High-Resolution Melting Analysis of *MED12* Mutations in Uterine Leiomyomas in Chinese Patients

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Objectives: Somatic mutations in mediator complex subunit 12 (*MED12*) have emerged as a critical genetic change in the development of uterine leiomyomas. Studies, however, have focused largely on cohorts consisting of Caucasian patients. In this study, uterine leiomyomas from Chinese patients were examined for *MED12* mutations. In addition, polymerase chain reaction (PCR)-based high-resolution melting analysis (HRMA) was compared with direct sequencing as a potentially more sensitive method for the detection of *MED12* mutations. **Methods:** Tissue samples with the pathologies of uterine leiomyoma ($n=181$) and other endometrial diseases ($n=157$) were collected from Chinese patients at the Taizhou People's Hospital and Taizhou Polytechnic College (Taizhou City, China). Genomic DNA was prepared from all samples. Both PCR-based HRMA and PCR-based direct sequencing were used to detect *MED12* mutations. **Results:** PCR-based HRMA and direct sequencing revealed *MED12* mutations in 95/181 (52.5%) and 93/181 (51.4%) uterine leiomyomas, respectively. Nearly half of these mutations (46/93) were found in a single codon, codon 131. The coincidence rate between the two methods was 98.9% (179/181) so that no statistically significant difference was evident in the application of the methodologies ($\chi^2=0.011$, $p=0.916$). In addition, *MED12* mutations were identified in 1/157 (4.17%) case of other endometrial pathologies by both methods. **Conclusions:** *MED12* mutations were closely associated with the development of uterine leiomyomas, as opposed to other uterine pathologies in Chinese patients, and PCR-based HRMA was found to be a reliable method for the detection of *MED12* mutations.

Introduction

UTERINE LEIOMYOMA is a benign smooth muscle neoplasm, but may cause severe complications, such as uterine bleeding, abdominal pain, and infertility (Mäkinen *et al.*, 2011b). While the development of uterine leiomyomas is closely associated with the steroid hormones, estrogen and progesterone, the genetic basis for the disease remains unclear. Recent studies with whole-exome sequencing, however, have identified a high frequency of mutations in the mediator complex subunit 12 (*MED12*) gene in uterine leiomyomas of Caucasian patients (Taatjes, 2010; Mäkinen *et al.*, 2011a; McGuire *et al.*, 2012).

MED12 is only 1 of 26 subunits that make up the mediator complex transcription factor. The subunit regulates the expression, in an RNA polymerase II-dependent manner, (Taatjes, 2010) of target genes, which are generally involved in cell growth and differentiation (Zhang and Emmons, 2000; Ding *et al.*, 2008; Wu *et al.*, 2014). Mutations in *MED12* have therefore been found to lead to uncontrolled growth of cells and tumor development (Moghal and Sternberg, 2003; Phi-

libert and Madan, 2007). In human diseases, the incidence of *MED12* mutations in uterine leiomyoma (50–70%) has been found to be higher than in uterine leiomyosarcoma and uterine smooth muscle tumors of uncertain malignant potential (5–20%). Endometrial polyps, however, exhibited no *MED12* mutations (Kämpjärvi *et al.*, 2012).

Currently, polymerase chain reaction (PCR)-based direct sequencing is one of the commonly used methods for identifying gene mutations, and the method is the gold standard for detecting both previously identified and novel gene mutations. However, the detection of *MED12* mutations is difficult and time consuming due to diversity of the mutations. High-resolution melting analysis (HRMA) has proven to be an effective method for detecting genetic mutations and it has several advantages, such as low cost, simple operation, and high throughput (Tindall and Petersen, 2009). In this study, the goal was to directly compare HRMA technology with PCR-based sequencing to determine the frequency of *MED12* mutations in uterine leiomyomas and other endometrial diseases (adenomyosis, adenomyoma, cervical cancer, endometrial

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FIG. 1. Schematic representation of amplicons for *MED12* sequencing and HRMA. Exons 1–3 are designated by the gray boxes in the diagram. The location of the primers used for polymerase chain reaction (PCR)-based sequencing and HRMA are designated by the black and gray arrows, respectively. Both amplicons were designed to amplify exon 2 of the *MED12* gene. HRMA, high-resolution melting analysis.

cancer, and leiomyosarcoma) specifically from Chinese patients.

