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Many Tumors and Many Genes

Genetics of Uterine Leiomyomata

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Uterine leiomyomata, or fibroids, are smooth muscle tumors well known to the practicing gynecologist, women's pathologist, and a large number of women primarily of reproductive age. Their characteristic benign nature has resulted perhaps in a low profile as tumors of intense research interest. However, in the past few years positional cloning efforts have been used successfully to begin to approach an understanding of genes involved in the pathobiology of fibroids. Knowledge of these genes and their role in tumor biology may provide valuable insight into the molecular events that differentiate benign and malignant tumors, in addition to possibly impacting development of medical treatment for uterine fibroids.

Clinical Characteristics

Uterine leiomyomata are the most common pelvic tumors in women and are associated with a variety of symptoms including abnormal uterine bleeding, pelvic pain, urinary frequency, impaired fertility, and spontaneous abortion. It is obvious that many of these symptoms are due to the anatomic location of the tumors but other findings, such as impaired fertility, may result from presently unknown biochemical alterations in the endometrium mediated by a fibroid. Estrogen and progesterone undoubtedly play an important role in growth and development of these tumors, which are not seen before puberty, undergo a rapid increase in size during pregnancy, and then regress postmenopausally. The role of oral contraceptives in stimulating the growth of fibroids remains somewhat controversial and we have yet to know what effects hormone replacement therapy might have on these tumors. Fibroids occur in 20–25% of women of reproductive age, but this percentage may be a gross underestimate as studies by Drs. Cramer and Patel,¹ looking at the presence of uterine leiomyomata in serial sections from 100

hysterectomies indicated a prevalence rate of 77%. Lastly, but most importantly, these tumors are a major public health problem. Fibroids are the primary indication for greater than a third of all hysterectomies, accounting for more than 200,000 procedures in the United States annually and for an estimated one in five visits to the gynecologist.

Pathological Findings

Fibroids are benign tumors with less than 0.1% estimated to progress to malignancy. Rigorous studies of these tumors might provide insight into the molecular events that differentiate benign and malignant tumors. For example, in the case of leiomyomata and their presumed malignant counterpart, leiomyosarcomas, it remains to be determined whether these two neoplasms are in the same genetic pathway. One consideration for the observed low progression of the incredibly prevalent leiomyomata to the rare leiomyosarcoma is that there are so many additional genetic events that must occur that a malignant tumor rarely arises. Histologically, uterine leiomyomata are characterized by well-differentiated whirling bundles of smooth muscle cells that comprise fairly distinct nodules. Consistent with their benign nature, mitoses are scant and normal in appearance. In comparison to the normal myometrium the fibroid appears more cellular, although in tissue culture the two are indistinguishable. Fibroids can be found in intramural, subserosal, or submucosal locations in the uterus. An average uterus contains six or seven fibroids, which have often been referred to as "potato-like" due to the diversity in shape and form of these tumors.

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Genetic Studies

Epidemiology

It is estimated that there is a threefold to ninefold greater prevalence of uterine leiomyomata among black females than white females, and a roughly threefold increase has been confirmed recently in an analysis of data from the Nurses' Health Study II.² This finding indicates that there might be some genetic factor which predisposes one to develop fibroids. A genome-wide scan could be performed to look for a major susceptibility locus for fibroids, similar to that reported for prostate cancer for which a locus on chromosome 1 was identified.³ Interestingly, twin pair correlations for hysterectomy in monozygotic twins are about twice that reported in dizygous twins,⁴ consistent with the genetic relationship between monozygotic and dizygotic twins, indicating that there is a genetic liability for hysterectomy. Because fibroids are the most common indication for hysterectomy, it seems likely that they play a role in this correlation although there may be other factors as well.

Biochemical and Molecular Studies

Concerning biochemical and molecular studies, G6PD isozyme analysis has been used to establish clonality of these tumors⁵ and we have used the polymorphic androgen receptor locus (HUMARA) to confirm those studies and to make some additional observations.⁶ Clonality was evaluated in 36 fibroids from 16 patients. Twenty-seven of the 36 fibroids had been karyotyped previously and these tumors fell into three groups: normal 46,XX tumors, karyotypically abnormal, and mosaic tumors of 46,XX and karyotypically abnormal cells. The assay takes advantage of the facts that essentially one X chromosome in a normal human female is inactivated and that there is a methylation sensitive restriction enzyme site upstream of the polymorphic CAG repeat in the gene. Oligonucleotide primer pairs were designed flanking these repeats and used to amplify the tumor DNA. In informative individuals (*ie* those with different numbers of CAG repeats), clonality was determined following digestion of the amplified DNA with *Hha*I. In a monoclonal tumor, DNA from only the inactive X chromosome would remain whereas in a polyclonal tumor, products from either X chromosome would be found, representing either the maternal or paternal X chromosome randomly inactivated. In addition to confirming the monoclonal origin of

