Family with Bannayan-Riley-Ruvalcaba Syndrome: Linkage...

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ABSTRACT

The purpose of this study was to locate the gene responsible for Bannayan-Riley-Ruvalcaba syndrome in an affected family. DNA was isolated from 13 members of the family and linkage studies were conducted. Twelve markers encompassing a specific region on chromosome 10 (10q23) were used in linkage analysis. DNA segments specific for each primer set were amplified using PCR (polymerase chain reaction) techniques and then analysis of the products was conducted using the ABI genetic analyzer. Alleles were then assigned to each family member and linkage was tested (LINKAGE 5.0). A maximum LOD score of 0.98 was obtained at marker D10S573. This gave an indication of linkage in the family but no definitive conformation was possible. Crossover events in the family were also examined in an attempt to localize the gene responsible for Bannayan-Riley-Ruvalcaba syndrome. All segments on chromosome 10 were ruled out for the BRR gene except the region between markers D10S215 and D10S2470.
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS ................................................. i

ABSTRACT .......................................................... ii

TABLE OF CONTENTS ................................................ iii

LIST OF ILLUSTRATIONS ............................................. iv

LIST OF TABLES ..................................................... v

INTRODUCTION AND LITERATURE REVIEW

BANNAYAN-RILEY-RUVALCABA SYNDROME ......................... 1
COWDEN SYNDROME .................................................. 8
SIMILARITIES BETWEEN BRRS AND CS ............................ 9
PTEN/MMAC1 GENE .................................................. 11
LINKAGE STUDIES ................................................... 12
OUR RESEARCH ...................................................... 13

MATERIALS AND METHODS

DIAGNOSIS OF FAMILY MEMBERS ................................ 17
DNA ISOLATION ..................................................... 18
SAMPLE HANDLING AND PROCESSING ............................... 20
ESTIMATION OF DNA CONCENTRATION ............................ 22
PCR AMPLIFICATION ............................................... 22
AGAROSE GEL ELECTROPHORESIS ................................. 25
PCR PURIFICATION .................................................. 27
ABI ANALYSIS ...................................................... 28
LINKAGE ANALYSIS ................................................ 30

RESULTS ............................................................... 32

DISCUSSION .......................................................... 43

REFERENCES .......................................................... 49
LIST OF ILLUSTRATIONS

Fig. 1  Markers and Positions Relative to Chromosome 10 ....................... 16

Fig. 2  Pedigree of Bannayan-Riley-Ruvalcaba Syndrome Family ............... 33

Fig. 3  Picture and Characteristics of Affected Individual III:2 .............. 34

Fig. 4  Picture and Characteristics of Affected Individual III:9 .............. 35

Fig. 5  Haplotypes Showing Possible Region for BRR Syndrome Gene ........ 42
LIST OF TABLES

Table 1. Major Signs of Bannayan-Riley Ruvalcaba Syndrome .................... 7

Table 2. Overlap of BRR Syndrome and Cowden Syndrome in Family ............ 15

Table 3. Affected Alleles Passed in Family ........................................ 38

Table 4. LOD Score Table For Specific Markers in Family ........................ 39
INTRODUCTION AND LITERATURE REVIEW

BANNAYAN-RILEY-RUVALCABA SYNDROME

Bannayan-Riley-Ruvalcaba (BRR) syndrome is a genetic condition characterized by a variety of signs such as macrocephaly, multiple lipomas, and skeletal abnormalities. Manifesting as a rare syndrome which appears during childhood, BRR syndrome shows an autosomal dominant pattern of inheritance with variable penetrance and expressivity. Slight male predominance has been found in the disorder. The BRR syndrome is considered to be one disorder in a family of macrocephaly-dominated genetic syndromes.

Over the past twenty years, similar conditions have been described in the literature pertaining to disorders inherited in an autosomal dominant fashion. Riley and Smith reported a case in 1960 with macrocephaly, pseudopapilledema, and multiple hemangiomas. In 1971, Bannayan described a patient with macrocephaly, multiple lipomas, and hemangiomas (Bannayan 1971). Zonana and coworkers independently described a patient similar to the patient described by Bannayan in 1976 and suggested the disorder followed an autosomal dominant pattern of inheritance. In 1980, Ruvalcaba et al. diagnosed two male patients with Sotos syndrome. Characteristics of the patients included hamartomatous intestinal polyps and
freckles on the penis (Ruvalcaba et al. 1980). Cohen proposed that the syndrome described by Ruvalcaba (1980) was not Sotos syndrome and designated a new name for this condition, Ruvalcaba-Myhre syndrome (Cohen 1982). At this point, three similar genetic syndromes (Riley-Smith, Bannayan-Zonana, and Ruvalcaba-Myhre) had been designated by their respective authors. Dvir et al. then described a patient with characteristics of each of the three previously described syndromes in 1988. These characteristics included macrocephaly, pseudopapilledema, lipomas, and spotted pigments on the penis (Dvir et al. 1988). Because of this new evidence, Dvir et al. proposed the three conditions were actually different expressions of a single heterofamilial disorder and proposed the name macrocephaly, hamartomas, and papilledema syndrome (1988). In 1992, Gorlin et al. described a family which provided additional support for the unification of the three syndromes into one due to clinical overlap of many signs (Gorlin et al. 1992). The name eventually adopted was suggested by Cohen in 1990. The new syndrome was called Bannayan-Riley-Ruvalcaba syndrome (Cohen 1990).

The Bannayan-Riley-Ruvalcaba syndrome has only recently been classified as a distinct genetic entity, thus research pertaining to this condition is still at an early stage. Although BRR syndrome can be distinguished from other
Macrocephaly and hamartomatous disorders, such as Cowden syndrome (CS), classification is difficult for individual patients with BRR syndrome. This difficulty arises in the fact that no classification standard has been proposed for diagnosis of BRR syndrome.

One important sign most often associated with BRR syndrome is macrocephaly. Macrocephaly refers to an increased occipto-fronto circumference (OFC), at least 2 standard deviations above the mean for both age and sex (Gorlin et al. 1992). The OFC in patients diagnosed with macrocephaly is usually greater than the 95th percentile. Once macrocephaly and autosomal dominant inheritance are established, further classification of a family may proceed. There are several autosomal dominant macrocephaly syndromes, including Bannayan-Riley-Ruvalcaba syndrome and Cowden syndrome. Several authors have implied that these syndromes may represent phenotypic variability at a single genetic locus (DiLiberti 1991). Although the autosomal dominant macrocephaly syndromes have many overlapping clinical expressions, a definitive classification of each syndrome may be made according to distinct and specific manifestations.

