1996) to examine allele-specific methylation of H19 in a BWS patient with a p57^{KIP2} mutation. The primers spanned both a polymorphic invariably unmethylated *Hha*I site and a *Hpa*II site that is normally methylated only on the paternal chromosome. Patient 1 was heterozygous for the a and b alleles, and her mother was homozygous for the a allele (fig. 4). Therefore, the a allele of patient 1 is of maternal origin, and the b allele is of paternal origin. Digestion with *Hpa*II prior to PCR abolished the a allele only. Thus, the H19 gene is normally methylated, at least at one imprint-specific site, on the paternal chromosome in patient 1, and the p57^{KIP2} mutation in this patient did not disturb the imprint-specific methylation of H19 at that site.

Discussion

By direct sequencing in 40 BWS patients, we have analyzed the entire coding region and intron-exon boundaries of the p57^{KIP2} gene, in order to detect mutations unequivocally. We have found only two, although both of these are likely to result in loss of function, since they are both frameshift mutations disrupting most of the coding sequence of the gene.

There are three major implications of these data. First, mutations of p57^{KIP2} account for a small fraction of BWS—only 5% of the large number of patients studied here, which is even less than the frequency (22%) in

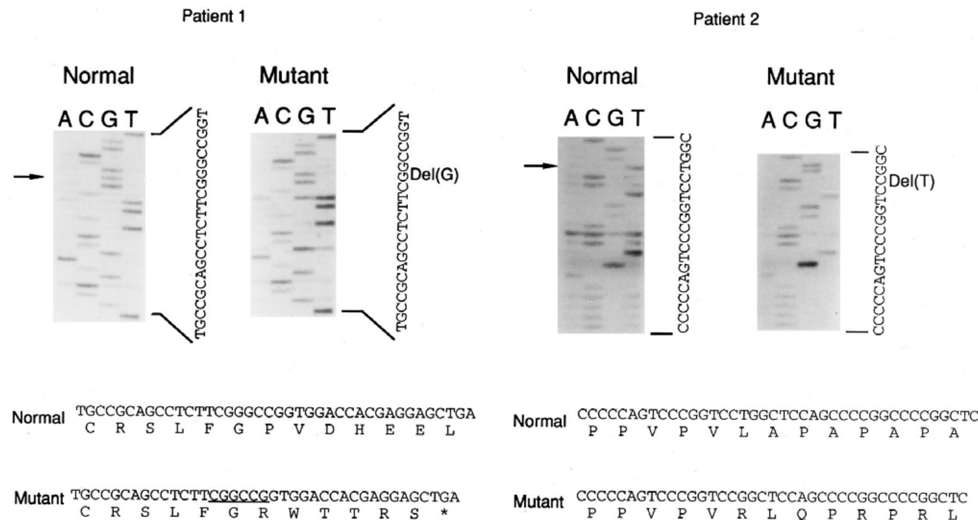

Figure 2 p57^{KIP2} mutations in BWS patients. *Left,* Patient 1. The PCR fragment from primer set 1 was subcloned in PCR2.1. Four subclones from both normal and mutant alleles were sequenced, giving identical results. The mutant allele showed deletion of a single nucleotide G, as indicated. Note the generation of an *Eag*I site, CGGCCG (underlined in the figure) in the mutant allele, and the frameshift creating a truncated protein with 24 N-terminal amino acids plus 6 new amino acids. *Right,* Patient 2. The PCR fragment from primer set 4 was subcloned. Two subclones from both normal and mutant alleles were sequenced. A single nucleotide-T deletion was detected in the mutant allele, as indicated.

the smaller series (nine patients) reported recently by Hatada et al. (1996). What could account for this low frequency? One possibility is that the mutations are rare null polymorphisms unrelated to BWS. This possibility seems unlikely, since similar mutations were not seen, by us or others, in non-BWS patients. In one of our patients (patient 1) and in one of the Japanese patients, the mother also carried the mutation, suggesting a functional defect, since the maternal allele of p57^{KIP2} is normally expressed. The family of patient 1 studied here also shows cosegregation of the mutation with the disease over three generations. A second possibility is that most BWS arises by virtue of imprinting errors leading to silencing of p57^{KIP2}. We earlier described biallelic expression of p57^{KIP2} in a patient with BWS, which could be due either to increased expression of the paternal

