Microarray analysis of gene/transcript expression in Prader-Willi syndrome: deletion versus UPD

D C Bittel, N Kibiryeva, Z Talebizadeh, M G Butler

Background: Prader-Willi syndrome (PWS), the most common genetic cause of marked obesity, is caused by genomic imprinting and loss of expression of paternal genes in the 15q11-q13 region. There is a paucity of data examining simultaneous gene expression in this syndrome.

Methods: We generated cDNA microarrays representing 73 non-redundant genes/transcripts from the 15q11-q13 region, the majority within the PWS critical region and others distally on chromosome 15. We used our custom microarrays to compare gene expression from actively growing lymphoblastoid cell lines established from nine young adult males (six with PWS (three with deletion and three with UPD) and three controls).

Results: There was no evidence of expression of genes previously identified as paternally expressed in the PWS cell lines with either deletion or UPD. We detected no difference in expression of genes with known biallelic expression located outside the 15q11-q13 region in all cell lines studied. There was no difference in expression levels of biallelically expressed genes (for example, OCA2) from within the 15q11-q13 region when comparing UPD cell lines with controls. However, two genes previously identified as maternally expressed (UBE3A and ATP10C) showed a significant increase in expression in UPD cell lines compared with control and PWS deletion subjects. Several genes/transcripts (for example, GABRA5, GABRB3) had increased expression in UPD cell lines compared with deletion, but less than controls indicating paternal bias.

Conclusions: Our results suggest that differences in expression of candidate genes may contribute to phenotypic differences between PWS subjects with deletion or UPD and warrant further investigations.

Prader-Willi syndrome (PWS) is characterised by infantile hypotonia, feeding difficulties, hypogonadism, small hands and feet, mental deficiency, hyperphagia leading to obesity in early childhood, and a particular facial appearance. The chromosome 15q11-q13 region is known to contain imprinted sequences that are differentially expressed depending on the parent of origin. Imprinted expression is coordinately controlled in cis by an imprinting centre (IC) which is functional in the germline and early postzygotic development. The IC regulates the establishment of parental specific allelic differences in DNA methylation, chromatin structure, and expression.

A deletion of the paternal 15q11-q13 region is found in about 70% of PWS subjects, maternal disomy 15 or UPD in approximately 25%, and an imprinting mutation in 2-3%. The 15q11-q13 region contains about 4 million base pairs of DNA and as many as 50-100 genes/transcripts. Recent evidence supports the existence of at least 30 presumed genes in the region. To date, several genes have been located in this region and shown to be paternally expressed. The lack of expression of these genes causes PWS. In addition, at least two genes in this region are maternally expressed in some tissues (UBE3A, ATP10C). Deletions in UBE3A have been shown to cause Angelman syndrome, an entirely different clinical syndrome.

Numerous transcripts have been identified within and nearby the PWS critical region, 15q11-q13. Analysis of gene expression has identified several candidate genes which may play a role in PWS. In particular, SNRPN is a prime candidate gene. Recent evidence has shown that the SNRPN locus is highly complex, composed of a very long variable precursor transcript with multiple functions. SNRPN, IPW, PAR5, and multiple small nuclear RNAs (snRNAs) are also associated with the SNRPN locus. The snRNAs associated with the SNRPN locus (for example, HBII-85) have been proposed to confer most of the PWS phenotype. However, this hypothesis remains to be verified. Regardless, it is still unclear how subtle changes in gene expression resulting from the loss of both imprinted genes and reduced expression of non-imprinted genes lead to the clinical manifestations associated with PWS.

Analyses of the genetic subtypes of PWS have shown a number of cognitive and behavioural differences between PWS subjects with deletions compared to those with UPD. We previously reported significantly higher verbal IQ scores in PWS subjects with UPD compared to subjects with deletions. The UPD subgroup scored significantly higher than the deletion subgroup in four subcategories of verbal testing including information, arithmetic, vocabulary, and comprehension. Several reports have indicated that PWS subjects with UPD had less severe manifestations than subjects with deletions of specific maladaptive behaviours commonly associated with PWS. These differences must be related to the underlying differences in gene expression of the genetic subtypes.

