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Genome-wide Analysis of Loss of Heterozygosity and Copy Number Amplification in Uterine Leiomyomas using the 100K Single Nucleotide Polymorphism Array

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Abstract

PURPOSE—Uterine leiomyomas (fibroids) are benign smooth muscle tumors commonly found among reproductive-aged women. Though benign, these tumors are the leading indication for hysterectomies in the United States and cause significant morbidity. Despite the importance of this tumor in women's health, relatively little is known about the molecular etiology.

METHODS—In this study, we used the Affymetrix 100K single nucleotide polymorphism (SNP) chip to assess whether the pattern and frequency of genome-wide loss of heterozygosity (LOH) and copy number amplifications is associated with clinical heterogeneity.

RESULTS—Thirty-seven tumors with varying sizes and histology from eleven patients were analyzed. LOH was observed in 4/37 tumors (10.8%) and significantly associated with large-sized tumors ($p < 0.0014$). Two tumors revealed hemizygosity on chromosome 7q, a region that has been consistently reported to have LOH. Additionally, we detected one novel region of LOH, 16p13.11 in one tumor (2.7 %). Copy number amplifications were observed on all chromosomes; however, most were low-level amplifications and only detected in a single tumor. One region of amplification at 3p26.3 was detected in four tumors.

CONCLUSIONS—Despite the use of a high-density SNP platform, our results suggest that genome-wide LOH and copy number amplifications are infrequent events and generally do not determine clinical and histologic characteristics of this disease.

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Conflict of interest statement

All authors have no conflict of interest.

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Keywords

uterine leiomyoma; single nucleotide polymorphism array; LOH; copy number amplification

INTRODUCTION

Uterine leiomyomas (fibroids) are benign smooth muscle tumors commonly seen in reproductive-age women. Although benign, these tumors may be associated with a range of clinical symptoms including profuse abnormal bleeding, severe pelvic pain, reproductive dysfunction and infertility (Stewart, 2001). As a result, they represent a significant health problem and are the leading indication for hysterectomy in the United States, with an estimated 20% of African-American women having hysterectomies for uterine leiomyomata (Day Baird D et al., 2003; Sandberg, 2005; Stewart, 2001; Walker and Stewart, 2005). Despite the high prevalence and significant morbidity, little is known about the molecular etiology of this disease or whether molecular changes correlate with clinical phenotype.

Cytogenetic changes have been reported in 40%-50% of the excised tumors examined and often occur at specific chromosomal regions (Ligon and Morton, 2000; Sandberg, 2005). The most common chromosome aberrations are a translocation involving chromosomes 12 and 14, t(12:14)(q14-15;q23-24) and deletions on chromosome 7q. Other less frequently occurring chromosome abnormalities include trisomy 12, deletions on 3q, and rearrangements involving 6p21-23 and 10q23. Comparative genomic hybridization (CGH) studies have shown chromosome gains on chromosomes 9q, 14q and 19p and 19q as well as losses on chromosomes 1p, 4p and 4q, 12q and Xq (Levy et al., 2000; Packenham et al., 1997). Interestingly, uterine leiomyomas with abnormal karyotypes have been associated with larger uterine tumor size compared to tumors with normal karyotypes (Rein et al., 1998). Moreover, tumors with deletions on 7q tend to be smaller compared to those tumors carrying t(12:14)(q14-15;q23-24) (Hennig et al., 1999). Consequently, it has been suggested that different cytogenetic subgroups may indicate multiple genetic pathways involved in fibroid growth (Sandberg, 2005).

Loss of heterozygosity (LOH) is a common feature of genetic instability or allelic imbalance. The detection of LOH has been useful for the identification of tumor suppressor genes and patterns of LOH may characterize specific tumor types, stage and growth characteristics (Lieberfarb et al., 2003; Lindblad-Toh et al., 2000). The presence of LOH is also frequently correlated with chromosome abnormalities. However, despite the frequency of reported cytogenetic changes in uterine leiomyomas, there have been few molecular studies examining the presence of LOH in this tumor type. Previous reports have used traditional polymerase chain reaction (PCR) based microsatellite marker analysis and found little evidence of LOH outside the 7q15-31 or 15q26 regions (Canevari et al., 2005; Mao et al., 1999; van der Heijden et al., 1998). While microsatellite analysis is a reliable and inexpensive method for detection of LOH, the relatively small number of loci available for genotyping limits LOH detection capabilities. Furthermore, both CGH and PCR-based microsatellite analyses are designed to detect only those LOH regions with chromosomal loss (hemizyosity), not LOH regions where there is concomitant gene duplication and the copy number remains at 2 (Zhao et al., 2004). Recent studies investigating a variety of other tumor types have reported that 50–70% of observed LOH occurs in copy-neutral regions (copy number = 2) where the loss of one allele is followed by the duplication of the remaining allele (Beroukheim et al., 2006; Huang et al., 2004). Therefore, it is possible genomic regions of LOH exist that have not been detected via CGH or microsatellite analysis.

