Missense mutations in exon 2 of the MED12 gene are involved in IGF-2 overexpression in uterine leiomyoma

S. Di Tommaso, A. Tinelli, A. Malvasi, and S. Massari

ABSTRACT: Uterine leiomyoma (UL), the most common benign tumour found in females, is associated with many recurrent genetic aberrations, such as translocations, interstitial deletions and specific germline mutations. Among these, mutations affecting exon 2 of the mediator complex subunit 12 (MED12) gene are commonly detected in the majority of ULs. Mutational analysis of the MED12 gene, performed on 36 UL samples, revealed that 12 leiomyomas (33.4%) exhibited heterozygous missense mutations in codon 44 of exon 2 of the MED12 gene, four leiomyomas (11.1%) showed internal in-frame deletions, and two leiomyomas (5.5%) exhibited deletions involving intron 1—exon 2 junction, which caused a predicted loss of the splice acceptor. No mutations were detected in uterine myometrium (UM) and pseudocapsule (PC) samples, including those from women with a MED12 mutation in UL. These data showed that the PC is a healthy tissue that surrounds the UL to maintain UM integrity. Analysis of insulin-like growth factor 2 (IGF-2) and collagen type IV alpha 2 (COL4A2) mRNA expression levels in the same set of ULs revealed that only those with MED12 missense mutations expressed significantly higher levels of IGF-2 mRNA. In contrast, MED12 gene status does not appear to affect mRNA expression levels of the COL4A2 gene. On the basis of this finding, we suggest that the MED12 status stratifies the ULs into two mutually exclusive pathways of leiomyoma genesis, one with IGF-2 overexpression and the other with no IGF-2 activation. The occurrence of IGF-2 overexpression could be therapeutically targeted for the non-surgical treatment of leiomyomas.

Key words: COL4A2 gene / IGF-2 gene / MED12 gene / pseudocapsule / uterine leiomyoma

Introduction

Uterine leiomyomas (ULs) are the most common gynaecological pathology, which affect 25–50% females of reproductive age, with strong differences among races (Bulun, 2013). ULs can develop both as a single lesion or as multiple masses, with a diameter that is variable from a few mm to more than 10 cm. Larger ULs (>2 cm) are surrounded by a pseudocapsule (PC), which is an echogenic structure in functional continuity with the uterine myometrium (UM) (Tinelli et al., 2009) characterized by a strong angiogenic profile (Di Tommaso et al., 2013). Although asymptomatic in most cases, ULs can sometimes be associated with a distortion of the uterine morphology and volume. This leads to an alteration of uterus functionality, which results in abnormal bleeding, abdominal pain, infertility and multiple pregnancy loss (Cook et al., 2010). Although pharmacological treatment, which mainly consists of administration of GnRH agonist and ulipristal acetate (Hoellen et al., 2013), is used to reduce UL growth, surgery that is performed both by myomectomy or hysterectomy is the only well-studied effective treatment available (Mettler et al., 2012). Despite its high prevalence, the precise pathophysiology underlying UL still remains unknown.

Genomic aberrations have been suggested to play a key role in the pathogenesis of leiomyomas. Cytogenetic anomalies have been observed in ~40% of ULs. These anomalies include a translocation between chromosome 12 and 14 involving the high-mobility group AT-hook 2 (HMG2) and RAD51 genes, interstitial deletions in 7q and trisomy of chromosome 12 (Canevari et al., 2005). Rarely, ULs are associated with a familial hereditary leiomyomatosis and renal cell cancer (HLRCC) syndrome, which is caused by heterozygous germline mutations in fumarate hydratase (Sudarshan et al., 2007). A recent study performed by genome-wide sequencing reported that 70% of ULs harbour mutations affecting the mediator complex subunit 12 (MED12) gene (Mäkinen et al., 2011). MED12 is a co-factor of the mediator complex, which is a multiprotein complex of 1.2 MDa consisting of 26 different subunits that act as a molecular bridge between RNA polymerase II and transcription factors and that favour preinitiation complex assembly at the promoter sites of most protein-coding genes (Cantin et al., 2003; Baek et al., 2006).