Most analyses of the gene expression patterns of the 15q11-q13 region have focused on identifying imprinted genes and transcripts. However, the imprinted sequences cannot by themselves account for the phenotypic differences observed between the UPD and deletion subtypes within PWS, since paternally expressed sequences would presumably be silent in both the deletion and UPD subtypes.

In this report, to improve our understanding of gene expression within or close to the 15q11-q13 region, we report our experience using a custom made cDNA microarray comprising mostly of sequences within the PWS critical region (PWSCR). This report represents the first application of microarray technology to examine the simultaneous expression of multiple genes/transcripts from genetic subtypes of a
human condition resulting from haploinsufficiency and/or genomic imprinting.

**SUBJECTS AND METHODS**

**Subjects**
All subjects were matched for age, cognition, obesity status, and sex. Our study subjects included young adult males, six with PWS (three with 15q11-q13 deletion (mean age 28 years) and three with UPD (mean age 27 years)), and three non-syndromic comparison males with obesity of unknown cause (mean age 26 years). Chromosomal status was confirmed by FISH and microsatellite analysis using standard techniques in the PWS subjects.

**Methods**
Transcripts were chosen for analysis by searching the Unigene and UniSTS databanks. A total of 129 transcripts and genes were identified and subsequently reduced to 73 non-redundant sequences of mRNA from lymphoblast cell cultures derived from each of the nine adult males. Equal quantities of mRNA from lymphoblast and brain cDNA were spotted. Four additional EST or transcript sequences from the uniSTS databank could not be derived from lymphoblast or brain cDNA: WI-18351, HO2863, sts-N58001, STSG15842. Signals amplified from brain cDNA but not detected on array: GABRG3, A007E33, WI-16777, sts-N35112, sts-Y00757.

<table>
<thead>
<tr>
<th>Proximal to D15S1035</th>
<th>SHGC-32610, MYLE, A002B45, NIB1540</th>
</tr>
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<tbody>
<tr>
<td>D15S1035-D15S122</td>
<td>ZNF127, WI-15987, NDN, MAGEL2, SGC44643, WI-15028, WI-13791, SNRPN, AA258222, R99003, PAR5, aSG12920, WI-6780, sh2N1972, WI-14946, IPW, PAR1, SHGC-3208, WI-6654, Cde0bi12, WI-11918</td>
</tr>
<tr>
<td>D15S122-D15S156</td>
<td>UBE3A, WI-16777, AT1P10C, GABRB3, GABRB5, GABRB3</td>
</tr>
<tr>
<td>D15S156-D15S165</td>
<td>OCA2, HERC2, WI-6527, sh2N135, sh2N3492, A007E33, STSG3316, STSG10131, STSG15956, SGC33431, SGC35687</td>
</tr>
<tr>
<td>D15S165-D15S144</td>
<td>st3-N3547, SHGC-3121, WI-18157, Cda1F10, A006O15, aSG248278, D13638</td>
</tr>
<tr>
<td>D15S144-D15S118</td>
<td>WI-15231, SGC3497, SGC32006, SGC35611, A005N08, WI-14003, BCD2917</td>
</tr>
<tr>
<td>Distal to D15S118</td>
<td>WI-18493, 3B449, R22446, WI-17962, H18148, Bda9812, H69794, WI-15193, SHGC-30973, SHGC-13414, SHGC-17227, FBRILLIN, Fb3a09, Cde0bi10, UTE-P875, Bdaoc505, STSG4005</td>
</tr>
</tbody>
</table>

*Signals unable to amplify from lymphoblast or brain cDNA: WI-18351, HO2863, sts-N58001, STSG15842. Signals amplified from brain cDNA but not detected on array: GABRG3, A007E33, WI-16777, sts-N35112, sts-Y00757.

A CyScribe reverse transcription labelling kit (Amersham, Piscataway, NY) and hybridisations were done in 3 x SSC at 65°C overnight in individual hybridisation chambers. The following day the slides were washed in 2 x SSC, 0.1% SDS, followed by four washes in 0.1 x SSC and one wash in distilled water with a final rinse in 100% ethanol. The slides were scanned with an Affymetrix 428 Slide Scanner and the data analysed with Jagger V.2 software (Affymetrix, Santa Clara, CA).