Table 1. Cytogenetics of uterine leiomyomata

60% are 46,XX
40% are chromosomally abnormal
del(7)(q22q32)
t(12;14)(q15;q23-24)
rearrangements of 6p21
trisomy 12
rearrangements of 10q22
rearrangements of 13q21-22
deletions of 3q

uterine leiomyomata, we observed that tumors which were mosaics were also clonal.¹⁰ This leads to the interesting interpretation that the cytogenetic aberrations may be secondary and that the clonal expansion of tumor cells occurs prior to the acquisition of the cytogenetic aberration.

Cytogenetics

We and others have described tumor-specific chromosome aberrations in uterine leiomyomata^{7,8} and these aberrations have been invaluable in targeting areas of the genome in which genes involved in the pathobiology of these tumors might reside. About 40% of tumors are karyotypically abnormal, suggesting that genetic aberrations at the submicroscopic level might be present in karyotypically normal fibroids. A variety of cytogenetic subgroups have been described that predict different genetic mechanisms (Table 1). A trisomy (*eg*, trisomy 12) predicts a gene dosage mechanism whereas a translocation (*eg*, t(12;14)(q15;q23-24)) predicts a gain-of-function mutation in the form of a fusion protein, or potentially dysregulation of a particular gene product. A deletion (*eg*, del(7)(q22q32)) is consistent with a loss-of-function mutation, typified by tumor suppressor genes. Thus, it appears that there are probably many different genetic pathways by which a fibroid can grow and develop.

Phenotypic-Genotypic Correlations

Because fibroids are genetically heterogeneous, it is interesting to ask whether there are any phenotypic correlations among the different cytogenetic subgroups. Recently, we evaluated the possible relationship between the size of a tumor and its karyotype⁹ (Table 2). Our sample consisted of 73 karyotypically normal and 41

Table 2. Summary of myoma size and cytogenetic analysis

Cytogenetic analysis	Myoma diameter (cm), Mean $\pm$ SD	Myoma diameter (cm), Median	Calculated myoma volume (cm ³), Mean $\pm$ SD
Normal (<i>n</i> = 73)	5.9 $\pm$ 4.2 ^a	5.4	334 $\pm$ 1123 ^c
Abnormal (<i>n</i> = 41)	7.6 $\pm$ 5.3	7.0	642 $\pm$ 1638
Abnormal (mosaic) (<i>n</i> = 21)	5.0 $\pm$ 3.0 ^b	4.5	136 $\pm$ 165 ^d
Abnormal (non-mosaic) (<i>n</i> = 20)	10.2 $\pm$ 5.9 ^{a,b}	10.0	1173 $\pm$ 2246 ^{c,d}

a,b,c,d Values with the same superscripts were significantly different, *P* < 0.01.

karyotypically abnormal tumors. Although a trend was noted for association of a larger tumor with an abnormal karyotype, there was not a statistically significant difference. However, if the abnormal tumors were classified into tumors which were either mosaics or nonmosaics, a significant difference was observed between the normal and karyotypically abnormal nonmosaic tumors. Underlying this observation is the finding that most mosaic tumors involve the deletion 7 subgroup and are actually smaller in size than chromosomally normal tumors, although the difference is not statistically significant. It is possible that loss of genetic material from chromosome 7 results in less than optimal growth in a fibroid.¹⁰

Positional Cloning of a Gene at 12q15 Involved in Fibroids

To begin to identify genes involved in fibroids, we undertook a positional cloning project focused on chromosome 12 in the q15 breakpoint region. This region was frequently rearranged in fibroids in a consistent translocation with chromosome 14 in q23-24. Of particular interest and potential relevance were numerous chromosome aberrations involving this region in a variety of other mesenchymal tumors including lipomas,¹¹ pulmonary chondroid hamartomas,^{12,13} and endometrial polyps^{14,15} among others, suggesting either that this region contains a single gene involved in all of these tumors or that multiple genes involved in neoplasia reside within 12q15.