The most common clinical findings for BRR syndrome include "macrocephaly with normal ventricular size, multiple subcutaneous or visceral lipomas and vascular malformations,
and skeletal abnormalities” (Fargnoli et al. 1996). Associated with the major findings of BRR syndrome (Table 1) are a host of other associated characteristics. These are found throughout the body and in various systems, including the craniofacial features, the skin, the gastrointestinal system, and the skeletal system.

Craniofacial features of BRR syndrome include macrocephaly, as noted above, and prominent Schwalbe lines or enlarged corneal nerves in approximately 35 per cent of affected individuals (Fargnoli et al. 1996). Down-slanting palpebral fissures are found in approximately 60 per cent of the reported cases while strabismus or amblyopia are found in only 15 per cent (Gorlin 1992). A high palate and prolonged drooling may also be seen in some patients (Fargnoli et al. 1996).

Characteristics associated with the skin are diverse in BRR syndrome. A defining characteristic of the syndrome found in most affected males is pigmented macules found on the penile shaft. A small number of cafe-au-lait spots can be found on the trunk and lower extremities. Accessory nipples are also quite common (Cohen 1990).

Hamartomatous polyps are found in the distal ileum and colon in approximately 45 per cent of patients (Gorlin 1992). The term hamartoma refers to a benign, hyperplastic, and disorganized growth. Although malignant tumors are not
hallmarks of BRR syndrome (Marsh et al. 1997), they have been noted in patients presenting with the disorder (Cohen 1990). Tumors found include hemangiomas, lipomas, meningiomas, and lymphangiomas (Higginbottom et al. 1982). Mesodermal hamartomas are found in many patients with BRR syndrome and most are subcutaneous. The hamartomas present themselves as discrete lipomas in 75 per cent of cases, hemangiomas in 40 per cent of the cases, and lymphangiomas in 10 per cent of the cases (Cohen 1990).

Joint hyperextensibility, pectus excavatum, and scoliosis have been reported in some patients with BRR syndrome (1990). Overgrowth is usually present during early stages of development, but final adult height is usually within normal range for affected individuals (Hamartoses 1994). Birth weight is usually in excess of 4000 grams (Cohen 1990).

Muscle biopsies have shown neutral fat accumulation in 60 per cent of individuals. This occurs predominantly in enlarged Type I skeletal muscle fibers and has been treated successfully with carnitine (Higginbottom et al. 1982).

A variety of other abnormalities have been associated with BRR syndrome through expanding recognition of affected individuals. The most important of the additional abnormalities associated with this condition include mental retardation (Saul et al. 1982), seizures (Cohen 1990),
central nervous system vascular malformations, and thyroid tumors (Fargnoli et al. 1996).
Table 1. MAJOR SIGNS OF BANNAYAN-RILEY-RUVALCABA SYNDROME

<table>
<thead>
<tr>
<th>Affected Region</th>
<th>Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Face</td>
<td>macrocephaly, high/prominent forehead, downslanting fissures, high-arched palate</td>
</tr>
<tr>
<td>Central Nervous System</td>
<td>macrencephaly, hypotonia/drooling, developmental delay, MR, seizures, tumors</td>
</tr>
<tr>
<td>Skin</td>
<td>CAL, nevi, lentigines of the penis/vulva, acanthosis nigricans, tags, verrucous facial lesions</td>
</tr>
<tr>
<td>Eye</td>
<td>visible corneal nerves, prominent Schwalbe lines, pseudopapilledema, strabismus/amblyopia</td>
</tr>
<tr>
<td>Skeletal System</td>
<td>pectus excavatum, limb asymmetry, broad thumbs/distal phalanges, supernumerary nipples, macrodactyly, scoliosis, myopathy</td>
</tr>
<tr>
<td>Hamartomas (mesodermal)</td>
<td>nevus flammeus, cavernous hemangioma, AV fistula, angiokeratoma, lipoma, lymphangioma, leiomyoma, gastrointestinal polyps</td>
</tr>
<tr>
<td>Thyroid</td>
<td>Hashimoto’s, adenomas, carcinoma</td>
</tr>
</tbody>
</table>

*table adapted from Shodair Children’s Hospital medical genetics laboratory, Helena, MT.*
COWDEN SYNDROME

Cowden syndrome is an autosomal dominant macrocephaly syndrome similar to BRR syndrome. CS was first described by Lloyd and Dennis in 1963 (Hanssen et al. 1995). Recently, the gene responsible for CS has been localized to 10q23 and when isolated was given the name PTEN/MMAC1. CS shows variable expression and is characterized by hamartomas of multiple organ systems derived from ectoderm, mesoderm, endoderm (Marsh et al. 1997). It can be considered a familial cancer syndrome with a high risk of breast and thyroid carcinoma. Other cancers include adenocarcinoma of the colon, liposarcoma, squamous cell cancer, melanoma, teratocarcinoma, neuroma, and meningioma. Benign tumors are also common in CS.

Main characteristics of CS include macrocephaly, hamartomas, central nervous system manifestations, thyroid abnormalities, multiple, early-onset uterine leiomyoma, and mental retardation (Eng 1997). The hallmark signs of CS, found in almost all cases, are facial trichilemmomas and papillomatous papules.

Similar to the other autosomal dominant macrocephaly syndromes, CS is rare and many cases are apparently sporadic. Until 1993, there were only 160 reported cases of CS in the world (Eng 1997).
Hamartomas are present throughout the patient and can be found in any organ or germ layer. Such locations for these hamartomas include the skin, breast, thyroid, oral mucosa, and intestinal epithelium. Pathognomonic hamartomatous features include multiple smooth facial papules, acral keratosis, and multiple oral papillomas (Nelen et al. 1996). Other features of CS include central nervous system manifestations, such as megalencephaly, epilepsy, and dysplastic gangliocytomas of the cerebellum (Nelen et al. 1996). Lhermitte-Duclos disease, a central nervous system disorder, has recently been associated with CS as well as BRR syndrome (Eng 1997).

SIMILARITIES BETWEEN BRRS AND CS

Bannayan-Riley-Ruvalcaba syndrome and Cowden syndrome are autosomal dominant genetic syndromes that share many common manifestations. It has been hypothesized that the similarities may represent different alleles of the same genetic locus (10q23) or mutations of two separate genes in a common pathway (Fargnoli et al. 1996). More recently, the overlap of signs combined with clinical, molecular, and cytogenetic findings suggest that both BRR syndrome and CS are caused by a loss of function of the PTEN gene (Arch et al. 1997). If this is true, the two syndromes are allelic; different mutations in the same gene could be responsible
for the different syndromes. This idea is supported by a patient described with BRR syndrome who had a deletion of the PTEN gene, a gene known to cause CS (Arch et al. 1997).