To date, there has been no comprehensive genome-wide LOH examination of uterine leiomyomas using single nucleotide polymorphism (SNP) chip platforms. The Affymetrix 100K SNP chip genotypes and quantifies >115,000 SNPs, and thus provides an accurate and technically feasible assay to detect regions of LOH and amplification. In the present study, we use the Affymetrix 100K SNP chip to examine genome-wide LOH and copy number changes in 37 uterine fibroid samples collected from 11 patients in The Fibroid Growth Study (Davis et al., 2009). We evaluated surgically removed tumors with varying sizes and histology to determine whether the pattern and frequency of molecular changes correlate with clinical and histologic characteristics of individual tumors.

METHODS AND MATERIALS

Uterine Leiomyoma Samples and DNA extraction

We selected uterine leiomyomas from a group of subjects originally recruited for The Fibroid Growth Study (FGS) (Davis et al., 2009). In the present study, we selected eight subjects having at least two large tumors (>4.5 cm in diameter) and two medium tumors (2–4.5 cm in diameter) for a total of 32 tumors. Five additional tumors with atypical leiomyoma histology were also analyzed. In sum, eleven patients and thirty-seven uterine leiomyomas were evaluated for LOH and copy number amplification. Matched lymphocyte DNA from each patient served as an internal control for copy number and heterozygosity. DNA was extracted from tissue samples using the Qiagen DNeasy Tissue Extraction kit (Qiagen Inc., Valencia, CA), and from lymphocytes using the Autopure Robot LS (Gentra Systems, Minneapolis, MN). Purified DNA was quantified via 260/280 λ absorbance using the Nanodrop® ND-1000 spectrophotometer (Wilmington, DE).

Affymetrix 100K SNP chip assay

All array experiments were carried out according to the manufacturer's recommendations (Affymetrix, Inc., Santa Clara, CA). Briefly, two 250 ng aliquots of genomic DNA were digested with *Xba*I or *Hind*III (New England Biolabs, Boston, MA). Digested DNA was ligated to a universal adaptor using T₄ DNA ligase (NEB) and PCR amplified using Ampli-Taq Gold (Applied Biosystems, Foster City, CA). Four independent 100 μ l PCR reactions were pooled, concentrated and fragmented with DNase I to generate 250–1000 bp products. The fragmented products were end-labeled with biotin at free 3'-OH using terminal deoxynucleotidyl transferase and dideoxy biotinylated nucleoside triphosphate. The biotinylated fragments were denatured and hybridized to the arrays overnight at 48°C. Following hybridization, the chip arrays were washed and scanned with the Gene Chip Scanner 3000.

Real-time PCR

Copy number amplification for *CHL1* was verified with the ABI Taqman® Copy Number assay (Life Technologies, Carlsbad, California) matched to the Affymetrix chip region. RNaseP was used as the reference gene and standard assay protocols were followed, as recommended by the kit manufacturer. Copy number was quantified using the ABI Copy Caller™ software.

Data Analysis

The genotype (AA, AB, BB) of each SNP was determined based on signal intensity for each allele using a dynamic model mapping algorithm incorporated in software GeneChip Genotyping Analysis software (GTYPE) (Di et al., 2005). If a genotype could not be determined, the SNP was classified NO_CALL. The Affymetrix Copy Number Analysis Tool® (CNAT) version 3.0 was used to estimate the copy number for each SNP allele. This

program works by comparing the sample signal intensities of SNP alleles to the mean signal intensities of SNP alleles established from a large reference set of ethnically diverse individuals. Copy number change and LOH were analyzed using software Partek® (St. Louis, MO) version 6.3 beta.

For SNP-based array platforms, LOH is defined as the loss or conversion of contiguous, heterozygous SNP alleles to homozygosity, and is considered either a hemizygous or copy-loss event ($AB \rightarrow A_ \text{ or } _B$) or a copy-neutral event ($AB \rightarrow AA \text{ or } BB$). The boundaries of a chromosomal region having undergone LOH are defined by comparing contiguous heterozygous alleles in the normal control to those corresponding heterozygous alleles in the tumor. For our study, LOH was determined by a Hidden Markov Model implemented in Partek software for paired samples. We compared lymphocyte normal control samples against their matched tumor samples. Consequently, when a series of heterozygous SNPs (AB) in the normal control underwent a genotype change ($AB \rightarrow AA, A_ \text{ or } BB, B_$) in the tumor, the region was identified as LOH.