In particular, the MED12 subunit is a member of the CDK8 submodule, together with CDK8, cyclin C and MED13 proteins. Several studies have suggested that this sub-complex acts as a negative or
positive regulator of transcription depending on the cellular context (Taartjes, 2010; Conaway and Conaway, 2011).

Despite the high incidence of MED12 mutations in ULs, their functional roles and the temporal relationship between MED12 mutations and leiomyoma genesis have not been well characterized. Recently, Mäkinen et al. (2011) have suggested, based on bioinformatics pathway analysis, a potential association between de-regulation of the Wnt/β-catenin pathway in UL and MED12 mutations. Although many studies have appeared to confirm a close association between the MED12 factor and Wnt pathway (Rocha et al., 2010), immunohistochemistry data do not reveal any constitutive activation of the pathway in mutated ULs (Pérot et al., 2012). Furthermore, Mehine et al. (2013) reported RAD51B as the most up-regulated gene in MED12-mutated ULs, although they suggest that RAD51B expression may be a cellular response to replication stress, rather than a potential up-stream event.

However, the gene expression data obtained by microarray studies indicate that de-regulation of many other genes has also been implicated in the development and growth of ULs. These genes are involved in a number of different pathways, such as cell proliferation, cellular differentiation and extracellular matrix production (Arslan et al., 2005). In particular, the overexpression of insulin-like growth factor 2 (IGF-2) (Catherino et al., 2003; Wang et al., 2003; Hoffman et al., 2004; Arslan et al., 2005) and collagen type IV alpha 2 (COL4A2) (Weston et al., 2003) represents the most consistent evidence from gene arrays to date. At present, the correlation between MED12 mutations and the deregulation of these genes has not been established.

In the present study, we aimed to define the type and frequency of mutations in the MED12 gene in UL samples. Moreover, the expression levels of IGF-2 and COL4A2 in ULs harbouring specific MED12 mutations and in mutation-free samples were analysed to investigate the link between MED12 status and de-regulation of specific pathways involved in leiomyoma growth.

Materials and Methods

Sample acquisition and preparation

Samples of UL, UM and PC were collected from 36 females who underwent a laparoscopic intracapsular myomectomy (LIM) at the Department of Gynaecology and Obstetrics of Vito Fazzi University affiliated Hospital, Lecce (Italy). LIM was performed using a standardized method described by Tinelli et al. (2012a, b). The protocol was performed in accordance with the 1964 Declaration of Helsinki and was explained to the patients. A full written informed consent to participate in the prospective, non-randomized, observational study (level of evidence II-2), which was approved by the IRB, was obtained prior to surgery. The following exclusion criteria were applied: abnormal uterine bleeding, endometrial hyperplasia, adenomyosis, uterine polyps, cervical intraepithelial neoplasia, uterine or cervical cancer and confirmed or suspected primary adnexal pathology. None of patients had major medical problems, such as cardiovascular disease, diabetes, autoimmune disease. Moreover, none of the patients used hormonal medications. All operations were performed in the first 10 days of the menstrual cycle. During the operation, the surgeons obtained leiomyoma specimens for analysis, taken to ~20 mm in depth, including the full thickness of the UL, the UL-PC interface and the surrounding UM. Samples of the PC were obtained using scissors as soon as good haemostasis was achieved, while the UM tissues were biopsied at a distance of 2 cm from the UL. The samples were stored in saline solution prior to transport to the laboratory in a dry ice container, were then divided into pieces of 100–200 mg and stored at −80 °C for subsequent analysis.

Nucleic acid extraction

Genomic DNA from all ULs and corresponding UM and PCs was extracted from 100 to 200 mg of freshly frozen tissue using the ArchivePure DNA tissue kit (5-Prime) according to the manufacturer’s instructions.

Trizol reagent (Invitrogen) was used for total RNA extraction according to the manufacturer’s instructions, from samples stored at −80 °C and then placed into RNA-later prior to the start of the procedure. The RNA was suspended in RNase-free water, and the quality of the extracted RNA was confirmed using electrophoresis based on the identification of 18S and 28S ribosomal RNA (rRNA) bands. A NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) was used to determine the DNA and RNA purity and concentration. Only high-quality samples were used (A260/A280 > 1.95).