Materials and Methods

Ethics statement

All protocols in this study were approved by the Institutional Review Board of the Taizhou People's Hospital at Taizhou Polytechnic College (Taizhou City, China), and written informed consent was obtained from all participants.

Clinical characteristics

Tissue samples were collected from patients who underwent surgical resection for uterine leiomyomas ($n=181$) and other endometrial diseases ($n=157$; adenomyosis, adenomyoma, cervical cancer, endometrial cancer, and leiomyosarcoma) in the Department of Obstetrics and Gynecology in Taizhou People's Hospital. Fresh tissues were snap-frozen and stored in liquid nitrogen until use. Pathological diagnosis was performed on all samples. The age range of patients was from 28 to 65 years (mean age, 42 years). Clinical features of patients with uterine leiomyomas included hypermenorrhea (120/181, 66.3%) and irregular menstrual cycles (43/181, 23.8%).

Genomic DNA isolation

Frozen tissue samples (1–20 mg) were homogenized, and genomic DNA was extracted with the AxyGen genome extraction kit, according to the manufacturer's protocols (AxyGen Scientific, Inc., Union City, CA).

PCR-based direct sequencing for the *MED12* gene

The primers for sequencing were designed based on the gene sequences from the NCBI GenBank database. The amplified fragment contained *MED12* intron 1 to exon 2 (Fig. 1). In a total volume of 50 μ L, the reaction consisted of DNA template (5 μ L), 10 $\times$ Tag Buffer (5 μ L), 2.5 mM dNTPs (1 μ L), 50 pM forward primer (0.5 μ L), 50 pM reverse primer (0.5 μ L), Taq Polymerase (1 μ L), and DEPC H₂O (37 μ L; Jiangsu Futai Biotechnology Co. Ltd., Taizhou, China). Sequencing was performed with the following cycle parameters: 95°C for 5 min, 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and finally at 72°C for 5 min (Table 1). Sequencing was performed with the PCR primers and reactions were run on the ABI3730 sequencer (Applied Biosystems, Life Technologies, Grand Island, NY, USA).

HRMA method for detecting *MED12* mutations in exon 2

The primers used are shown in Table 1. The reaction (20 μ L) consisted of genomic DNA (2 μ L), 10 $\times$ Tag Buffer (2 μ L), 2.5 mM dNTPs (1 μ L), evagreen (1 μ L), and 20 pM for each primer (0.5 μ L), Hot Star (0.2 μ L), and ddH₂O (12.8 μ L; Roche Applied Science, Basel, Switzerland). PCR was carried out with the following cycle parameters: denaturation: 95°C for 5 min; amplification: 50 cycles at 95°C for 10 s, 55°C for 15 s, and 72°C for 25 s (Table 1); dissolution: 95°C for 1 min, 40°C for 1 min, 65°C for 1 s, and 95°C to detect fluorescence 40 times every second. PCR products were analyzed by Roche LightCycler 480 (Roche Applied Science).

Statistical analysis

SigmaPlot software 12.0 was used to perform statistical analysis. The differences in the incidence of *MED12* mutations between the two groups and the two methods were assessed by the χ^2 test where $p<0.05$ was considered statistically significant.

Results

Both PCR-based sequencing and HRMA were used to detect the frequency of *MED12* mutations in patients with uterine leiomyoma and to compare methodologies. In the 181 patients with uterine leiomyomas, *MED12* mutations were

TABLE 1. PRIMERS USED FOR POLYMERASE CHAIN REACTION AMPLIFICATION OF THE *MED12* GENE

Primer name	Sequence (5'-3')	Product length (bp)	Parameters for PCR
<i>MED12</i> _{SEQ}	Forward: GCCCTTTCACCTTGTTCTCTC	290	94°C, 30 s 55°C, 30 s 72°C, 30 s
<i>MED12</i> _{SEQ}	Reverse: GTCCCTATAAGTCTTCCCAAC		35 cycles
<i>MED12</i> _{HRM}	Forward: AAACGCCGCTTTCCTGCCTC	98	95°C, 10 s 55°C, 15 s
<i>MED12</i> _{HRM}	Reverse: CACTGCCATGCTCATCCCCA		72°C, 25 s 50 cycles

The first denaturation was carried out at 95°C for 3 min, and the final elongation was performed at 72°C for 10 min. PCR, polymerase chain reaction.