Copy number amplifications were defined as having at least two contiguous SNPs where copy number had increased by one or more alleles in the tumor when compared to the normal lymphocyte control. The genomic context of SNPs (i.e. dbSNP RS identification, physical position and gene(s) nearest to SNPs) showing evidence of LOH or copy number amplification was obtained using Affymetrix and The University of California Santa Cruz gene annotation databases. A generalized linear mixed model (GLMM) (SAS version 9.1.3) was used to determine if significant correlation exists between tumor size and frequency of copy change or LOH events adjusted for histology (fixed effect), individual and age. Copy number change and LOH events were assumed as Poisson distributed.

RESULTS

Clinical characteristics for the fibroids are summarized in Table I. Seven of the 11 patients had tumors with typical leiomyoma histology characterized by relatively monomorphic whorls of well differentiated smooth muscle cells, while four patients had tumors with necrosis, hemorrhage, cystic change or myxoid degeneration.

Genotyping call rates, the percent of SNPs successfully analyzed per array, ranged from 87% to 99% with a median call rate of 97%, and did not differ between fibroids and lymphocytes. The frequency of observed heterozygous SNPs (informative loci) differed slightly between patients, ranging from approximately 34,000 to over 38,000 SNPs.

Approximately half of the 37 tumors exhibited no genetic events; five tumors had 6 or more events.

Copy number amplifications

Fifteen tumors showed copy number amplification. Over 68 different chromosomal loci with copy number amplifications were identified however, most of the affected loci occurred in only two tumors from a single patient (Table I). Copy number increases ranged from 1.0 to 3.0 extra alleles in the tumor when compared to the normal lymphocyte control, and extended from a few hundred base pairs to over 1Mb in length. One amplified locus at 3p26.3 was detected in four tumors and verified by real-time PCR. Amplification characteristics for 3p26.3 are summarized in Table II.

We also evaluated whether an association existed between atypical leiomyoma histology and the presence of LOH and copy number amplification. For this analysis, we examined all

available tumors from the original parent study (n=11) with histologic phenotypes including: necrosis, hemorrhage, cystic change or myxoid degeneration; no association was detected.

Loss of Heterozygosity

LOH was identified in 4 of 37 tumors, three with hemizygous LOH and one with copy neutral LOH; one tumor had three separate LOH events. Hemizygous or copy-loss LOH was detected in the 7q21.3-7q34 region for 2 of 37 tumors (5.4%). Figure 1 shows the loss of chromosomal material in the 7q regions when these two tumors are compared to their matched lymphocyte controls. Hemizygosity was also detected in a third tumor, which spanned over half the length of chromosome 1, affecting the entire genomic regions of 1p34-1p31.3 and 1q43-1q44. Moreover, this same tumor had an additional region of hemizygous LOH on 16p13.11 approximately 1Mb in length. Lastly, a copy-neutral region approximately 500 Kb in length was mapped to 3q26.1. A statistically significant association ($p < 0.0014$) was found between large-sized tumors (4.5 cm diameter) and the presence of LOH. These tumors and affected chromosomal regions are summarized in Table III.

DISCUSSION

Uterine leiomyomas are the most common gynecological tumors, and though nonmalignant, often cause profound clinical sequelae resulting in significant personal and economic burden (Flynn et al., 2006; Hartmann et al., 2006; Lee et al., 2007). Despite the considerable clinical morbidity, little is known about the molecular etiology surrounding development and growth of this common tumor. It is estimated that almost half of surgically resected uterine leiomyomas exhibit karyotypic abnormalities. Whether these chromosomal changes are secondary events rather than primary etiologic determinants of tumor development is not known (Flake et al., 2003). Unlike malignant tumors, where karyotypic abnormalities are closely correlated with genetic instability as manifest by LOH and copy number amplification (Lengauer et al., 1998), there is limited evidence of such instability in leiomyomas, outside of a few reports of changes in 7q and 15q26 (Canevari et al., 2005; Mao et al., 1999; van der Heijden et al., 1998). These studies utilized a traditional genome-wide, PCR-based microsatellite approach and older CGH platforms, and while these approaches are very reliable, both methods have limitations which may potentially fail to identify areas of LOH containing important candidate genes involved in tumor development.