Major characteristics common to both BRR syndrome and CS include facial papules (trichilemmomas and verrucae), pigmented macules of the genitalia, cafe-au-lait spots, lipomas, and vascular malformations. Additional shared characteristics include macrocephaly, high arched palate, goiter, adenoma, carcinoma, hamartomatous polyps, and mental retardation (Fargnoli et al. 1996).

Characteristics found in BRR syndrome but not CS include down slanting palpebral fissures, joint hyperextensibility, seizures, prominent Schwalbe lines, and pseudopapilledema. Thyroid carcinoma is found in BRR patients. Hypotonia, lipid storage myopathy, and persistent drooling are also characteristics of BRR syndrome exclusively (Fargnoli et al. 1996).

Cowden syndrome can be distinguished from BRR syndrome in its characteristic adenoid facies, ductal papillomas, adenocarcinomas, menstrual irregularities, myopia, and wide variety of associated malignant tumors. Cowden syndrome can also be distinguished from BRR syndrome through major differences in skin lesions (Fargnoli et al. 1996).
A candidate tumor suppressor gene, PTEN, was isolated and characterized by researchers in 1997 (Arch et al. 1997). This gene was localized between regions 10q23.1 and 10q24.1. PTEN stands for Phosphatase and Tensin Homolog Deleted on Chromosome 10. Independently, another group of researchers isolated the same gene and called it MMAC1 (Steck et al. 1997). They found the MMAC1 gene to encode a widely expressed 5.5 kb mRNA producing a polypeptide of 403 amino acids with a PTPase catalytic domain. According to the researchers, loss of function of the tumor suppressor gene at 10q23.3 appears to be associated with oncogenesis of multiple human cancers (Steck et al. 1997). Other researchers speculate that disruptive germline mutations in the PTEN gene may cause a proliferative and disorganizational tendency, leading to hamartoma formation (Liaw et al. 1997). Several regulation processes PTEN may be involved in include cell growth regulation, tumor cell invasion, angiogenesis, or metastasis (Li et al. 1997).

A susceptibility gene for CS was located and assigned to a 1-cM interval between markers D10S215 and D10S541. This is the same region in which the candidate tumor suppressor gene PTEN is found (Law et al. 1997). Nelen et al. confirmed the PTEN gene was, in fact, the susceptibility...
gene for CS and refined the interval between D10S1761 and D10S541 (Nelen et al. 1997).

PTEN/MMAC1 is the gene that is responsible for CS. If BRR syndrome is allelic to CS as hypothesized by many researchers, the PTEN gene would also be responsible for BRR syndrome. Different mutations could lead to distinct conditions, namely BRR syndrome, CS, or other possible syndromes associated with the gene.

**LINKAGE ANALYSIS**

Linkage studies can be used to predict inheritance and map genetic disorders, a concept discussed by Mendel at the frontier of genetic studies. When linkage is present, specific alleles for different genes are co-segregated or co-inherited by offspring. For a DNA segment included within or in close proximity to the actual gene, a specific marker allele will be inherited by all the affected members of the family (Johnson 1988). A "phase" is established by noting the marker allele inherited with the gene. In addition, the further the marker is located away from the gene of interest, the greater the likelihood of a crossover, in which the specific allele is not coinherited with the syndrome. "The physical distance between the marker and the disease gene is proportional to the probability that a
crossover (recombination) will occur during meiosis and disrupt the phase relationship” (Johnson 1988).

OUR RESEARCH

Bannayan-Riley-Ruvalcaba syndrome is a member of the family of autosomal dominant macrocephaly syndromes but has only recently been described as an encompassing entity of the Bannayan-Zonana, Riley-Smith, and Ruvalcaba-Myhre syndromes. It can usually be distinguished from Cowden syndrome. The problem in diagnosis of BRR syndrome is variable expressivity for major characteristics. Many associated characteristics for the syndrome have also been described in the literature and make classification of affected individuals more complicated. Cowden syndrome has been studied extensively and a set standard exists for diagnosis of affected individuals. No such standard has been developed for BRR syndrome thus, at this time, diagnosis must be made without the aid of standards.

Similarity in a variety of characteristics between BRR syndrome and CS in the family we studied led myself, Dr. Johnson, and Linda Beischel to believe the two syndromes may be allelic (see Table 2). Both syndromes have autosomal dominant inheritance, macrocephaly, hamartomas, and a host of similar signs. The gene responsible for CS, PTEN, was localized to region 10q23 and characterized at the early
stages of our research. We focused on this area as a potential site for the BRR syndrome gene. If the two syndromes are in fact allelic, a mutation in the PTEN gene would be the cause of BRR syndrome. Another possibility is that a distinct gene located close to the PTEN gene could be responsible for the syndrome.

With these ideas in mind, we ran a linkage study on the affected family with markers encompassing the region of the PTEN gene on chromosome 10 (Fig 1). The purpose of this study was to localize the gene responsible for Bannayan-Riley-Ruvalcaba syndrome using linkage studies in an affected family, hypothesizing that the PTEN gene is the cause of BRR syndrome.
Table 2. OVERLAP OF BRR AND COWDEN SYNDROMES IN FAMILY

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Family</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dominant inheritance</td>
<td>11/14</td>
</tr>
<tr>
<td>Macrocephaly</td>
<td>7/10</td>
</tr>
<tr>
<td>Lipomas</td>
<td>4/10</td>
</tr>
<tr>
<td>GI polyps</td>
<td>2/10</td>
</tr>
<tr>
<td>Facial papules</td>
<td>1/10</td>
</tr>
<tr>
<td>PPK/keratoses</td>
<td>0/10</td>
</tr>
<tr>
<td>Skin tags (acrochordons)</td>
<td>4/10</td>
</tr>
<tr>
<td>Vascular hamartomas</td>
<td>3/10</td>
</tr>
<tr>
<td>Pectus excavatum</td>
<td>2/10</td>
</tr>
<tr>
<td>High arched palate</td>
<td>7/10</td>
</tr>
<tr>
<td>&quot;Scrotal&quot; tongue</td>
<td>3/10</td>
</tr>
<tr>
<td>Thyroid CA/adenoma/goiter/Hashimoto’s</td>
<td>3/10</td>
</tr>
<tr>
<td>CNS tumors</td>
<td>1/10</td>
</tr>
<tr>
<td>Penile lentigines</td>
<td>2/3</td>
</tr>
<tr>
<td>Ovarian cysts</td>
<td>1/6</td>
</tr>
<tr>
<td>Uterine fibroids</td>
<td>1/6</td>
</tr>
</tbody>
</table>