In order to maximise the total number of comparisons, the arrays were performed in sets following a simple looping design as described by Churchill (for example, set 1 compared control subjects to deletion subjects, set 2 compared control subjects to UPD subjects, and set 3 compared deletion subjects to UPD subjects, see table 2). This looping design for the total of nine subjects required the use of 18 custom made microarray slides. Within each set, each individual target was labelled with both Cy3 and Cy5 and compared to different subjects, respectively. Thus, each target was arrayed five times, replicated twice for each colour of dye and hybridised in four different combinations. The average signal intensity per subject was calculated from 20 replicate spots per gene/transcript for Cy3 and 20 replicate spots per gene/transcript for Cy5.

Quantitative reverse transcription-PCR (RT-PCR) was performed using a QuantiTect Sybr Green RT-PCR kit (Qiagen, Valencia, CA) according to the manufacturer’s directions. Briefly, an equal quantity of total RNA from a representative individual subject from each group (control, UPD, deletion) and primers specific for the gene/transcript being quantified were added to a reaction mix containing all components necessary for reverse transcription and PCR. The reaction was carried out using an ABI 7000 system beginning with a 30 minute step at 50°C to allow for reverse transcription, followed by 15 minutes at 95°C. The PCR was performed for 45 to 50 cycles during which the intensity of the Sybr green fluorescence was measured at the extension step of each PCR cycle. The point at which the intensity level crossed the threshold (Ct, defined as the narrowest point between individual reactions in the logarithmic phase of the reaction) was used as recommended by the manufacturer’s guidelines.

<table>
<thead>
<tr>
<th>Label</th>
<th>Comparison strategy among subjects</th>
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<tbody>
<tr>
<td>Set 1</td>
<td>Cy3 C1 D1 C2 D2 C3 D3</td>
</tr>
<tr>
<td>Cy5 D1 C2 D3 C1</td>
<td></td>
</tr>
<tr>
<td>Set 2</td>
<td>Cy3 C1 U1 C2 D2 U2 C3 U3</td>
</tr>
<tr>
<td>Cy5 U1 C2 U3 C1</td>
<td></td>
</tr>
<tr>
<td>Set 3</td>
<td>Cy3 D1 U1 D2 U2 D3 U3</td>
</tr>
<tr>
<td>Cy5 U1 D2 U3 D1</td>
<td></td>
</tr>
</tbody>
</table>

C = control (obese comparison) subject. D = PWS subject with 15q11-q13 deletion. U = PWS subject with UPD.

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The custom array used for our analyses contained several genes both within (for example, OCA2) and outside (for example, GAPDH, B-actin, and fibrillin) the PWSCR and known to be biallelically expressed. The expression levels of these genes were all very similar in the individual subjects and the groups examined (fig 2A). In addition, the PWSCR is known to contain a large number of imprinted genes/transcripts which express only from the paternal allele. For the most part, we could not detect any expression from these sequences in the deletion or UPD subjects but clear signals were obtained from the comparison subjects (fig 2B). Three of the imprinted sequences, WI-15028, WI-15987, and STS-N21972, had signal intensities in the UPD cell cultures which were slightly above the threshold of detection which was defined as the average signal intensity from the negative probes plus two standard deviations. In addition, WI-15987 had a detectable signal in the deletion cell cultures. This may be because of non-specific cross hybridisation which we were unable to reduce given the constraints of array hybridisation and washing.

Fourteen of the genes/transcripts examined produced signal intensities from the PWS cell lines which were inconsistent with equal expression from both alleles (biallelic) or expression from the paternal allele (maternally imprinted) (fig 2C). The genes/transcripts with unusual expression patterns (confirmed by quantitative RT-PCR) can be divided into four groups. The first group had significantly less expression in cell cultures derived from UPD subjects than either of the other two groups (fig 2C, first panel). These three transcripts were all located outside the PWSCR suggesting that the expression from the maternal allele is reduced relative to the paternal allele. The second group contained genes/transcripts whereby expression from deletion subjects was less than half that of the controls and expression from the UPD cell lines was greater than the deletion lines but significantly less than the controls (fig 2C, second panel). This suggests paternal bias in the allelic expression pattern of these genes/transcripts. The third group contained two transcripts from outside the PWSCR in which the intensity levels from the deletion cell cultures were significantly greater than either the control or UPD cell cultures (fig 2C, third panel). The fourth group contained genes/transcripts with expression levels significantly higher in the UPD cell cultures, suggesting that expression was exclusively, or at least primarily, from the maternal allele (fig 2C, fourth panel). Two of the genes in the fourth group, UBE3A and ATP10C, have been shown to be maternally expressed in some tissues. Several of the genes were chosen for validation by quantitative RT-PCR. Fig 3 shows a representative example of GABRB3 and a single set of curves generated by real time PCR using an ABI 7000 system. Table 3 shows C values generated from at least six replicated quantitative RT-PCR reactions for each subject. The calculated fold change in expression is also shown. The expression data of all sequences were in agreement with the microarray data. Only a few significant differences identified with microarray were not observed with quantitative RT-PCR analysis (for example, WI-6527).