The present study describes the first report utilizing a genome-wide SNP-based platform to evaluate LOH and copy number amplifications in uterine leiomyomas. Our study design reflected scientific hypotheses based on strategic tumor selection from the original parent study, The Fibroid Growth Study. Thus, while the number of analyzed tumors was smaller compared to earlier LOH studies, the Affymetrix 100K SNP array provided increased genomic resolution for identifying small regions of LOH and copy number amplifications compared to microsatellite marker analysis or older CGH platforms.

Our study revealed a total of 10.8% of tumors with LOH, with over 80% of LOH events being hemizygous (copy-loss). We identified one novel region of loss on 16p13.11 and one copy-neutral LOH event on 3q26 which was previously detected by CGH and microsatellite analysis (Canevari et al., 2005). The most commonly affected region of LOH in our sample set was in the 7q21.3-7q34 region (Table III) with a frequency of 5.4% (2/37) and is comparable to other reports. However, allelic loss in the 7q chromosomal region and as well as other loci seems to vary significantly depending on the tumor sample set and appears to be independent of the number of tumor samples analyzed. Microsatellite analysis conducted by van der Heijden reported a 34% LOH (11/34 tumors) frequency on 7q while Levy et al. found losses on 7q in 25% of studied tumor (3/12 tumors) by CGH (Levy et al., 2000; van

der Heijden et al., 1998). Other studies have reported much lower frequencies of LOH on 7q ranging from 9% to 6.7%, analyzing 94 and 165 tumors respectively (Mao et al., 1999; Vanharanta et al., 2005). Additional regions with allelic loss include 15q26.3 and 7p15.3-22.2 although these specific loci have not been consistently reported (Canevari et al., 2005).

Though the frequency of LOH in this study was low, we found a significant association between the presence of LOH and large tumor size, indicating LOH may affect tumor growth or be a reflection of genetic alterations accumulating over time. This finding complements published cytogenetic studies which have also found associations between increased tumor size and chromosomal abnormalities (Hennig et al., 1999; Kataoka et al., 2003; Rein et al., 1998). In the present study, the mean tumor diameter is 9 ± 2.45 cm, and is similar to tumor diameter sizes reported by Hennig et al. for tumors showing the 12q14-15 translocation. However, tumors harboring 7q deletions in that study were significantly smaller compared to those with the 12q14-15 translocation and similar in size with tumors having normal karyotypes. Though none of the tumors in our sample set have been karyotyped, we did not detect any deletions or aneuploidy similar to earlier CGH analysis; SNP array analysis is not useful for identifying balanced chromosomal rearrangements.

Chromosomal gains were more common than LOH with 41% of tumors showing at least one amplified locus. Overall, amplifications were low-level, did not involve large genomic regions and primarily due to two tumors from one patient. Since most amplified loci occurred in only one tumor, no detectable pattern or frequency of copy number amplification was identified. One region of amplification on 3p26.3 was confirmed in four different tumors; however, the nearest gene located to this amplification, *CHL1*, does not seem likely to be involved in fibroid development or growth. Finally, we did not detect any association between atypical leiomyoma histology and the presence of copy number changes, however, the number of analyzed tumors was small, and this finding will need to be confirmed.

Although the Affymetrix 100K SNP chip provides an excellent method to determine LOH and copy number changes, more recent improvements in probe design and selection for whole genome SNP and CGH platforms have increased the precision of genomic scanning. Thus, it is possible that small areas of LOH or copy number changes went undetected in our analysis.

In conclusion, our data are consistent with earlier findings which suggest little evidence of large scale LOH or copy number variation in the genomes of most uterine leiomyomas. Thus, molecular mechanisms other than genetic instability most likely underlie the initiation and progression of this common tumor.

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Figure 1. LOH on chromosome 7q

Two tumors with LOH on 7q are compared to their respective normal lymphocyte control. Each square represents an individual SNP. The arrow designates the expected copy number of 2.