*table adapted from Shodair Children's Hospital medical genetics laboratory, Helena, MT.*
Figure 1. MARKERS USED IN LINKAGE STUDY AND POSITIONS RELATIVE TO CHROMOSOME 10

D10S676
D10S1432
D10S1427
GATA134F03
D10S573
D10S215
D10S541
GATA115E01
D10S1242
D10S1229
D10S677
D10S1240
MATERIALS AND METHODS

DIAGNOSIS OF FAMILY MEMBERS

Thirteen members from a single family were used in this study. DNA was taken from each of the 13 members and genetic analysis (ABI) was performed. Prior to analysis of DNA, each member was classified as affected or unaffected with Bannayan-Riley-Ruvalcaba Syndrome. This classification was assessed by Dr. John Johnson, the Director of Medical Genetics at Shodair Children’s Hospital in Helena, MT, and confirmed by colleagues. Diagnosis was determined by certain characteristics present in the Bannayan-Riley-Ruvalcaba Syndrome. Since well defined criteria for diagnosis have yet to be established with this syndrome, the classification was subjective. However, certain characteristics were considered to be hallmarks of the syndrome (macrocephaly, lipomas, hamartomas, cafe au lait spots, high palate). To be classified as affected, each individual had to have either macrocephaly and a second prominent sign or two prominent signs of the syndrome at a minimum. In the family of 13, 10 individuals were classified as affected with Bannayan-Riley-Ruvalcaba syndrome. Details of the family are discussed further in the INTRODUCTION AND LITERATURE REVIEW (Table 2).
DNA ISOLATION

Prior to testing and analysis, DNA was isolated from whole blood samples of each individual in the Bannayan-Riley-Ruvalcaba family described in this research. The Puregene DNA Isolation Kit D-5000 (Gentra systems, Minneapolis, Minn) was used to isolate DNA from leukocytes in the peripheral blood. The kit consists of "RBC Lysis solution" (Ammonium Chloride, EDTA, Sodium Bicarbonate), "Cell lysis solution" (Tris, EDTA, SDS), "Protein Precipitation Solution" (Ammonium Acetate), "DNA Hydration Solution" (Tris, EDTA), and RNase A Solution (4 mg/ml).

Briefly, this procedure included addition of a solution of low salt concentration to whole blood in order to lyse red blood cells. A cell lysis solution, containing SDS detergent, was then added to lyse white blood cells. Extraneous proteins were then extracted using a solution of high salt concentration. Finally, DNA was precipitated from the remaining solution using alcohol. The precipitated DNA was re-dissolved in a dilute salt solution for storage.

In application of the procedure, aseptic techniques were used to avoid contamination of the sample and the technician. The whole blood sample was poured into a sterile plastic centrifuge tube. Volume was estimated to the nearest 0.5 ml from markings on the side of the tube and was considered "one volume" for subsequent steps.
A total of three volumes of RBC Lysis Solution was added to the blood sample, mixed by inversion, and incubated at room temperature for 10 min. A small amount of this solution was used to rinse out the collection tube. The mixed solution was then centrifuged for 10 min at 3200 RPM (Program 3, IEC Centra, 2000G). After completion of this step, the supernatant was discarded into the sink, retaining only the visible white cell pellet and approximately 0.2 ml of residual liquid. Next, the centrifuge tube was vortexed vigorously to evenly resuspend the cell pellet. One volume of Cell Lysis Solution was added while pipetting vigorously to lyse white blood cells. 0.005 ml RNAse solution was then added to the cell lysate per 1 ml of original volume, inverted 25 times to mix, and incubated in a 37° C water bath for 15 min.

After incubation, the sample was cooled to room temperature and one-third volume Protein Precipitation Solution was added to the lysate. This mixture was vortexed vigorously for 20 sec and centrifuged at 3200 rpm for 10 min. Next, one volume of isopropanol was poured into a new centrifuge tube in addition to the supernate from the protein precipitation step. The precipitated protein pellet was discarded while the supernate and isopropanol were mixed by gently inverting 50 times. This generated white threads of DNA in a visible clump. Using a sterile plastic
inoculating loop (Nunc), the precipitated DNA was transferred into a 1.5-ml microfuge tube containing 1 ml of 70% ethanol in water. The sample was centrifuged for one min (Eppendorf centrifuge 5415) at 14000 RPM to pellet the DNA sample. After centrifugation, the supernate was poured off and excess drops were removed with a sterile swab. The sample was allowed to air dry for 10 min.

In final preparation, one-tenth volume DNA Hydration Solution was added to the pellet and mixed to dissolve. This solution was heated to approximately 50° C for 1 hr to speed dissolution. After dissolution, the DNA concentration was estimated using the Hoefer fluorometer (see method) and the sample was stored at 4° C.

SAMPLE HANDLING AND PROCESSING

Working in a molecular genetics lab requires precision and care. Aseptic techniques must be consistently used in order to protect both the sample and the technician. The following section pertains to sample handling and processing and is taken from the Shodair Hospital Medical Genetics Laboratory Procedure Manual.

In general, any material which contains or did contain nucleated cells may be a source of DNA. When using whole blood specimens, standard universal precautions should be
employed for phlebotomy and all laboratory procedures. Such precautions include use of latex gloves and other protective equipment, proper disposal of blood products, and safe handling of needles.

Prior to any research taking place, each specimen is entered into the general laboratory log book, the computer database, and the DNA log book. Pertinent information includes the patient’s name, date of birth, type of specimen, date and time arrived, when drawn, referring physician or laboratory, and test requested. In addition to this, each specimen is assigned a sequential identification number, which begins with the last two digits of the year (97-001, etc.).

DNA samples are stored in microfuge tubes at 4° C in plastic boxes, organized by year. Each sample is labeled with the sample number, the patient’s name, and the DNA concentration. DNA is stable indefinitely at 4° C, although the concentration often increases due to solvent evaporation.
ESTIMATION OF DNA CONCENTRATION

After extraction, the concentration of each DNA sample was estimated. This was accomplished using the Hoefer fluorometer (TKO 100) in accordance with instructions provided by the manufacturer. Cuvettes were rinsed with water and ethanol before and after each use.