DISCUSSION

The regulatory mechanisms which control gene expression in the 15q11-q13 region are complex and not clearly understood. Procedures used to examine gene expression in this region previously have been primarily non-quantitative. We have used a custom cDNA microarray to analyse and compare gene expression in lymphoblastoid cells from young adult male subjects with PWS with either 15q11-q13 deletion or UPD and obese comparison males. This has allowed a semi-quantitative analysis of expression of the genes/transcripts located in or close to the PWSCR.
For the most part, sequences from outside the PWS critical region (PWSCR) produced signal intensities that were similar in each of the three groups (see fig 2A for representative examples). Genes and transcripts previously identified as imprinted produced no detectable signal in the cell lines from PWS patients (fig 2B) with the exception of WI-15028, WI-15987, and N21972 which were slightly above the threshold in PWS subjects, probably as a result of cross hybridisation.

The remaining genes and transcripts can be divided into four categories (fig 2C). First, three transcripts had significantly less expression in the UPD cell lines than in either the deletion or control cell lines (fig 2C, panel 1). These were all located outside the PWSCR and may indicate that these transcripts have paternal bias in their expression pattern. Since the expression from the UPD cell lines was reduced relative to both the control and deletion lines, the expression from the
maternal allele must be significantly less than the expression from the paternal allele. If confirmed, this observation suggests that a mechanism exists for regulating allele specific expression which does not require the presence of the paternal imprinting centre.

The second group of transcripts contained several sequences from within the PWSCR which had significantly less expression than half the control level of expression in the deletion lines (fig 2C, panel 2). Furthermore, signal intensities were significantly less in the UPD cell cultures compared to the control cell cultures. Taken together, these data suggest paternal bias in the expression of these sequences. When confirmed, these sequences express at higher levels from the paternal allele than the maternal allele. This suggests that the imprinting mechanism may play a role regulating quantitative allele specific expression. Interestingly, this group of genes includes the GABA receptor subunit genes where a decreased expression was seen in GABRB3 in both the deletion and UPD subjects compared to controls in the microarray and quantitative RT-PCR studies (for example, −2.64 fold change in deletion compared with controls and −1.63 for UPD compared with controls). Although deletion and UPD subjects showed reduced expression compared with controls in both the microarray and quantitative RT-PCR studies, as well as biallelic expression, there appeared to be less expression from the maternal allele indicating paternal bias.

GABA is an amino acid neurotransmitter that is widely distributed throughout the central nervous system. It has been estimated that up to 40% of the neurones in brain and spinal cord use GABA as their neurotransmitter making it, quantitatively at least, the most important inhibitory neurotransmitter in brain.19 GABAergic mechanisms have been implicated in a number of symptoms associated with PWS including hunger,20 compulsivity,21 metabolism,22 and visual perception and memory.23 24 GABAergic mechanisms may have a direct effect on these characteristics or there could be indirect effects owing to feedback from other GABA influenced systems.

The five subunits that comprise an individual GABA-A receptor are drawn from a family of 18 proteins, each of which is a distinct gene product.24 These subunits are divided into six classes, α, β, γ, δ, ρ, and ε, based on their homologies. There are six α subunits (α1-6), four β subunits (β1-4), four γ subunits (γ1-4), two ρ subunits (ρ1-2), one δ subunit, and one ε subunit. Given the number of possible pentameric combinations, including homomers that can be drawn from these 18 subunits, thousands of distinct GABA-A receptor subtypes are possible. However, only a dozen or so have been identified in mammalian central nervous system, with most of these containing a combination of α, β, and γ subunits in various stoichiometries.25 The most prevalent GABA-A receptor in brain is a pentamer composed of α, β, and γ subunits.