To facilitate the positional cloning project it was necessary to develop a high-density physical map of this region of chromosome 12 and in this effort we collaborated with Drs. Donald Moir, Thomas Dorman, and Jen-i Mao (Genome Therapeutics Corp., Waltham, MA), and Drs. Raju Kucherlapati, Sung-Joo Yoon, Kate Montgomery, and Kenneth Krauter (Albert Einstein College of Medicine, New York, NY) who provided various yeast artificial chromosomes (YACs) and cosmids from chromosome 12, which we mapped using fluorescence in situ hybridization (FISH) on tumor metaphase chromosomes. Thirty-nine YACs and six cosmids were mapped with respect to t(12;14).¹⁶ YAC 981f11 was found to bridge the translocation breakpoints in uterine leiomyomata, pulmonary chondroid hamartoma, and lipoma, making it highly likely that this YAC would contain the DNA sequence of interest in these tumors.

HMGIC, a Positional Candidate Gene

Following identification of YAC 981f11 at 12q15, we collaborated with Drs. Kucherlapati and Kiran Chada (University of Medicine and Dentistry of New Jersey, Piscataway, NJ) to evaluate a potential candidate gene, known as *HMGIC*, because it mapped within the YAC. Most studies of *Hmgic* had been conducted in the mouse, and many of the findings made this gene an especially attractive candidate. Interestingly, *Hmgic* was a very large gene spanning approximately 200 to 250 kb, and a large gene was consistent with a large target in which numer-

ous tumors rearrange potentially. Insertional mutagenesis in a transgenic mouse, knocking out this gene in essence, resulted in a mini-mouse that was about 60% smaller than the wild-type littermate.¹⁷ The mini-mouse had a large reduction in adipose tissue,¹⁸ an intriguing finding with respect to frequent rearrangements in lipomas at 12q15. *Hmgic* was expressed at highest levels during embryonic days 12.5 to 14 in mesenchymal tissues, which was also of interest with regard to the various mesenchymal tumors under investigation. *HMGIC* encodes a member of a family of high mobility group proteins,¹⁹ which are accessory transcription factors, and contains 109 amino acids with 5' DNA-binding AT hook motifs and a 3' acidic domain of unknown function.²⁰⁻²¹ A very highly homologous family member known as *HMGII* is located on chromosome 6 in band p21,²² of note because one of the cytogenetic subgroups in fibroids involved 6p21.

HMGIC in Lipomas

Probes from the 5' and 3' regions of *HMGIC* were used separately in FISH experiments to metaphases from three lipomas with rearrangements of one chromosome 12 homolog at 12q15;²³ the three lipomas included tumors with t(3;12)(q29;q15), t(12;13)(q14-22;q21-32) and t(12;15)(q15;q24). Hybridization signals for the 5' and 3' *HMGIC* probes were detected in each case on the normal chromosome 12 as expected. Additionally, signals were found on the derivative chromosome 12 with the 5' probe in the t(3;12) and t(12;15) lipomas but the 3' probe was present on the translocation partner chromosome, indicating an intragenic rearrangement in *HMGIC*. One of the lipomas, involving a t(12;13), turned out to have a more complex rearrangement such that the 5' probe was detected on the der(13) and the 3' probe was deleted. Thus, the rearrangement on chromosome 12 in the t(12;13) lipoma most likely occurred 5' of the *HMGIC* gene.

Two possible mechanisms were considered: a fusion transcript resulting from a chromosomal rearrangement and a truncation of the 3' region of the gene by chromosomal rearrangement or gene deletion. 3' RACE experiments identified fusion transcripts in both lipomas with intragenic rearrangements and, in both cases, an in frame splice had occurred precisely at the end of the third exon of *HMGIC* fusing heterologous sequence from the partner chromosome with the 5' region of *HMGIC*.²³ Monochromosomal somatic cell hybrids were used to confirm that the heterologous sequence was derived from the translocation partner chromosome, in these two lipomas either chromosome 3 or 15.