Solution A (5 ml TNE), 0.005 ml Hoechst dye 33258 (1 mg/ml), with water to total volume of 50 ml, was made fresh for each use. After the instrument warmed up for 10 min, 2 ml of Solution A were placed in the clean cuvette. The fluorometer was adjusted to read "000" (+/- 001). 0.002 ml of the low DNA standard (100 μg/ml) was then mixed with the 2 ml of Solution A. The resulting solution gave a reading of 100 +/- 10. Two ml of Solution A were then added to the cuvette, the reading was adjusted to 0, and 0.002 ml of the test DNA sample were added and mixed. The results were recorded and if the concentration of any sample read over 400-μg/ml, the measurement was repeated using 0.001 ml of sample. This reading was doubled and then recorded.

PCR AMPLIFICATION

PCR amplification was performed on each member of the Bannayan-Riley-Ruvalcaba family described in this research. Overall, multiple markers were run for each family member
but for a given set of PCR amplifications, a single marker was run on each testable family member. Certain members of the family were diagnosed with Bannayan-Riley-Ruvalcaba syndrome while others were not, as described previously.

The procedure for PCR amplification was taken from the Shodair Hospital Medical Genetics Laboratory Procedure Manual. PCR set-up takes place in the laminar flow hood to minimize contamination from amplified PCR products and the technologist. However, the amplified PCR products are analyzed in the main lab area. A set of pipettes are reserved for PCR setup and are cleaned with dilute ethanol and bleach before each use. Pipette tips with aerosol filters (Rainin CP-10F) are used whenever possible, and always for addition of DNA template and DNA polymerase to the reactions. DNA samples are diluted to 63 ng/μl in 1X TAQ polymerase buffer. PCR reactions are performed in 200-μl microtubes which fit the Perkin-Elmer 2400 thermocycler (Perkin-Elmer N801-05333). Reaction volume is normally 25 μl.

First, a master mix was prepared which contained all necessary reactants except DNA and polymerase. These reactants were pooled together and then distributed to each tube in a specific amount. Each reaction tube contained the equivalent of 2.5 μl of 10X PCR (Taq polymerase) buffer containing 1.5 mM MgCl₂, 0.5 μl dNTP (40 μmol final
concentration dATP, dCTP, dGTP, dTTP; 2 mM stock), 0.25 μl each of forward and reverse primer as appropriate for the specific reaction, 0.5 μl of dye (RG6, R110, or Tamra) necessary to differentiate samples in ABI analysis, 2.5 μl RediLoad dye (for enhancement of PCR and visualization of product), and water were added to bring the solution to a total volume of 23 μl. Two μl (125 ng) of the appropriate DNA sample were then added to each tube. One μl of Taq polymerase (1.25 U of a 1:4 dilution of 5 U/μl) was added after starting the thermal cycling reaction in order to achieve a "hot start".

This procedure was repeated for each primer pair (obtained from Research Genetics) to be used for amplification. Research Genetics provides primers with desired sequences in its catalog ("Map Pairs"). Primers for some amplifications were labeled with fluorescent dye at the time they were synthesized to allow specific analysis on the ABI.

Once the reaction tubes were prepared, an appropriate thermocycler program was selected. The tubes were placed in the thermocycler after a denaturation temperature of 94° C was reached. After a five min denaturation, polymerase was added to the mixture while the reaction was paused at 80° C ("hot start"). After addition of the polymerase, the
thermocycler program resumed and began to cycle. First, the mixture was heated to 94° C for 30 sec to denature the DNA. Next, the mixture was cooled to 58° C for 30 sec to allow the primers to anneal. The mixture was then heated to 72° C for 30 sec to allow the DNA to amplify (new DNA synthesis). Twenty five cycles were run for this program. Finally, the temperature was held at 72° C for 7 min and the DNA was then cooled to 4° C upon completion of the run.

**AGAROSE GEL ELECTROPHORESIS**

Agarose gel electrophoresis was run after each PCR amplification process to assess the integrity of isolated DNA. In addition, the presence and size of PCR products was checked prior to ABI analysis. The procedure for agarose gel electrophoresis was taken from the Shodair Hospital Medical Genetics Laboratory Procedure Manual.

First, gels were prepared by melting agarose into the appropriate volume of buffer solution in a microwave oven. The concentration of agarose used was 1.5% of LE (FMC) in TAE buffer. After melting for 1-3 min and swirling the beaker under cold running water, the mixture was poured into a gel casting apparatus where it was allowed to solidify with a sample loading comb in place. The gels required approximately 40 ml of molten agarose solution. After
solidification, the gel was overlaid with a thin layer of buffer and the comb was removed. Rubber dams, which help to maintain gel integrity, were also removed from the gel. Buffer troughs were then filled and the gel was covered by a minimum volume of electrophoresis buffer. Next, PCR reactions (5 μl of 25 μl) were loaded into the sample wells. DNA size markers of appropriate size were run when possible to allow for sample fragment size estimation ("100 bp ladder" from Research Genetics).

The PCR gels were run in 1X TAE buffer at 80 volts for about 30 min. During the run, the gel was cooled with an ice pack. Immediately after the run, the gel was stained in ethidium bromide (2 μg/ml; stock is at 10 mg/ml; 1/5000 dilution in the soaking solution). Results were visualized in the dark room on a Fisher 300-nm UV transilluminator. Pictures of each gel were taken using a Fisher Polaroid camera with "deep yellow 15" filter and Polaroid 557 film. The F stop value for this camera is 16 and exposure time is approximately 2 sec. The distance from the gel to the camera is set with hoods of assorted sizes which completely cover the gel and block out light.
PCR PURIFICATION

Once PCR amplification has taken place and products have been analyzed using agarose gel electrophoresis, PCR products must be purified prior to ABI analysis. Purification was accomplished with the QIAquick system (Qiagen). In the QIAquick system, DNA is bound to a solid matrix in a buffer of high salt concentration while non-DNA products are washed away. Concentrated DNA is then eluted in a buffer of low salt concentration. The QIAquick protocol, using QIAquick spin columns in a microcentrifuge, is designed to purify DNA products from primers, nucleotides, polymerases, and salts.