Reports suggesting that GABA and GABA-A receptors play a critical role in central nervous system development26-28 are of

Table 3 Quantitative RT-PCR validation of selected genes/transcripts studied using microarrays

<table>
<thead>
<tr>
<th>Gene/transcript</th>
<th>Control</th>
<th>Deletion</th>
<th>UPD</th>
</tr>
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<tbody>
<tr>
<td>WI-15193</td>
<td>CT (SD)</td>
<td>27.8 (0.15)</td>
<td>28.1 (0.88)</td>
</tr>
<tr>
<td></td>
<td>(U&lt;D&amp;C) Fold change†</td>
<td>-1.23</td>
<td>-1.87</td>
</tr>
<tr>
<td>WI-18493</td>
<td>CT (SD)</td>
<td>27.1 (0.15)</td>
<td>27.0 (0.19)</td>
</tr>
<tr>
<td></td>
<td>(U&lt;D&amp;C) Fold change‡</td>
<td>+1.07</td>
<td>-1.87</td>
</tr>
<tr>
<td>GABRB3</td>
<td>CT (SD)</td>
<td>24.7 (0.20)</td>
<td>26.1 (0.38)**</td>
</tr>
<tr>
<td>(Paternal bias)</td>
<td>Fold change‡</td>
<td>-2.64</td>
<td>-1.63</td>
</tr>
<tr>
<td>WI-6527</td>
<td>CT (SD)</td>
<td>37.8 (0.50)</td>
<td>37.3 (0.14)</td>
</tr>
<tr>
<td>(D&gt;C &amp; U) Fold change‡</td>
<td>+1.41</td>
<td>-1.32</td>
<td></td>
</tr>
<tr>
<td>SHGC-32610</td>
<td>CT (SD)</td>
<td>27.4 (0.19)</td>
<td>27.9 (0.24)**</td>
</tr>
<tr>
<td>(Maternal bias)</td>
<td>Fold change‡</td>
<td>-1.41</td>
<td>+2.14</td>
</tr>
<tr>
<td>D38449</td>
<td>CT (SD)</td>
<td>32.5 (0.51)</td>
<td>32.6 (0.21)</td>
</tr>
<tr>
<td>(Maternal bias)</td>
<td>Fold change‡</td>
<td>-1.07</td>
<td>+6.06</td>
</tr>
</tbody>
</table>

**Significant difference compared to the control, p<0.05.
***Significant difference compared to the control, p<0.001.
†CT is the threshold PCR cycle which represents the cycle at which the fluorescence signal exceeds a baseline level. The threshold baseline is set at the narrowest point in the linear part of the amplification curve following recommended guidelines. The numbers represent an average (SD) calculated from six replicate RT-PCR reactions per subject.
‡Fold change in expression equals 2^X, where X = the CT from the Deletion or UPD and C = the CT from the Control.
§Significant difference compared to the control, p<0.01.

1CT is the threshold PCR cycle which represents the cycle at which the fluorescence signal exceeds a baseline level. The threshold baseline is set at the narrowest point in the linear part of the amplification curve following recommended guidelines. The numbers represent an average (SD) calculated from six replicate RT-PCR reactions per subject.
2Fold change in expression equals 2^X, where X = the CT from the Deletion or UPD and C = the CT from the Control.
3Significant difference compared to the control, p<0.05.
4**Significant difference compared to the control, p<0.01.
5***Significant difference compared to the control, p<0.001.
particular relevance to the present finding. Changes in subunit expression and, therefore, the composition of GABA-A receptors, appears to be essential for proper neurogenesis. Notably, brain α1, and γ subunit gene expression diminishes during development, suggesting that these proteins themselves, or as components of GABA-A receptors, may regulate neuronal development and the establishment of central nervous system pathways. The concept of abnormal fetal brain development in PWS was proposed as early as 1977. Our data suggest that even though the GABA receptors do not appear to be expressed solely from the paternal allele there may be a greater than expected reduction in expression from these genes, even in cases of UPD. Presumably, even a modest decrease in the synthesis of these proteins could have a significant effect on brain development, with permanent consequences on the central nervous system function. This speculation is supported by the finding that the β subunit is preferentially expressed in the hypothalamus and that a reduction in its production during development could lead to obesity and hypogonadism, hallmarks of PWS. Whereas β subunit proteins are found mainly in the hypothalamus, the γ subunit message tends to be localised in the thalamus and α message is expressed predominately in the hippocampus. Thus, a decline in the production of one or more of these subunits could account, at least in part, for the somatic and behavioural abnormalities associated with PWS.

