Gynecologic Oncology xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

Gynecologic Oncology

journal homepage: www.elsevier.com/locate/ygyno

HMGA2 and *MED12* alterations frequently co-occur in uterine leiomyomas

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HIGHLIGHTS

- *MED12* and *HMGA2* appear to be independently altered in fibroids.
- 50% of fibroids in our sample set showed simultaneous alterations in both genes.
- *HMGA2* and *MED12* alterations frequently coexist in fibroids.

ARTICLE INFO

Article history:
Received 16 March 2018
Received in revised form 6 July 2018
Accepted 8 July 2018
Available online xxxx

Keywords:
Leiomyoma
MED12
HMGA2
Mutation
Gene expression
Sequencing

ABSTRACT

Objective. Around 70% of uterine leiomyomas show *MED12* mutations while overexpression of *HMGA2* mRNA is also highly frequent in fibroids. However, previous studies suggested that alterations in both genes are mutually exclusive. In the present study, we searched for mutation in *MED12* and analyzed the expression of *HMGA2* in 20 uterine leiomyomas and their matched myometrium.

Methods. Normal and tumor tissue obtained from premenopausal women who underwent hysterectomy were collected after surgery and DNA, RNA and proteins were isolated and analyzed for *MED12* mutations using Sanger sequencing, *HMGA2* mRNA expression by quantitative PCR and HMGA2 protein detection by western blot and immunohistochemistry.

Results. 75% of the tumors displayed *MED12* mutation while 65% of them showed overexpression of *HMGA2* mRNA in leiomyomata compared to myometrial tissues ($p = 0{,}0008$). Interestingly, 50% of the tumors showed mutations in *MED12* and overexpression of *HMGA2* mRNA simultaneously, suggesting that alterations in both genes are relatively frequent in uterine leiomyomas.

Conclusions. Contrary to the present findings, former studies showed that mutations in *MED12* and overexpression of *HMGA2* are mutually exclusive. Here, we observed that overexpression of *HMGA2* mRNA in tumors measured by quantitative PCR and compared to myometrium is a common phenomenon in fibroids and is frequently associated with *MED12* mutations. In addition, the common clonal origin of tumors overexpressing *HMGA2* mRNA and its expression in few myometrial tissue points to *HMGA2* up-regulation as an early event in leiomyoma tumorigenesis.

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1. Introduction

Uterine leiomyomas (UL), also called fibroids or myomas, are the most common gynecological tumors, with an incidence of 40% at the age of 35 and nearly 70%–80% around the age of 50 [1]. Severe symptoms develop in 15–30% of patients causing irregular, prolonged or heavy vaginal bleeding, pain in the back of the legs and pelvic discomfort [1]. In addition, these tumors affect normal uterine function, such as embryo implantation, pregnancy and labor, contributing to female infertility and pregnancy-related problems [2].

Despite the high prevalence and significant health problems, relatively little is understood about the etiology of UL. Although women frequently develop multiple leiomyomas, it seems clear by chromosomal and molecular analyses that each UL is an independent monoclonal

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https://doi.org/10.1016/j.ygyno.2018.07.007
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process that arises from the proliferation of a single cell [3]. Until now, it is not clear whether these masses of proliferating cells could originate from a single myometrial smooth muscle cell (SMC) or from a somatic stem cell (SSC) that have the ability for self-renewal and tumor growth under the influence of ovarian hormones [4]. In either case, a genetic mutation may contribute to the neoplastic transformation of the normal tumor-initiating cell [4,5]. In this sense, two frequently altered genes in UL and candidates for initiating tumorigenesis are high mobility group AT-hook 2 (*HMGA2*) and mediator complex subunit 12 (*MED12*) [4,5].

Approximately 40 to 50% of leiomyomas have detectable cytogenetic aberrations. Of these, 20% correspond with rearrangement of 12q14–q15 affecting *HMGA2* [6]. This gene is an architectural transcription factor that induces conformational changes in chromatin structure affecting growth, differentiation, apoptosis and cellular transformation [7]. Most of the breakpoints found in the UL are located upstream of the *HMGA2* gene promoter, thus primarily affecting its expression rather than its protein sequence and giving rise to full-length *HMGA2* overexpression [8]. Although the highest expression levels for *HMGA2* were observed in tumors with rearrangements affecting the region 12q14-q15 [7,9], leiomyomas without such aberrations also showed higher *HMGA2* mRNA levels compared to matched myometrium. In fact, Klemke et al. [7] found that in nearly all paired samples analyzed (51 myometrial tissues and 107 corresponding UL) the leiomyomas showed higher *HMGA2* expression than the corresponding myometrium regardless of the presence or absence of chromosomal abnormalities.

The expression of *HMGA2* is widely restricted to the embryonic stage and decreases to undetectable levels in adult tissues [10]. Deregulated expression in adult tissues has been associated with growth of mesenchymal tumors [11,12].