Computer analysis of DNA sequence from the t(3;12) fusion transcript with *HMGIC* identified a motif known as a LIM domain. This was a very interesting domain for consideration in the fusion transcript. LIM domains are 50 to 60 amino acid motifs rich in cysteine and histidine organized into adjacent zinc fingers separated by a two-residue linker.²⁴ They are highly conserved among divergent species; were first identified in three proteins, lin11, ISL1, and MEC3;²⁵⁻²⁷ and have important developmental

functions including patterning, cell fate decision, and differentiation. Many LIM-containing proteins are presumed to be transcription factors with their activity thought to be regulated by protein-protein interactions through the LIM dimerization domain. Sequences of the two LIM domains from the chromosome 3-derived sequence were a perfect match with totally conserved cysteine, histidine, and aspartic acid residues seen in other proteins with LIM domains, in addition to being consistent with the presence of an aromatic residue adjacent to the first histidine and a leucine located carboxyl terminal to the central cluster of histidines and cysteines.²³ Interpretation of the sequence analysis of the chromosome 15 fusion transcript was not as straightforward as that of the chromosome 3 fusion transcript as no known motif was detected; however, the sequence is potentially a transactivation acidic domain. The carboxyl terminal end of the predicted protein is highly acidic and rich in serine and threonine residues, and such domains have been implicated in transcriptional activation. In summary, the two fusion transcripts had a very similar structure in that a heterologous sequence from either chromosome 3 or chromosome 15 was spliced in frame following exon 3 of *HMGIC*. That splicing resulted in the 5' portion of *HMGIC* containing exons 1–3 with AT hook domains fused directly with a new 3' region containing either a LIM domain or a putative transactivation domain.²³

HMGIC in Uterine Leiomyomata

FISH experiments with metaphase chromosomes from a uterine fibroid with a t(12;14) failed to detect any rearrangement. Both 5' and 3' probes were found on the derivative 14 chromosome, indicating that the breakpoint occurred proximal in *HMGIC* on chromosome 12 and might involve yet a different gene. However, following the observations in lipomas, we reconsidered our observations in uterine fibroids and reasoned that dysregulation of *HMGIC* might be mediated by an alteration in gene expression, either temporally or by elevated levels. A potentially relevant observation is that one of the characteristic cytogenetic aberrations in uterine fibroids is trisomy 12, which would potentially upregulate *HMGIC* expression by a gene dosage mechanism. In addition, one fibroid from our karyotyped series contained a copy of the derivative 14 chromosome from a t(12;14) rearrangement, but no copy of the derivative 12. Two cytogenetically normal copies of chromosome 12 were present and FISH experiments were consistent with three copies of *HMGIC*. Seven additional fibroids and one pulmonary chondroid hamartoma all with t(12;14) rearrangements were studied by FISH using a variety of genomic probes from the *HMGIC* locus. In all of these cases the translocation breakpoints were upstream of *HMGIC* and in one tumor a deletion of some of the 5' sequences had occurred as well.²⁸ Breakpoints were found at 10 to >100 kb upstream of *HMGIC*. To substantiate a model predicting dysregulation of *HMGIC* expression, a Northern blot of RNA prepared from uncultured tissue from a series of five fibroids with t(12;14) rearrangements and their

matched normal myometrium was performed. Expression of *HMGIC* was detected in four fibroids and in none of the matched myometrial RNAs. One fibroid containing a t(12;14) lacked a detectable signal but was a mosaic with normal 46,XX cells; decreased sensitivity to detect *HMGIC* expression accounted for this discrepancy as *HMGIC* message was detectable by subsequent RT-PCR experiments. In addition, no hybridization was found in a karyotypically normal fibroid.