TABLE 2. *MED12* MUTATIONS IN PATIENTS WITH UTERINE LEIOMYOMAS

Mutation site	Number, n	Percentage, %
EX2, c.130G->A, c.146C->T	6	6.45
EX2,c.131G->T	23	24.73
EX2,c.131G->A	21	22.58
EX2,c.130G->C	8	8.60
EX2,c.130G->A	7	7.53
EX2,c.130G->T	8	8.60
EX2,c.128A>C	6	6.45
EX2,c.127Ins27	2	2.15
EX2,c.118_132Del15	2	2.15
EX2,c.141_165Del15	2	2.15
EX2,c.131_147Del17	2	2.15
EX2,c.117_134Del18	2	2.15
Intron1, IVS1-10_135Del46	2	2.15
Intron1, IVS1-8T>A	2	2.15
Total	93	100

TABLE 3. *MED12* MUTATIONS IN PATIENTS WITH UTERINE DISEASES

Disease	Case, n	Mutation, n	Percentage, %
Uterine leiomyomas	181	93	50.28
Adenomyosis	47	0	0
Adenomyoma	55	0	0
Cervical cancer	16	0	0
Endometrial cancer	15	0	0
Leiomyosarcoma	24	1	4.17

detected in 93 cases (51.4%) by PCR-based sequencing. The distribution of *MED12* mutations between exon 2 and intron 1 was 89 (95.7%) and 4 (4.3%), respectively (Table 2). Single-nucleotide mutations were identified in exon 2 in 79 cases, and insertion/deletion mutations in exon 2 and intron 1

in 14 cases (Table 2). Interestingly, 46/93 of these mutations occurred at a single codon, codon 131. When the cases were examined by PCR-based HRMA, *MED12* mutations were detected in additional two cases, 95/181 (53.3%). In contrast, *MED12* mutation was detected in only a single case by either method in the 157 patients with other endometrial diseases (Table 3). The incidence of *MED12* mutations by either method in uterine leiomyomas was higher compared with other endometrial diseases, and this result was statistically significant (direct sequencing: $\chi^2 = 82.183$, $p < 0.001$; PCR-based HRMA: $\chi^2 = 84.667$, $p < 0.001$).

Mutant and wild-type sequences were easily distinguishable in most cases in the sequencing chromatograms and melting curves from PCR-based direct sequencing and HRMA, respectively (Fig. 2). The coincidence rate between these two methods in this cohort of 181 patients with uterine leiomyoma was 98.9% (179/181). Only two cases were mismatched between the two methods (Fig. 3). In both these cases, a mutation at codon 131 (G→A) was detected with HRMA, whereas only the wild-type sequence was apparent with direct sequencing. After further examination, a weak signal on the sequencing chromatogram was consistent with a mutation at codon 131. Therefore, when the mutation frequency was statistically analyzed based on the method employed, no significant differences in the incidence of *MED12* mutations detected by PCR-based direct sequencing versus HRMA were found ($\chi^2 = 0.0111$, $p = 0.916$).

Discussion

The underlying genetic map for uterine leiomyomas had been largely uncharacterized until several large-scale genomic sequencing projects identified the presence of *MED12* mutations in these tumors (Taatjes, 2010; Mäkinen *et al.*, 2011a; McGuire *et al.*, 2012). In this study, *MED12* mutations were investigated in uterine leiomyomas and other

FIG. 2. Mutated or wild-type *MED12* sites as detected by gene sequencing and HRMA. The melting curves of HRMA are shown on the left, while the chromatograms of PCR-based sequencing are shown on the right. The curves of wild-type samples are in gray and mutant samples are in black. Black arrows indicate the position of the mutation in the chromatogram.

