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ARTICLE

High levels of hypusinated eIF5A in leiomyoma and leiomyosarcoma pathologies: a possible novel therapeutic target

BIOGRAPHY

Dr Ciarmela is Associate Professor of Human Anatomy at Università Politecnica delle Marche, Italy. Her research focus is uterine physiology and pathophysiology, and the role of growth factors. The aim of her research is to understand the pathogenesis of female diseases and to develop potential therapeutic agents.

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KEY MESSAGE

Hypusinated eIF5A was expressed in normal myometrium, neoplastic benign leiomyoma and neoplastic malignant leiomyosarcoma. The inhibition of eIF5A hypusination, with GC-7 treatment, reduced cell proliferation in myometrium, leiomyoma and leiomyosarcoma cell lines and reduced expression of fibronectin in leiomyoma and leiomyosarcoma cells, identifying eIF5A as a potential future therapeutic target.

ABSTRACT

Research question: Is the hypusinated form of the eukaryotic translation initiation factor 5A (EIF5A) present in human myometrium, leiomyoma and leiomyosarcoma, and does it regulate cell proliferation and fibrosis?

Design: The hypusination status of eIF5A in myometrial and leiomyoma patient-matched tissues was evaluated by immunohistochemistry and Western blotting as well as in leiomyosarcoma tissues by immunohistochemistry. Myometrial, leiomyoma and leiomyosarcoma cell lines were treated with N1-guanyl-1,7-diaminoheptane (GC-7), responsible for the inhibition of the first step of eIF5A hypunization, and the proliferation rate was determined by MTT assay; fibronectin expression was analysed by Western blotting. Finally, expression of fibronectin in leiomyosarcoma tissues was detected by immunohistochemistry.

Results: The hypusinated form of eIF5A was present in all tissues examined, with an increasing trend of hypusinated eIF5A levels from normal myometrium to neoplastic benign leiomyoma up to neoplastic malignant leiomyosarcoma. The higher levels in leiomyoma compared with myometrium were confirmed by Western blotting ($P = 0.0046$). The inhibition of eIF5A hypusination, with GC-7 treatment at 100 nM, reduced the cell proliferation in myometrium ($P = 0.0429$), leiomyoma ($P = 0.0030$) and leiomyosarcoma ($P = 0.0044$) cell lines and reduced the expression of fibronectin in leiomyoma ($P = 0.0077$) and leiomyosarcoma ($P = 0.0280$) cells. The immunohistochemical staining of leiomyosarcoma tissue revealed that fibronectin was highly expressed in the malignant aggressive (central) part of the leiomyosarcoma lesion, where hypusinated eIF5A was also highly represented.

Conclusions: These data support the hypothesis that eIF5A may be involved in the pathogenesis of myometrial benign and malignant pathologies.

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INTRODUCTION

Uterine fibroids are benign tumours with high prevalence, associated with significant health problems and enormous economic impact on the health system. Relatively little is known about their cause and pathophysiology, resulting in a lack of effective therapeutic strategies (*Buttram Jr and Reiter, 1981; Cramer and Patel, 1990*).

Uterine leiomyosarcoma is a rare but aggressive cancer recognized and diagnosed with histological criteria of hypercellularity, severe nuclear atypia and high mitotic rate (>15 mitotic figures for 10 high power fields) (*Kurman and Norris, 1976*; *Lu et al., 2017*). For a better and certain diagnosis, and adequate treatment, specific biomarkers are strongly needed (*Dall'Asta et al., 2014*). In fact, the diagnosis of myometrial disorders can be controversial (*Gockley et al., 2014*), and they are often classified as smooth muscle tumours of uncertain malignant potential.

It is known that genetic and epigenetic factors, sex steroids, growth factors, vascularization, cytokines, chemokines, inflammation and components of the extracellular matrix (ECM) are factors involved in the pathogenesis of leiomyoma (*Ciarmela et al., 2011*;*2022*; *Islam et al., 2018*; *Zannotti et al., 2021*). The components of the ECM, such as collagen1A1, fibronectin and versican, are overexpressed in leiomyoma (*Malik and Catherino, 2012*; *Giuliani et al., 2019*) and their upregulation is induced by activin A (*Islam et al., 2014*). Indeed, leiomyoma has been classified as a fibrotic tissue (*Protic et al., 2016*). The ECM proteins transmit mechanical signals from outside to the inside of the cell by the transmembrane integrin proteins. It is important to consider the interconnection between ECM, cell membrane and intracellular environment (*Ciarmela et al., 2011*; *Protic et al., 2016*; *Islam et al., 2018*). The cell membrane is actively involved in processes such as the diffusion of nutrients and oxygen, the reception and propagation of signals for cellular adaptation to physiological and pathological environments (*Ciarmela et al., 2011*; *Protic et al., 2016*; *Islam et al., 2018*). We have previously shown that omega-3 fatty acids can modulate the lipid profile, modifying the architecture of the cell membrane and can also downregulate the expression of genes involved in the mechanical signals process and in the accumulation of lipids in the cells of leiomyoma (*Islam et al., 2018*). As the interconnection between ECM, cell membrane and intracellular environment also affects gene expression, molecules, such as translation factors, could play an important role in the cause of leiomyoma.