Recently, exome sequencing has identified somatic mutations in the RNA polymerase II transcriptional Mediator subunit 12 (*MED12*) in as many as 67–70% of uterine leiomyomas from Caucasian women [13,14]. This finding has now been validated in other populations and ethnic groups, with a mutation frequency ranging from 52% to 80% [15–20]. All mutations resided in exon 2 or in the preceding exon–intron boundary, and the majority of them affected a triplet encoding glycine 44. Although *MED12* is located on chromosome X and cells undergo random X-inactivation early during development, analysis of cDNA sequence from UL demonstrated that mutations were present in the active allele. *MED12* is part of an evolutionary conserved multiprotein complex called Mediator, which participates in the regulation of transcription [21]. MED12, along with MED13, Cyclin C and CDK8 or CDK19, forms Mediator subcomplex known as a CDK8. It has been previously shown that uterine leiomyoma-linked mutations in MED12 disrupt its direct interaction with components of the CDK8 module, which may contribute to leiomyoma formation [22].

Mutually exclusive driver mutations in *MED12* and *HMGA2* have been previously proposed [5,23,24], suggesting the existence of two molecularly distinct subtypes of UL. Interestingly, the very high frequency of *MED12* mutation in UL and the fact that *HMGA2* mRNA is overexpressed in most UL compared to matched myometrium, suggest that both alterations may coexist in a significant percentage of UL. To further shed light on the molecular pathogenesis of fibroids, we searched for mutation in exon 2 of *MED12* using Sanger sequencing and analyzed the expression of *HMGA2* by quantitative PCR (qPCR) and western blot in a set of 20 uterine leiomyoma and 18 matched myometrium samples. In addition, immunohistochemistry analysis was also carried out to detect both proteins in normal and tumor tissue.

2. Materials and methods

2.1. Patients

Eighteen female patients aged 36–49 years, admitted to Hospital Quironsalud between 2006 and 2012 were enrolled in this study after giving informed consent. Ethical approval was granted by the Committee for Clinical Research Ethics of the Hospital Universitario de Canarias. The clinicopathological features of patients are shown in Supplementary Table S1. Samples analyzed included 16 intramural, submucous, or subserous leiomyoma specimens from 16 women, as well as the matched myometrial tissue; two tumors (intramural and submucous) obtained from one woman, and her matched myometrial tissue; and two tumors (intramural and subserous) obtained from another woman, and her matched myometrial tissue. Myometrial samples were taken as far away as possible from leiomyomata. All patients underwent hysterectomy for menorrhagia without any previous hormonal treatment for at least 3 months. Well-circumscribed, firm and white-greyish leiomyomas, were chosen for the study. The histopathological analysis using standard H&E staining and performed by a pathologist indicated tumors with benign histology with no sing of malignancy, nuclear atypia, mitotic figures or necrosis. Regarding the menstrual phase, participants enrolled in this study included 7 in proliferative phase and 11 in secretory phase. The proliferative and secretory phases were assigned based on the date of the last menstrual period and confirmed by histological assessment.

2.2. Nucleic acid isolation and reverse transcription

A piece of approximately 1 cm^2 of myometrium and tumor tissue were taken immediately after surgery, submerged in 2 mL of RNAlater during 1 day at 4 °C and stored at −20 °C until processed.

Total RNA and DNA were extracted using a combination of Tri-Reagent (Sigma-Aldrich, St Louis, MO, USA) and the RNeasy Mini Kit (Qiagen, London, UK). Fifty milligrams of tissue were homogenized in 1 mL of Tri-Reagent using TissueRuptor (Qiagen, London, UK). After adding 200 μL of chloroform and mixing, samples were centrifuged at 12,000 ×*g* for 15 min at 4 °C. The upper aqueous phase was used to extract RNA as outlined below while the interphase and organic phase were used to precipitate DNA according to the manufacturer's instructions (Sigma-Aldrich, St Louis, MO, USA).

To isolate RNA, one volume of ethanol 100% was added to the aqueous phase, mixed, and then the RNA was cleaned and eluted using the RNeasy Mini Kit according to the manufacturer's instructions. Residual genomic DNA was removed by incubating the RNA samples with RNase free DNase I and RNasin (Promega Corp. Madison, WI, USA) according to the manufacturer's instructions. The effectiveness of the DNase treatment was assessed in samples with no reverse transcriptase added (RT-negative). Integrity of RNA was checked by agarose gel electrophoresis and ethidium bromide staining. Finally, RNA was quantified by absorbance using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The interphase and organic phase were used to precipitate DNA according to the manufacturer's instructions (Sigma-Aldrich, St Louis, MO, USA).

Retro-transcription was carried out using 2 μg of RNA, and first-strand complementary DNA was synthesized using Moloney murine leukemia virus reverse transcriptase, RNase H Minus, Point Mutant according to manufacturer's instructions (Promega Corp. Madison, WI, USA).

2.3. MED12 mutation detection

Gene-specific primers were designed using Primer3 (http://frodo.wi.mit.edu/primer3) [25] and Primer-BLAST (http://www.ncbi.nlm.nih.gov). The secondary structures of PCR primers and products were calculated using mfold web server [26]. A total of six primer pairs were used in this study (Supplementary Table S2).

Amplification of *MED12* DNA was performed with primers located in intron 1 and intron 2 covering the hot spot region where 99% of mutations have been described. To check for the presence of mutations in cDNA, a primer pair located in exon 1 and exon 2 was used.

PCR mixes contained 0.25 pmol of each primer, 1 unit of Phire Hot Start DNA Polymerase (Finnzymes, Thermo Fisher Scientific, Waltham,

Please cite this article as: L.J. Galindo, et al., *HMGA2* and *MED12* alterations frequently co-occur in uterine leiomyomas, Gynecol Oncol (2018), https://doi.org/10.1016/j.ygyno.2018.07.007

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