In our third group, two transcripts from outside the PWS region expressed at higher levels in the deletion than in either UPD or controls (fig 2C, panel 4) indicating maternal bias of expression. UBE3A and ATP10C are known to be maternally expressed, although UBE3A only in the brain. Our data are in agreement with a previous report suggesting that SGC32610 may be maternally biased in its expression pattern. Our data further suggest that, although expression from the paternal allele is detectable, a significantly greater proportion of the RNA is produced by the maternal allele.

Our data may suggest that the expression of genes and transcripts in and around the PWS region is influenced by chromatin structure and context, as well as the imprinting centre. The dynamic interactions suggested by the microarray data reinforce the observations of the complex nature of expression in the 15q11-q13 region. Finally, we recognise that the targets applied to our microarray were isolated from lymphoblastoid cell cultures and gene expression in cell culture may not be in complete concordance with gene expression in brain tissue. Nevertheless, our results suggest differences in expression of candidate genes which may contribute to differences observed between PWS subjects with deletions and UPD. These sequences warrant further investigation to determine what contribution they make to the phenotype of persons with Prader-Willi syndrome.

ACKNOWLEDGEMENTS

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Japanese researchers have suggested that searches for rogue genes in multiple colon cancer should be broadened to include the p53 mutation. This follows their discovery of a new p53 germline mutation in a patient with multiple primary colon cancers. The case was of a 73 year old man with one advanced colon carcinoma and five polyps in an area including the caecum and ascending colon. Histological analysis confirmed an advanced carcinoma, two early carcinomas, and three adenomas. The advanced carcinoma yielded a novel germline p53 mutation of GCC (Ala)→GTC (Val) at codon 189, plus a somatic mutation at codon 245, somatic APC mutations, and a somatic K-ras-2 mutation on genetic analysis. The early carcinomas and adenomas yielded the germline p53 mutation and somatic APC mutations resulting in stop sequences. One early carcinoma had a somatic K-ras-2 mutation.

The case came from a series of 15 patients without germline mutations in the APC gene or DNA mismatch repair genes who had multiple primary colorectal cancers and colorectal polyps without microsatellite instability. Tissue samples from the tumours and adjacent healthy tissue were taken from resected colon for histological and genetic analysis. The researchers looked for germline p53 mutations because these occur with tumours in multiple organs in Li-Fraumeni syndrome, though it was not known if they might occur with multiple colon cancers. Whether this new germline mutation proves to be pathogenic or a rare polymorphism remains to be determined.

A new lead to understanding normal eye development has emerged from a molecular genetic study mapping the disease locus of a rare complex optical syndrome to a region within the locus for nanophthalmia. The study was confined to three generations of one English family, six of whom were affected with an autosomal dominant condition (MRCS) in which microcornea, progressive rod-cone dystrophy, cataract and posterior staphyloma segregate together.

The affected phenotype was consistent with nanophthalmia, plus other characteristics and suggested that the disease locus might occur on chromosome 11. Genetic testing with microsatellite markers associated with autosomal dominant nanophthalmia excluded CMIC and CHX10 loci on chromosome 14q and NNO2 on chromosome 15, but linkage results suggested a 5.0 cM genetic interval within the NNO1 locus as the most likely site.

Six affected members and three unaffected members of one family were tested from 11 affected and three unaffected members of one family who agreed to participate. Each had comprehensive medical and ophthalmological examinations and gave venous blood for DNA amplification and genotyping with microsatellite markers associated with nanophthalmia by a positional candidate gene approach. Autosomal dominant nanophthalmia has been assigned a locus at NNO1 between chromosomes 11p12 and 11q13 by a previous linkage study in one family. So it seemed a useful candidate to test for mutation in a family with autosomal dominant MRCS. The researchers are seeking to confirm their results by testing more affected families and to screen for other candidate genes in this region.

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