First-strand cDNA synthesis was performed by reverse transcription of 2 µg of total RNA per sample using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT; Invitrogen) according to the manufacturer’s protocol. We reverse transcribed using the oligo-dT primer for real-time PCR applications; for RT–PCR experiments on the MED12 transcript, which is known to show low expression, the first-strand cDNA was synthesized using a MEDI2-specific primer (L1: 5′-TCTGCGGCTTCTTGAT TCT-3′).

Polymerase chain reaction and RT–PCR

DNA (50–100 ng per sample) was amplified using a primer pair (2 µl each of a 10 µM dilution) that encompasses the frequently mutated region of MED12 and based on the first and second intron of the gene (U3: 5′-GCCC TTTCACCTGTGTCCT-3′; L4: 5′-TCTGCGGCTTCTTGAT TCT-3′); product size 292 bp) in a reaction mixture containing 2 µl of MgCl2 (50 mM), 1 µl of dNTP mix (10 mM) and 1 µl of Taq Platinum (Invitrogen) in the recommended buffer adjusted to a final volume of 50 µl.

For the RT–PCR reaction, 5 µl of first-strand cDNA was amplified in the same conditions as described above, with the exception that the primer pair (10 µM each) was designed against the first and third exons of the MED12 gene (U2: 5′-GCCTCCCGATGTTTACCC-3′; L2: 5′-TCTGCGCTCTTTGACTT GGGGT-3′; product size 232 bp).

Reactions for both genomic and cDNA amplification were performed for 35 cycles, each consisting of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 10 min, with an initial denaturation step of 95 °C for 5 min and a final elongation step of 72 °C for 10 min.

All PCR products obtained from genomic and cDNA amplification were purified using the Kit 5 Prime PCR Extract Mini Kit (Eppendorf) and then sequenced using a commercial service, provided by BMR Genomics (Padova-Italy).

Quantitative RT–PCR

The quantitative RT–PCR method was used to quantify mRNA transcript levels for IGF-2 and COL4A2 in our sample collection. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA level was used as an internal control. The primer sequences, annealing temperatures and amplicon lengths are reported in Table I.

The primer pairs for each target gene were initially tested on a randomly selected cDNA using standard PCR. The PCR product of the correct length was purified using the PureLink PCR Purification Kit (Invitrogen-Life Technologies) and cloned into a TA-vector. Each clone was sequenced using a commercial service to confirm the specificity of the insert and then used to obtain a standard curve.

The transcriptional levels were determined in triplicate using the Smart Cycler apparatus (Cepheid®). Each reaction was performed with cDNA equivalent to 100 ng of RNA, 10 µM of each forward and reverse primer, and sybr Green real-time PCR mix (Euroclone) according to the
mutations, which are summarized in Table III. Exon 2 of the MED12 gene were interpretable, and we detected 18

temperature specific for each primer pair for 30 s, and extension at 72

enrolled women are summarized in Table II.

Statistical analyses

Data on the expression levels of the target genes in each sample were obtained in triplicate and used to calculate the mean values and SDs. Analysis and presentation of the data were achieved using MedCalc software. The data were described by their medians and interquartile ranges; the degree of variability among groups was determined using the Kruskal–Wallis non-parametric algorithm, while the statistical significance of pairwise comparisons was assessed using the Mann–Whitney test. Significance was established at P-values of < 0.05.

Four different missense mutations were found in 12 ULs (33%). These mutations include c.130G>T (1/31) and c.130G>A (1/31), which involve the first base of codon 44, c.131G>T (3/31) and c.131G>A (7/31), which affect the second base of the same codon. Four cases exhibited in-frame deletions ranging in size from 12 to 24 bp. Two additional deletions span the intron 1–exon 2 junction (25 and 46 bp in size), which caused a predicted loss of the splice acceptor. All mutations were in the heterozygous state. The remaining 18 UL samples did not harbour any mutation in the analysed region.

Table I Primers for mRNA detection used in quantitative RT–PCR.