First, five volumes of Buffer PB were added to and mixed with one volume of the PCR reaction. The QIAquick spin column was then placed in a 2-ml collection tube. The sample was then applied to the QIAquick column and centrifuged for 60 sec (14,000 rpm) to bind DNA. Immediately after centrifugation, flow-through was discarded into the sink and the QIAquick column was reinserted into the same 2-ml collection tube. 0.75 ml Buffer PE (ethanol must be added to Buffer PE before use) was then added to each column and the mixture was centrifuged for an additional 60 sec (14,000 rpm). The flow-through was again discarded into the sink and the QIAquick column was placed into the same 2-ml collection tube. The column was then
centrifuged for an additional minute at maximum speed. Next, the QIAquick column was placed into a clean 1.5-ml microfuge tube. Finally, to elute the purified DNA, 30 μl of elution buffer TE (10mM Tris-HCl, 1 mM EDTA, pH 8.0) were added to the center of the QIAquick column. This mixture was allowed to stand for 1 min before it was centrifuged at maximum speed for 1 min.

**ABI ANALYSIS**

After the purification step was completed for each PCR amplified DNA sample, capillary electrophoresis (ABI analysis) was performed to size the fluorescently-labeled products of PCR amplifications. Sizing accuracy was within one base-pair. Samples were run and analyzed to determine which alleles the family members carried. This procedure gave precise results and provided the opportunity to probe for possible linkage and Bannayan-Riley-Ruvalcaba gene location. The procedure used in this research was taken from the Shodair Hospital Genetics Laboratory Procedure Manual, which was derived from several user manuals provided by the manufacturer (ABI).

Samples were prepared prior to ABI analysis. One μl of the sample (post PCR purification) was mixed with 12 μl of DI Formalin and 0.5 μl of Genscan 500 ROX (standard). Each
solution was added to ABI microtubes provided by the manufacturer for ABI analysis and then capped, heated at 100° C for 3 min to denature the DNA, and placed in an ice bath until ready to load in the ABI analyzer.

The ABI machine was then set-up according to manufacturer specifications (ABI). First, the polymer was equilibrated and mixed by inversion. At this time, the 10X Genetic Analyzer EDTA buffer was diluted to 1X and brought to room temperature. The block was then rinsed with water. Next, the capillary and capillary detector window were inspected and cleaned. Following the menu prompts on the computer, the autosampler was recalibrated. To continue cleaning the analyzer, a clean syringe was selected and polymer (ABI) was injected. Taking care that all bubbles were removed, the syringe was screwed into the right side of the analyzer pump block. Next, the manual control menu on the computer was opened and Buffer Valve Close was selected. The waste valve below the syringe was then opened manually and pressure was applied to the syringe in order to remove bubbles prior to closing the valve. Buffer Valve Open was subsequently selected and the syringe was pushed manually in order to fill the polymer channel and remove any air bubbles. Syringe Down was finally selected from the manual control menu which moved the syringe drive toggle until the bottom of the drive touched the plunger. The buffer
reservoir was then filled with 1X Genetic Analyzer EDTA buffer and placed on the pump block. Finally, buffers were inserted in the autosampler as follows: 1X buffer in position 1 (in vial with adapter and septum), dH₂O in position 2 (in vial with adapter and septum), dH₂O in cap-less Eppendorf tube in position 3.

Once set-up was complete, a sample sheet and injection list were established in the computer program. A run module was chosen according to the manual and under the Genscan Injection List Defaults, the mod file (GS STR POP4A), the matrix file (FdNTP’s Matrix Bog...), and the length detector (30 cm) were established. The program was then started by clicking Run on the injection list. Analysis of the amplified DNA fragments was monitored periodically by checking programmed and normal values in the Status window, and checking the baseline in the Raw Data window.

**LINKAGE ANALYSIS**

Linkage analysis was done by a computer generated program, LINKAGE (version 5.0). The predicted percentage of recombination that gave the highest LOD score was 0.0 (θ=0). Results were expressed in the form of a LOD score, or logarithm of the odds. This score, a base 10 LOG ratio, can be described as a likelihood or probability of observing a
data set given a certain hypothesis. A LOD score of 2.0 implies a 100:1 chance of linkage while a LOD score of 3.0 implies a 1000:1 chance of linkage for the particular set of data. LOD scores of at least 3.0 are assumed to reflect actual linkage while a LOD score above 1.0 provides evidence of possible linkage in a family. A series of LOD scores was created according to several possible recombination values (Johnson 1988). The maximum value obtained was reported as the LOD score in this study.
RESULTS

The family used in this study was diagnosed as affected with Bannayan-Riley-Ruvalcaba syndrome by Dr. John Johnson. A medical history was taken for each family member including diagnostic phenotypes and pictures. A pedigree for the family was constructed and individuals I:5, III:6, and III:10 were diagnosed as unaffected. Individuals II:3, II:6, II:8, III:2, III:3, III:4, III:5, III:7, III:8, and III:9 were diagnosed as affected (Fig 2). Figures 3 and 4 show affected individuals and summarize their findings.

Thirteen members of the family volunteered for this linkage study and DNA was drawn from each member. Markers D10S676, D10S1432, D10S1427, D10S2475 (GATA134F03), D10S573, D10S215, D10S541, D10S2470 (GATA115E01), D10S1242, D10S1229, D10S677, and D10S1240 were used to perform the study. These markers encompassed the area of interest on chromosome 10 (10q23) and were located in close proximity in order to cut down on the chance of recombination between the marker and the hypothesized BRR syndrome gene. DNA specific for each marker was amplified using the PCR (polymerase chain reaction) technique.

Following PCR, each family member’s DNA was analyzed using ABI genetic analysis. Alleles were assigned to a specific individual for all markers. Polymorphisms were
Figure 2. PEDIGREE OF THE BANNAYAN-RILEY-RUVALCABA FAMILY
Figure 3. PICTURE AND CHARACTERISTICS OF AFFECTED INDIVIDUAL
III:2 (NOTE DEFINITE MACROCEPHALY)

*picture adapted from Shodair Children's Hospital medical
genetics laboratory, Helena, MT, with permission from
patient

HX: 7.5 year male
BW 8-2
Lipoma removed from back (keloid)
GE Reflux–Nissen, polypectomy
Strabismus, hyperopia, astigmatism
Special Education

PE: OFC 59 cm (>>98%)
High-arched palate, triangular face,
downslanting fissures
Pectus excavatum
Faint hyperpigmented macules on peri
Hypotonia/hyperextensibility

DX: AFFECTED
Figure 4. PICTURE AND CHARACTERISTICS OF AFFECTED INDIVIDUAL III:9

*picture adapted from Shodair Children’s Hospital medical genetics laboratory, Helena, MT, with permission from patient
tracked in each family member's DNA sequence. Difference in fragment sizes corresponded to difference in repeat sequences specific to each allele. For each marker, ABI analysis determined the size of the PCR amplified region within one base pair. Dinucleotide, trinucleotide, and tetranucleotide markers tracked respective repeat sequences (two, three, or four nucleotides) in the study.