FIG. 3. Melting curve and sequencing chromatogram at *MED12* mutation detected only by HRMA. The melting curves from the HRMA experiment are shown on the *left*, while the chromatogram of PCR-based gene sequencing is shown on the *right*. The curve of wild-type samples is in *gray* and mutant sample is in *black*. *Black arrows* indicate the position of the mutation on the chromatogram.

endometrial pathologies specifically from Chinese patients. The results revealed that the incidence of *MED12* mutations in uterine leiomyomas was significantly higher compared with other endometrial diseases and is consistent with previous studies worldwide (Taatjes, 2010; Mäkinen *et al.*, 2011a; Je *et al.*, 2012; Matsubara *et al.*, 2013). The incidence of *MED12* mutations in uterine leiomyomas was 51.4% (93/181) and 53.3% (95/181) by PCR-based direct sequencing and PCR-based HRMA, respectively. Furthermore, nearly half (46/93) of the mutations were found at codon 131. In addition, the low *MED12* mutation rate (0.6%) in other endometrial diseases (adenomyosis, adenomyoma, cervical cancer, endometrial cancer, and leiomyosarcoma) in this cohort was consistent with the reported low frequency of *MED12* mutations in leiomyosarcoma, colorectal diseases, uterine lipoleiomyomas, breast cancer, esophageal squamous cell carcinoma, hepatocellular carcinoma, leukemia, and other malignant tumors (Je *et al.*, 2012; Kämpjärvi *et al.*, 2012; Matsubara *et al.*, 2013). These data indicate that *MED12* mutations are closely associated with the development of uterine leiomyomas.

In recent years, PCR-based HRMA has been used to examine genetic mutations due to its low cost, simple operation, high throughput, and high sensitivity and specificity (Tindall *et al.*, 2009). It is suitable to detect single-base mutations as well as small base-pair insertions and deletions. In this study, PCR-based HRMA demonstrated a high agreement with PCR-based direct sequencing. However, mutations in two cases were only revealed by HRMA. After further examination, a weak signal on the sequencing chromatogram was consistent with a mutation at codon 131. The discrepancy between methods may reflect the proportion of contaminating normal DNA, a complication for the detection of mutations by direct sequencing. As somatic mutation generally drives the development of cancer, tumors are a mixture of mutated and normal cell types. Tumor DNA is therefore contaminated with the wild-type genome, and direct sequencing can yield false-negative results if the proportion of normal DNA is too high. Thus, the results of the present study indicate that the sensitivity of HRMA in the detection of *MED12* mutations is greater compared with direct sequencing.

HRMA, however, requires accurate PCR amplification. Any double-stranded molecules, such as primer dimers or nonspecific PCR products, will influence melting profiles. In addition, HRMA may also minimize the length of the target sequence to be examined. The sensitivity and specificity of HRMA have been reported to be 100% for amplicons < 200 bp, whereas increasing length of amplicons reduces the sensitivity and specificity slightly (Chou *et al.*, 2005; Whittall *et al.*, 2010). Therefore, in this study, PCR products were

designed to be 290 bp for sequencing, but only 98 bp for HRMA.

Why *MED12* is a frequent genetic target in uterine leiomyomas or other cancers may be due to its important role in cell growth and differentiation, which is mediated, in part, through the Nanog signaling pathway (Tutter *et al.*, 2009). Second, *MED12* also interacts with β -catenin to regulate the transcription of target genes in the *Wnt* signaling pathway, which has a well-described role in cancer development (Zhang *et al.*, 2000; Kim *et al.*, 2006). Furthermore, although *MED12* is a subunit of a transcription complex, its activity is not confined to the nucleus. In the cytoplasm, *MED12* negatively regulates TGF- β 2 through physical interaction. The suppression of *MED12* therefore results in activation of TGF- β 2 signaling (Huang *et al.*, 2012). Finally, uterine leiomyoma-linked *MED12* mutations have been shown to disrupt mediator-associated CDK activity (Turunen *et al.*, 2014). How specific *MED12* mutations found in human leiomyomas function in tumor development remains to be defined.

In conclusion, *MED12* mutations are closely associated with the development of uterine leiomyomas in Chinese patients. Furthermore, PCR-based HRMA is an effective method to detect *MED12* mutations.

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Author Disclosure Statement

No competing financial interests exist.

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