<table>
<thead>
<tr>
<th>Gene (Acc.Nr)</th>
<th>Primers</th>
<th>Position</th>
<th>Sequences</th>
<th>Amplicon length (bp)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH (NM_002046)</td>
<td>Forward</td>
<td>Ex 8</td>
<td>5′-GGGCATCTTGCGCTACACTGA-3′</td>
<td>165</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>Ex 9</td>
<td>5′-TCCACACCACTGGTGTAGTA-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF-2 (NM_000612)</td>
<td>Forward</td>
<td>Ex 6</td>
<td>5′-CTTACCGCCAGTGAGACC-3′</td>
<td>195</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>Ex 7</td>
<td>5′-GCTTGGCGGGGCTAGCAGC-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COL4A2 (NM_001846.2)</td>
<td>Forward</td>
<td>Ex 1</td>
<td>5′-GCTGGGTGAGGGAGTTGTG-3′</td>
<td>172</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>Ex 2</td>
<td>5′-TGGTGCCGATGATGGCCAGG-3′</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All of the sequences obtained for intervening sequence (IVS)1 and exon 2 of the MED12 gene were interpretable, and we detected 18 mutations, which are summarized in Table III.

Table II Basic characteristics of the 36 enrolled women.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>38.5 ± 6.1*</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.1 ± 2.9*</td>
</tr>
<tr>
<td>Parity</td>
<td>1.3 ± 0.5*</td>
</tr>
<tr>
<td>Subserous myomas, n (%)</td>
<td>20 (55.6%)</td>
</tr>
<tr>
<td>Intramural myomas, n (%)</td>
<td>16 (44.4%)</td>
</tr>
<tr>
<td>Single myomas, n (%)</td>
<td>15 (41.7%)</td>
</tr>
<tr>
<td>Multiple myomas, n (%)</td>
<td>21 (58.3%)</td>
</tr>
<tr>
<td>Leiomyoma size (cm)</td>
<td>6.6 ± 3.5*</td>
</tr>
</tbody>
</table>

*Mean ± SD.

Results

Mutational analysis

Thirty-six UL samples obtained from 36 patients were included in our mutational analysis on MED12 gene. The basic characteristics of the enrolled women are summarized in Table II.

All of the sequences obtained for intervening sequence (IVS)1 and exon 2 of the MED12 gene were interpretable, and we detected 18 mutations, which are summarized in Table III.

Expression analysis of IGF-2 and COL4A2 genes

The effect of MED12 mutation on the mRNA expression levels of IGF-2 and COL4A2 was investigated. On the basis of the different mutational status, the UL samples were divided into three groups: Group A consisted of samples without MED12 mutations, Group B consisted of samples exhibiting deletions in the MED12 gene and Group C consisted of samples harbouring missense mutations.

As shown in Fig. 2, analysis of IGF-2 mRNA expression level revealed a significant difference among the groups (P = 0.0004 by Kruskal–Wallis test).

In particular, the mean relative expression was significantly higher, as assessed by the Mann–Whitney tests, in Group C (0.017 units) compared with Group A (0.004 units, P = 0.0004) and Group B (0.003 units, P = 0.002). The difference between Groups A and B was not statistically significant (P = 0.76 by the Mann–Whitney test).

These data demonstrated a specific correlation between missense mutations in exon 2 of the MED12 gene and IGF-2 overexpression.
Conversely, the COL4A2 mRNA levels showed no significant differences among the three groups of UL (\( P = 0.132 \) by Kruskal–Wallis test; Fig. 2).

**Discussion**

We assessed mutations in the MED12 gene in 36 ULs and found that 50% of the samples presented heterozygous mutations in the hot spot IVS1 and exon 2 regions of the MED12 gene. The mutation frequency in such a series of samples was slightly lower compared with those reported by Mäkinen et al. (2011) (70%) and Markowski et al. (2012) (58%), but it is comparable with that reported by Je et al. (2012) (52.2%). This variability may be linked to both the number of samples included in the different studies (225, 80 and 67, respectively), as well as tumour sampling.