Table 1

Summary of tumor phenotypes and amplification/LOH events

Tumor number	diameter size (cm)	position	clinical histology ^a	Total number of events (amplifications/LOH)
16-1	7	intramural	5% necrosis, 1% cystic change	3/0
16-2	4.5	intramural	0.5% cystic change	0/0
22-1	7	subserosal	typical	0/1
22-2	5.3	subserosal	typical	0/0
22-3	3	intramural	typical	0/0
22-5	3.5	intramural	typical	0/0
32-1	12	intramural	typical	1/0
32-2	10.5	pedunculated	typical	4/0
32-5	3	intramural	typical	0/0
32-6	4	subserosal	typical	1/0
34-1	7	intramural	typical	5/0
34-5	3.5	submucosal	typical	0/0
34-6	2.2	unknown	typical	0/0
34-ex1	4.5	intramural	typical	0/0
49-1	9	intramural	typical	0/1
49-2	3.5	intramural	50% necrosis	38/0
49-3	4	intramural	90% hemorrhage	26/0
49-4	5.5	subserosal	80% hemorrhage	6/0
49-5	2.2	unknown	50% hemorrhage	0/0
51-1	7.5	subserosal	40% fatty degeneration/necrosis	0/0
51-2	6.8	subserosal	5% necrosis/myxoid degeneration	0/0
51-4	3	intramural	50% necrosis, 30% hemorrhage, 20% cystic change	0/0
51-7	3.6	subserosal	30% necrosis, 10% cystic change/myxoid degeneration	7/0
54-1	8.5	subserosal	typical	1/0
54-2	5.5	submucosal	typical	0/0
54-4	3	unknown	typical	0/0
54-8	3.5	subserosal	typical	2/0
64-1	9.5	intramural	typical	1/0

Tumor number	diameter size (cm)	position	clinical histology ^a	Total number of events (amplifications/LOH)
64-3	2.3	intramural	typical	0/0
64-ex2	3	intramural	typical	0/0
64-ex3	6	intramural	typical	0/0
73-1	6.5	intramural	typical	0/0
73-6	6	intramural	typical	11/0
73-10	3	intramural	typical	1/0
73-ex2	3	subserosal	typical	1/0
85-1	13	subserosal	0.5% necrosis, 0.2% hemorrhage	0/3
104-1	7	intramural	typical	0/1

^atypical refers to histologically unremarkable

Table 2

Copy number gains detected at 3p26.3 in 4 tumors

Chromosome location	Patient ID-Tumor number	# of amplified SNPs	dbSNP RS ID.	Approximate Size of copy gain (bp)	Nearest gene(s) to SNP(s)	Range of Copy number for each SNP
3p26.3	51-7	9	rs9284831	100,000	CHL1 Cell adhesion molecule with homology to L1CAM	3.2-3.4 (average = 3.2)
			rs10510176		"	
			rs10510177		"	
			rs6788514		"	
			rs10510178		"	
			rs1568086		"	
			rs10510179		"	
			rs10510180		"	
			rs1516342		"	
	34-1	12	(including 9 above)	121,700	CHL1 (continued) Cell adhesion molecule with homology to L1CAM	3.1-3.5 (average = 3.3)
			rs10510181		"	
			rs10510182		"	
	73-6	46	rs10510184	536,000	CHL1 (continued) Cell adhesion molecule with homology to L1CAM	3.0-4.4 (average = 3.4)
			(including 12 above)			
			rs10510185		"	
			rs6442315		"	
			rs6442427		"	
			rs10510186		"	
			rs7630843		"	
			rs7640724		"	
			rs7612619		"	
			rs4684335		"	
			rs10510164		"	
			rs162589		"	
			rs330891		"	
			rs7649544		"	
			rs9310982		"	

Chromosome location	Patient ID-Tumor number	# of amplified SNPs	dbSNP RS ID.	Approximate Size of copy gain (bp)	Nearest gene(s) to SNP(s)	Range of Copy number for each SNP
			rs10490841		"	
			rs2060311		"	
			rs3773377		"	
			rs2196039		"	
			rs7612327		"	
			rs7652221		"	
			rs3856878		"	
			rs10490842		"	
			rs2033518		"	
			rs10510192		"	
			rs10510193		"	
			rs1988234		"	
			rs2570024		"	
			rs2729070		"	
			rs2243813		"	
			rs1865160		"	
			rs9311410		"	
			rs9311442		"	
			rs1531913		"	
			rs2729207		"	
			rs2729257		"	
	73-10	46	"	536,000	"	3.2

Table 3

Chromosome regions with Loss of Heterozygosity

Patient ID-Tumor Number	diameter size (cm)	affected chromosome region(s)	copy-loss or copy-neutral
22-1	7	7q22-7q34	copy-loss
49-1	9	7q21.3-7q31.33	copy-loss
85-1	13	1p34-1p31.3	copy-loss
		1q43-1q44	copy-loss
		16p13.11	copy-loss
104-1	7	3q26.1	copy-neutral