The size of the fragment was recorded as the genotype of the individual at the marker site. These different fragment sizes, or alleles, were then followed from parents to offspring in an attempt to determine linkage of BRR syndrome to a specific DNA sequence of the chromosome. Individuals who were heterozygous for fragments on the two chromosomes, meaning they possessed two fragments of differing sizes, were informative at the marker locus. Individuals who were homozygous at a specific site were considered uninformative since the two chromosomes could not be distinguished. This allowed us to track inheritance of the gene (Johnson 1988).

The alleles assigned to each family member for all 12 markers tested were arranged in the pedigree on two chromosomes, paternal and maternal, in an attempt to determine linkage (Fig 2). Barring crossovers in certain individuals, a distinct set of alleles seemed to be passed
from affected parent to affected offspring throughout the family (Table 3).
Table 3. DISTINCT SET OF ALLELES PASSED BETWEEN AFFECTED INDIVIDUALS IN BRR FAMILY (BARRING CROSSOVERS)

<table>
<thead>
<tr>
<th>Marker</th>
<th>Allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>D10S676</td>
<td>3</td>
</tr>
<tr>
<td>D10S1432</td>
<td>4</td>
</tr>
<tr>
<td>D10S1427</td>
<td>2</td>
</tr>
<tr>
<td>GATA134F03</td>
<td>1</td>
</tr>
<tr>
<td>D10S573</td>
<td>4</td>
</tr>
<tr>
<td>D10S215</td>
<td>1</td>
</tr>
<tr>
<td>D10S541</td>
<td>1</td>
</tr>
<tr>
<td>GATA115E01</td>
<td>3</td>
</tr>
<tr>
<td>D10S1242</td>
<td>3</td>
</tr>
<tr>
<td>D10S1229</td>
<td>6</td>
</tr>
<tr>
<td>D10S677</td>
<td>3</td>
</tr>
<tr>
<td>D10S1240</td>
<td>3</td>
</tr>
</tbody>
</table>

Using the marker data presented in the pedigree, the affected haplotype could be followed as it was passed from the affected parent to the affected individual. In contrast, the unaffected haplotype could be followed through individuals not affected with BRR syndrome.

Data was then subjected to linkage analysis in an attempt to determine if linkage was, in fact, occurring at this specific region on chromosome 10. Alleles specific to each marker were entered into the computer for all family members. Next, each marker was run in a pair against the affected or unaffected status under the program LINKAGE 5.0.
<table>
<thead>
<tr>
<th>Marker</th>
<th>0.00</th>
<th>0.05</th>
<th>0.10</th>
<th>0.15</th>
<th>0.20</th>
<th>0.25</th>
<th>0.50</th>
</tr>
</thead>
<tbody>
<tr>
<td>676</td>
<td>0.38</td>
<td>0.30</td>
<td>0.22</td>
<td>0.15</td>
<td>0.09</td>
<td>0.04</td>
<td>0.00</td>
</tr>
<tr>
<td>1432</td>
<td>0.48</td>
<td>0.54</td>
<td>0.52</td>
<td>0.46</td>
<td>0.40</td>
<td>0.33</td>
<td>0.00</td>
</tr>
<tr>
<td>1427</td>
<td>0.27</td>
<td>0.25</td>
<td>0.21</td>
<td>0.17</td>
<td>0.13</td>
<td>0.09</td>
<td>0.00</td>
</tr>
<tr>
<td>6134</td>
<td>0.95</td>
<td>0.83</td>
<td>0.70</td>
<td>0.59</td>
<td>0.48</td>
<td>0.38</td>
<td>0.00</td>
</tr>
<tr>
<td>573</td>
<td>0.98</td>
<td>0.82</td>
<td>0.68</td>
<td>0.56</td>
<td>0.45</td>
<td>0.35</td>
<td>0.00</td>
</tr>
<tr>
<td>215</td>
<td>-0.10</td>
<td>-0.13</td>
<td>-0.15</td>
<td>-0.15</td>
<td>0.14</td>
<td>0.13</td>
<td>0.00</td>
</tr>
<tr>
<td>541</td>
<td>0.01</td>
<td>0.08</td>
<td>0.07</td>
<td>0.05</td>
<td>0.04</td>
<td>0.03</td>
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<td>6115</td>
<td>0.70</td>
<td>0.71</td>
<td>0.65</td>
<td>0.58</td>
<td>0.51</td>
<td>0.43</td>
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</tr>
<tr>
<td>6242</td>
<td>0.70</td>
<td>0.71</td>
<td>0.65</td>
<td>0.58</td>
<td>0.51</td>
<td>0.43</td>
<td>0.00</td>
</tr>
<tr>
<td>6229</td>
<td>0.47</td>
<td>0.35</td>
<td>0.25</td>
<td>0.17</td>
<td>0.11</td>
<td>0.06</td>
<td>0.00</td>
</tr>
<tr>
<td>677</td>
<td>0.47</td>
<td>0.44</td>
<td>0.41</td>
<td>0.36</td>
<td>0.31</td>
<td>0.25</td>
<td>0.00</td>
</tr>
<tr>
<td>1240</td>
<td>-0.27</td>
<td>-0.28</td>
<td>-0.28</td>
<td>-0.28</td>
<td>-0.26</td>
<td>-0.24</td>
<td>0.00</td>
</tr>
</tbody>
</table>
LINKAGE 5.0 produced LOD scores for each marker. The LOD scores varied according to given recombination values (\(\theta\)), or chance a crossover will occur as genetic information is passed on. A LOD score table was constructed for all markers (Table 4). The maximum LOD score was found in association with marker D10S573 (0.98, \(\theta=0\)). This provides an indication of linkage, a link between the trait and the segment of chromosome under study, but does not confirm that linkage is occurring. To be accepted as linked, the LOD score must be at least 3.0.