### Table III  Summary of somatic mutations in the human MED12 gene observed in 36 ULs.

<table>
<thead>
<tr>
<th>Mutation type</th>
<th>Nucleotide change</th>
<th>Predicted protein change</th>
<th>Number of mutated samples out of 36</th>
</tr>
</thead>
<tbody>
<tr>
<td>Missense</td>
<td>c.130G&gt;A</td>
<td>p.G44S</td>
<td>1 (2.7%)</td>
</tr>
<tr>
<td></td>
<td>c.130G&gt;T</td>
<td>p.G44C</td>
<td>1 (2.7%)</td>
</tr>
<tr>
<td></td>
<td>c.131G&gt;T</td>
<td>p.G44V</td>
<td>3 (8.3%)</td>
</tr>
<tr>
<td></td>
<td>c.131G&gt;A</td>
<td>p.G44D</td>
<td>7 (19.4%)</td>
</tr>
<tr>
<td>Deletion</td>
<td>IVS1–13_132del46</td>
<td>Loss of splice acceptor</td>
<td>1 (2.7%)</td>
</tr>
<tr>
<td></td>
<td>IVS1–21_104del25</td>
<td>Loss of splice acceptor</td>
<td>1 (2.7%)</td>
</tr>
<tr>
<td></td>
<td>c.102_113del12</td>
<td>p.Glu35_Ala38del</td>
<td>1 (2.7%)</td>
</tr>
<tr>
<td></td>
<td>c.122_145del24</td>
<td>p.Val41_Pro49delinsAla</td>
<td>1 (2.7%)</td>
</tr>
<tr>
<td></td>
<td>c.134_145del13</td>
<td>p.Phe45_Pro49delinsSer</td>
<td>1 (2.7%)</td>
</tr>
<tr>
<td></td>
<td>c.142_156del15</td>
<td>p.Gln48_Gly53delinsArg</td>
<td>1 (2.7%)</td>
</tr>
</tbody>
</table>

**Figure 1** Sequence chromatograms of three mutations in exon 2 of the MED12 gene. Examples of chromatograms revealing two heterozygous missense mutations (P1 and P2) and a heterozygous internal in-frame deletion (P3) identified in our UL samples, both at the genomic and mRNA level, compared with those obtained in the matching myometrium and pseudocapsule (PC).
However, consistent with these reports the mutations identified in the present investigation were predominantly missense mutations. The other mutations included four in-frame deletions and two deletions affecting the splice acceptor site in intron 1, which have not been previously reported.

When the mutational analysis was extended to the UMs and to the PCs corresponding to the mutated ULs, no mutations were detected in either tissue.

Recently, it was shown that the PC, a structure anatomically distinguishable from the UM and that surrounds the UL, displays a significant and specific gene expression profile promoting muscular repair and regeneration (Di Tommaso et al., 2013). These properties explain the optimal reconstruction of the damaged tissue and the high quality of the muscle scar exhibited by patients undergoing LIM, a surgical technique that preserves the PC during UL removal.

In this context, the discovery that the PC, as well as the UM, is free from mutations in the MED12 gene demonstrates that the PCs is a healthy tissue, created by the myometrium to cope with UL growth and to preserve the integrity of muscular myometrium surrounding the UL.

Sequencing analysis of the MED12 transcripts in our cohort of ULs revealed that all of the missense mutations and the four deletions included in exon 2 were expressed at the transcriptional level. Since MED12, being an X-linked gene, shows a monoallelic expression, these data indicate that the aberrant allele always escapes inactivation. However, the wild-type transcript was also detected although, in most cases, at lower levels, as also described by Mäkinen et al. (2011). In addition, only the wild-type transcript was detected in both samples with deletions affecting the splice acceptor site. The presence of a minor wild-type transcript in all samples may be due to non-tumour cell contamination. However, we could not exclude that MED12 wild-type allele expression derives from a subclone within the fibroid. Although many studies have described a clonal origin of UL (Zhang et al., 2006), other studies have shown that ULs could be heterogeneous (Han et al., 1994).

While the prevalence of exon 2 MED12 gene mutations in the majority of ULs has been ascertained, a major challenge remains in understanding the link between mutated MED12 and tumourigenesis. The most consistent studies have addressed this issue by comparing the expression patterns between mutated and non-mutated ULs.

The present study further segregated MED12-mutated ULs into two groups according to the mutation type status and demonstrated that only missense mutations in the MED12 gene are strictly associated with IGF-2 mRNA overexpression.