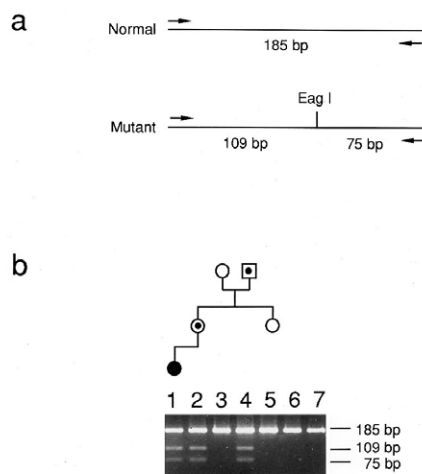

Figure 3 Cosegregation of the mutant allele with disease, in the family of patient 1. *a,* Restriction-digestion map of PCR fragment 1. The predicted size of the normal allele is 185 bp. The mutant allele acquires an *Eag*I site (see underlined sequence CGGCCG in fig. 2, *left*). Digestion of the PCR fragment with *Eag*I generates 109-bp and 75-bp fragments. *b, Eag*I digestion of PCR fragments from patient 1, other family members, and unrelated individuals. Lane 1, Patient 1. Lane 2, Mother. Lane 3, Maternal grandmother. Lane 4, Maternal grandfather. Lane 5, Maternal aunt. Lanes 5 and 6, Two unrelated individuals. The sizes of the three DNA fragments are indicated on the right side. Note that patient 1, her mother, and the maternal grandfather are all affected, and they are all heterozygous for the mutation.

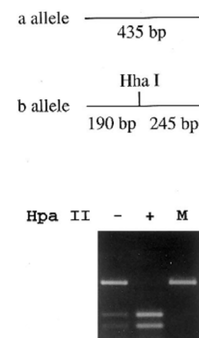

Figure 4 Paternal origin–specific methylation of H19 unaffected in patient 1. The diagram indicates the expected amplification products of two alleles, with use of primer set H19 forward and reverse primers. The a allele gives a 435-bp fragment, wheres the b allele gives two fragments, of 190 bp and 245 bp, because of the presence of a *Hha*I site. Minus signs (−) and plus signs (+) indicate, respectively, absence and presence of *Hpa*II digestion before PCR amplification; "M" denotes DNA from the mother of patient 1.

allele or to relative silencing of the maternal allele to a level comparable to that of the paternal allele (Thompson et al. 1996). However, the frequency of this alteration also appears to be low. A third possibility is that BWS involves epigenetic silencing of $p57^{KIP2}$ by an imprint-independent mechanism. We earlier observed reduced expression of $p57^{KIP2}$ in the hyperplastic tongue of six BWS patients, but the gene was also found to be transcriptionally inactive in the tongue of normal patients of comparable age (Thompson et al. 1996). We are currently examining the expression of $p57^{KIP2}$ in affected prenatal tissues, to test this hypothesis.

The second major implication of these data is that mutations in $p57^{KIP2}$ can be associated with BWS when they are transmitted from the father. The mother of patient 1 had most of the clinical features of BWS, including macroglossia, ear creases, and craniofacial anomalies. Although the proband, patient 1, had inherited the mutation from her mother, the mother had inherited her own mutant allele from her father, albeit with milder features. This is the first time that a nonsense mutation in an imprinted gene has been reported as causing disease when inherited from the presumed nonexpressing chromosome. The implication of this observation is that the paternal allele must also be expressed in at least some affected tissues at a critical stage of development. We have reported elsewhere that imprinting of $p57^{KIP2}$ gene is leaky (Matsuoka et al. 1996). Thus, haploinsufficiency due to mutation in either the maternal or the paternal allele may cause at least some features of BWS. Third, mutation in $p57^{KIP2}$ does not act indirectly by altering methylation of H19 gene, at least at the site examined here.

Finally, these data suggest that more than one gene is involved in the pathogenesis of BWS, which might explain the relatively low frequency of $p57^{KIP2}$ mutations. Thus, in addition to inactivation of the $p57^{KIP2}$ gene, upregulation of IGF2 also likely plays a role, since paternal duplication in some BWS patients (Mannens et al. 1994) would have no effect on $p57^{KIP2}$, and some BWS patients show loss of imprinting and biallelic expression of IGF2 in their nontumor tissues (Weksberg et al. 1993; Steenman et al. 1994). In addition, we have recently demonstrated that five BWS balanced germ-line chromosomal-rearrangement breakpoints located in 11p15.5 disrupt the maternally expressed K_vLQT1 gene (Lee et al. 1997), implying that inactivation of K_vLQT1 may also play a causative role in BWS. Some genetic alterations might thus affect the expression of multiple imprinted genes; for example, UPD involves duplication of the paternal copy and loss of the maternal copy of 11p15 (Henry et al. 1991), resulting, respectively, in duplication of the expressed allele of IGF2 and loss of the expressed allele of $p57^{KIP2}$ and K_vLQT1. Conceivably, disruption of K_vLQT1 might cause abnormal imprinting of both IGF2 and $p57^{KIP2}$. Consistent with this hypothesis, it has recently been found that at least one chromosomal-rearrangement patient shows loss of imprinting of IGF2 (Brown et al. 1996). The low frequency of $p57^{KIP2}$ mutations reported here, as well as our recent discovery of disruption of the K_vLQT1 gene in patients with chromosomal rearrangements, suggest that BWS can involve disruption of multiple 11p15.5 genes.

Acknowledgments

This work was supported by National Institutes of Health grant CA54358 and March of Dimes grant 6-FY96-1048. We thank J. Patey for preparing the manuscript.

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