Finally, to further localize the gene responsible for BRR syndrome, we turned again to the pedigree and haplotypes of both affected and unaffected individuals. A variety of crossovers, such as those in individuals II:3, II:8, III:4, III:5, III:9, and III:10, indicated gene location. Crucial crossovers in affected individuals III:4 and III:9 allowed us to propose a plausible region for the Bannayan-Riley-Ruvalcaba gene (see Figure 5). Three markers, D10S541, D10S2470, and D10S1242, were amplified and analyzed in individual III:4. III:4 possessed the unaffected haplotype at markers D10S2470 and D10S1242. Marker D10S541 was uninformative since III:4 was homozygous (1 allele) and transfer of either the maternal or paternal chromosome could not be distinguished. Individual III:9 possessed the unaffected haplotype outside of markers D10S215 and
D10S1229. III:9 was informative for both marker D10S2470 and D10S1242. Marker D10S541 was also uninformative in this individual. These findings indicate that the segment between markers D10S2470 and D10S215 likely contains the gene responsible for BRR syndrome, but because of the uninformative marker D10S541, placement of this gene cannot be concluded for certain (Fig 5).
Figure 5. HAPLOTYPES OF AFFECTED INDIVIDUALS SHOWING POSSIBLE REGION FOR BRR SYNDROME GENE

*table adapted from Shodair Children’s Hospital medical genetics laboratory, Helena, MT.
DISCUSSION

Researchers are currently testing the hypothesis that the gene responsible for Bannayan-Riley-Ruvalcaba syndrome is allelic with Cowden syndrome. A member of the autosomal dominant macrocephaly syndromes, BRR syndrome has a number of characteristics similar to CS. This, accompanied by current findings, gives a strong indication that BRR syndrome and CS are actually allelic, with mutations in the PTEN/MMAC1 gene causing each syndrome. Our results support the allelic hypothesis by indicating that the location for the BRR syndrome gene is at least in close proximity to that of the PTEN gene in the family under study.

Diagnosis of individuals as either affected or unaffected proved to be a difficult task prior to linkage analysis. No set criteria have been established for diagnosing individuals with BRR syndrome as they have for CS. Dr. Johnson was able to establish a criterion for diagnosis similar to that of CS (see METHODS). However, certain individuals were difficult to classify due to borderline characteristics. Individual III:9 posed a particular problem in classification because the only characteristic he possessed was pigmentation of the penis. Macrocephaly was not present. But due to the fact that this specific pigmentation is a major characteristic of BRR
syndrome and diagnosis of the rare syndrome had been established in the family, he was considered affected.

A problem prior to linkage analysis was placement of the markers in exact order on chromosome 10. We knew the general vicinity for each marker, as well as the exact location for most, but certain markers were not placed in the literature. For example, we knew markers D10S1432, D10S1427, and GATA134FO3 reside between D10S676 and D10S573, but we were not definite of their positions relative to each other. This also occurred with markers D10S1242, D10S1229, and D10S1240. Because of this uncertainty, a possible complication may have occurred for linkage results.

Initially, when linkage was run using LINKAGE 5.0, we predicted a possible LOD score greater than 3.0. However, when the two crucial crossovers and uninformative alleles were introduced into the analysis, the LOD score obtained was greatly reduced. A crossover occurred at marker D10S2470 and marker D10S541 was uninformative, decreasing the LOD score. Also, DNA from the fathers of many progeny was not obtained which blocked the ability for us to follow their homozygous alleles in the offspring. This was a second factor that lowered the LOD score. Finally, the LOD score was reduced because of a crucial double crossover in individual III:9. Crossovers occurred at D10S215 and D10S1229, leaving an affected segment spanning from markers
D10S541 to D10S1242. Further complicating matters, individual III:9 was considered affected due to pigmentation of the penis, a hallmark characteristic of BRR syndrome (see RESULTS). However, as stated earlier, this was the only feature of the syndrome he possessed thus affected status was questionable. If considered unaffected, the entire normal haplotype could conceivably have been passed to III:9. But III:9 was classified as affected, thus the double crossover lowered the overall LOD score to 0.98.

After linkage analysis was complete, we turned our attention to localization of the BRR syndrome gene. Even though the crossovers reduced the LOD score and opportunity to prove linkage, the crossovers had the potential to localize the gene in a specific region. Crossovers in several individuals allowed us to further delineate the location of the gene. The most important crossovers occurred in individuals III:9 and III:4. Only a small amount of DNA from individual III:9 was obtained thus it was used to analyze three specific markers: D10S541, D10S2470, and D10S1242. Individual III:4 possessed the unaffected haplotype at markers D10S2470 and D10S1242 but was uninformative at marker D10S541. Marker D10S541 was uninformative because III:4 and the mother, II:3, were homozygous for allele 1 at this position. Determination of which 1 allele was passed from mother to progeny could not
be assessed thus our information concerning this site was limited. Individual III:9 possessed the unaffected haplotype centromeric from marker D10S215 and telomeric from marker D10S1229. This left a possible affected haplotype segment between D10S541 and D10S1242 after the double crossover. Because individual III:4 had the unaffected allele for markers D10S2470 and D10S1242, these positions were ruled out. The remaining segment between markers D10S2470 and D10S215 likely contains the gene responsible for BRR syndrome although we cannot ascertain for certain because of the uninformative marker D10S541. This region encompasses the specific site for the PTEN/MMAC1 gene thus supporting the possibility that the PTEN/MMAC1 gene is responsible for BRR syndrome. If this is the case, the two similar syndromes would indeed be allelic.

Measures can be taken to care for and treat patients who are diagnosed with Bannayan-Riley-Ruvalcaba syndrome. Researchers suggest all patients diagnosed with this syndrome be tested for mutations in the PTEN locus, and the appropriate counseling be given to families concerning tumor risk and management (Arch et al. 1997). Patients should be monitored for gastrointestinal polyposis and thyroid neoplasms (Cohen 1990). The key is recognition of the syndrome and then families can be counseled for an autosomal dominant trait with high penetrance. Predictive testing
with linkage analysis is technically possible, but difficult and time consuming (Eng 1997). Muscle weakness is also a concern for individuals, as it was for our family, and this can sometimes be treated with the amino acid carnitine which facilitates metabolism and energy generation in muscles.

The findings in this study do support our hypothesis but do not substantially prove it. Our hypothesis included the claim that the gene responsible for BRR syndrome was, in fact, the PTEN/MMAC1 gene responsible for CS. We believe the two syndromes are allelic and result from different mutations of the same gene. Substantial similarity of characteristics between the two syndromes lends evidence toward this claim. The maximal LOD score was low but an indication of linkage did result. Further studies in this family with additional markers may be informative and prove to substantially increase the LOD score. Also, we were able to localize the gene responsible for BRR syndrome into a specific region of chromosome 10. This is the same region in which the PTEN gene is located which provides further evidence that the two genes may be the same. Although definite proof of linkage in the BRR family did not result from this research, we were able to provide an indication of linkage and localize the gene responsible for Bannayan-Riley-Ruvalcaba syndrome. Further research possibilities in this study include PCR amplification and analysis of other
markers not currently used or study using the same markers of other families diagnosed with BRR syndrome.
REFERENCES