The homogenous clinical characteristics of our patients suggest that such overexpression is linked to intrinsic features of the mutated ULs, rather than to the basal state of the patients considered in the analysis. Conversely, expression levels of COL4A2 did not differ among the three groups of ULs.

Recent genome-wide DNA methylation analysis indicated that an aberrant methylation pattern could be implicated in the deregulation of gene expression in ULs. In particular, a hypomethylation has been shown to be associated with the regulatory sequence of the COL4A2 gene (Maekawa et al., 2013). Thus, it can be assumed that COL4A2 expression in UL is not related to MED12 status but could be derived from an aberrant epigenetic regulation.

Overexpression of the IGF-2 gene occurs in many cancers, such as breast, hepatocellular carcinoma, ovarian and bladder cancer, and is often associated with a poor prognosis (Livingstone, 2013).

The most common mechanism for IGF-2 overexpression is via the loss of imprinting, however, other regulatory mechanisms have been reported, including induction by prolactin in breast carcinoma 15 and phosphatase and tensin homolog in hepatoma cells (Kang-Park and Lee, 2003). In these tumours, IGF-2 overexpression provides a way to predict prognosis and offers a potential molecular therapeutic target for treatment (Livingstone, 2013).

Many studies have described IGF-2 as one of the few genes that is constantly overexpressed in ULs (Catherino et al., 2003; Wang et al., 2003; Hoffman et al., 2004; Arslan et al., 2005). However, evaluation of the methylation status of the gene in ULs indicated that loss of imprinting events are not involved in the up-regulation that is commonly observed (Vu et al., 1995; Hashimoto et al., 1996).

This study adds to the current knowledge that IGF-2 overexpression is mostly restricted to those ULs which harbour a missense mutation in the MED12 gene. Peng et al. (2009) reported overexpression of IGF-2 in two-thirds of the ULs analysed, both at the mRNA and protein level. We suggest that this association could reflect the frequency of MED12 missense mutations among leiomyomas.
We describe the association between missense mutations in MED12 and specific gene deregulation, which suggests that mutant MED12 proteins could function either by abrogating the wild-type activity of the CDK8 sub-complex or by gaining new oncogenic function.

Knuesel et al. (2009) observed that the CDK8 sub-complex interacts via MED12–MED13 factors with the core mediator, thereby acting by occluding a large cavity within the complex, which corresponds to the PolII-binding site. If the activity of the CDK8 sub-complex is dependent on such an allosteric interaction, it is likely that missense mutations in codon 44 of the MED12 gene, converting a small, non-polar amino acid (such as the Gly44) into other more polar or bulkier amino acids, may prevent the proper organization of the entire complex. A mutated CDK8 sub-complex, which lacks its repressive function, could induce constitutive activity of the mediator complex and a strong up-regulation of IGF-2.

However, it has also been reported that the CDK8 sub-module can function independently of the mediator complex in regulating gene expression in specific conditions (Knuesel et al., 2009). For example, CDK8 protein has been shown to function as a positive regulator for specific p53-regulated genes, such as p21 and HMDS2 (Donner et al., 2007). Thus, it cannot be completely excluded that missense mutations in the MED12 subunit could confer new properties to the CDK8 sub-module, thereby acting as gain-of-function mutations.

In conclusion, our study showed for the first time the non-mutated MED12 gene condition in the PC, confirming its nature as a healthy tissue. Furthermore, our investigation indicated that MED12 gene status stratified the ULs into two subgroups characterized by the presence or absence of IGF-2 overexpression. Thus, it could be hypothesized that the subgroup of ULs with IGF-2 overexpression could be specifically sensitive to pharmacological treatments targeted towards this growth factor or other components of the IGF system.

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Authors’ roles

S.D.T. carried out the molecular studies, data acquisition, statistical analysis and wrote the manuscript; A.T. conceived the study, operated patients and revised the manuscript; A.M. revisited the manuscript critically; S.M. conceived and coordinated the study and contributed in drafting the article. All authors gave the final approval of the version to be published.

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Conflict of interest

The authors declare no conflict of interests related to this study.

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