HMGIY in Uterine Leiomyomata

Recognition of a highly related family member, *HMGIY*, mapped to 6p21, a site of consistent chromosomal rearrangement in benign mesenchymal tumors now known to involve *HMGIC*, led to an evaluation of a uterine fibroid with a pericentric inversion involving 6p21, designated 46,XX,inv(6)(p21q15).²⁹ In collaboration with Amy Williams and Dr. Tucker Collins (Brigham and Women's Hospital, Boston, MA) a FISH probe was developed for *HMGIY* and it was possible to show that this probe was split in the copy of chromosome 6 in which the inversion had occurred. This indicated that the breakpoint was within the vicinity of *HMGIY*. *HMGIY* is known to bind to specific AT-rich domains and promoters of several genes including the interferon β -1 promoter,^{30,31} E selectin,³² the interleukin-2 receptor α -chain,³³ the chemokine (MGS/GRO),³⁴ and the class II major histocompatibility complex gene HLA-DRA,³⁵ and it is necessary for inducible gene expression. Also of interest, *HMGIY* suppresses transcription of several genes including interleukin-4³⁶ and the immunoglobulin ϵ -heavy chain gene.³⁷ Three binding sites for *HMGIY* are present in the interferon β -1 promoter; one is known as positive regulatory domain 2, and is located within the center of an NF- κ B element. The other two sites flank ATF-2/c-jun sites and are known as positive regulatory domain.^{4,31} We developed an oligonucleotide to the positive regulatory domain 2 sequence, as well as a mutated oligonucleotide, to be used in electrophoretic mobility shift assays (EMSA) to detect *HMGIY* binding. In this assay a band was present following assay of protein from the leiomyoma with the inv(6) and no band was seen in protein from the myometrium (Figure 1). When the mutated oligonucleotide was used, the binding was almost completely abolished, whereas no diminution in band intensity was seen to the positive control sequence from the interferon β -1 promoter site. Matched leiomyomas and myometrium from nine different patients including a total of sixteen fibroids were studied for *HMGIY* expression. Interestingly, nine of these sixteen fibroids expressed *HMGIY* as shown by a band in the EMSA. Some fibroids from the same patient would express *HMGIY* whereas others would not, and an absolute correlation was seen with no expression of *HMGIY* in any of the myometrium. In summary, expression studies of *HMGIC* by Northern blot and *HMGIY* by EMSA revealed no myometrium to express either *HMGIC* or *HMGIY*. Five uterine leiomyomas with t(12;14) expressed *HMGIC*. Nine of seventeen uterine leiomyoma had detectable *HMGIY* binding activity including both

Figure 1. HMGIY DNA-binding activity is increased in a uterine leiomyoma with an inv(6p21q15).²⁹ (A) Sequence of oligonucleotides PRDII X 2 and mPRDII X 2 used in EMSA analysis. The duplicated NF- κ B site (PRDII)³⁰ from the human IFN- β promoter has been underlined. Bolded nucleotides show the HMGIY binding site and asterisks above the mPRDII X 2 sequence indicate those nucleotides that were changed to mutate the binding site. (B) EMSA analysis of protein extract from leiomyoma ST93-397. Lanes 1 to 4 contain ³²P-labeled PRDII X 2 oligonucleotide probe mixed with no extract (lane 1), 4 μ g of total protein extract from normal myometrium tissue ST93-398 (lane 2), 4 μ g of total protein extract from leiomyoma tissue ST93-397 (lane 3), and recombinant p50 (lane 4). Lanes 5 to 8 contain ³²P-labeled mPRDII X 2 mixed with no extract (lane 5), 4 μ g of total protein extract from normal myometrium ST93-398 (lane 6), 4 μ g of total protein extract from leiomyoma ST93-397 (lane 7), and recombinant p50 (lane 8). Recombinant p50 homodimer (a form of NF- κ B) was used to demonstrate the integrity of both wild-type and mutated PRDII X 2 oligonucleotides. Bound complexes are indicated by an arrow to the right of the figure. FREE indicates the location of unbound probe.

karyotypically normal and abnormal tumors with the highest expression seen in a fibroid with an inversion of chromosome 6. No uterine leiomyoma were found that expressed both *HMGIC* and HMGIY, and some tumors expressed neither. Thus, it is possible that there may be yet another gene which provides a similar function in these tumors as does *HMGIC* and HMGIY, accounting for the fact that neither have been found to be expressed in some. The finding that no tumors have been found to express both *HMGIC* and HMGIY may mean that these HMG proteins play similar, perhaps reciprocal, roles in the pathobiology of uterine fibroids.

In conclusion, uterine leiomyomata are characterized by several distinct cytogenetic subgroups implying that there are multiple genetic pathways which may lead to these tumors. The frequent finding of chromosomally normal fibroids and the observation of clonality in karyotypi-

cally mosaic primary cell cultures and in uncultured fibroid tissue from mosaic cases indicate that the characteristic cytogenetic aberrations are unlikely to be the primary pathogenetic event. Also, rearrangement of 12q15 in uterine leiomyomata is likely to involve an alteration in expression of *HMGIC*, an architectural factor which is a member of a family of high-mobility group proteins thought to be involved in transcription. Translocations at 12q15 in lipomas, other benign mesenchymal tumors, result in chimeric genes in which the 5' DNA-binding AT hook domains of *HMGIC* are fused with heterogeneous transcriptional regulatory domains. And lastly, rearrangement of 6p21 in uterine leiomyomata is likely to involve an alteration in expression of HMGIY, an architectural factor that is a family member of *HMGIC*.

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