In recent years, some studies have shown that eukaryotic translation Initiation Factor 5A (eIF5A) is involved in several and numerous physiological processes, including, ischaemic tolerance, metabolic adaptation, ageing, and development and differentiation of immune cells. It is a highly conserved protein, likely with a highly conserved physiological role in evolution. This factor undergoes a peculiar and unique post-translational modification called hypusination, a reaction catalysed by two enzymes: deoxyhypusine synthase (DHPS) and deoxyhypusine hydroxylase (*Park, 2006*). In the first step of hypusination, the enzyme DHPS catalyses the NAD and dependent transfer of the 4-aminobutyl portion of spermidine (a polyamine originating from the so-called polyamine pathway) to the ε-amino group of a specific lysine residue (Lys50) of the eIF5A precursor to form a deoxyhypusine residue. The metalloenzyme deoxyhypusine hydroxylase hydroxylates it at carbon 9 completing the synthesis of hypusine and the maturation of eIF5A (*Park et al., 2010*; *Qin et al., 2014*). The reaction is known to occur to almost all eIF5A in the cell (*Caraglia et al., 2013*).

The eIF5A factor is involved in different processes in eukaryotic cells, such as the elongation and termination phases of translation and the export of mRNA from the nucleus to the cytoplasm (*Maier et al., 2010*; *Park et al., 2010*; *Schuller et al., 2017*). For all these functions, hypusination of the factor is an absolute requirement (*Park and Wolff, 2018*). In humans, eIF5A exists in two isoforms: eIF5A1, abundant in most cells and tissues, and eIF5A2, which is tissue specific (*Jenkins et al., 2001*; *Clement et al., 2003*). N1-guanyl-1,7-diaminoheptane (GC-7), a spermidine analogue, can bind DHPS, inhibiting hypusination of eIF5A (*Caraglia et al., 2013*; *Nakanishi and Cleveland, 2016*).

Although eIF5A is involved in different physio-pathological mechanisms, the levels of hypusinated eIF5A and its function in myometrial healthy and pathological tissues have not been explored to date. Previous studies have shown that eIF5A can promote tumour cell proliferation, metastasis and chemoresistance. In particular, in pancreatic cancer, eIF5A1-PEAK1 (atypical kinase 1 enriched with pseudopodium) signalling regulates YAP1 (Y-associated protein 1)/TAZ (tafazine) protein expression and tumour cell growth (*Strnadel et al., 2017*). EIF5A2 overexpression improves cell motility of hepatocellular carcinoma and promotes tumour metastases (*Tang et al., 2010*). EIF5A1 may also promote leukaemia cell proliferation and may be a novel therapeutic target in BCR-ABL-positive leukaemia (*Balabanov et al., 2007*). EIF5A2 regulates chemoresistance in colorectal cancer through the epithelial–mesenchymal transition (*Bao et al., 2015*). The involvement of EIF5A1 in gynaecological cancers has also been reported. Blocking eIF5A1 modification in cervical cancer cells alters the expression of cancer-related genes and suppresses cell proliferation (*Memin et al., 2014*). eIF5A2 plays an oncogenic role in the development of ovarian cancer (*Guan et al., 2004*; *Yang et al., 2009*). Finally, eIF5A has recently been reported to be involved in inflammation and fibrosis in other diseases (*Kaiser, 2012*).

The aim of the present study was to evaluate the levels and localization of hypusinated eIF5A in myometrium, leiomyoma and leiomyosarcoma, and to establish the effect of inhibition of eIF5A hypusination on proliferation and fibronectin production of myometrial, leiomyoma and leiomyosarcoma cell lines.

MATERIALS AND METHODS

Tissue sample collection

A group of premenopausal women (n = 27; age range 33–46 years) was included in the present study collected between 2018 and 2022. They were admitted for surgical treatment for leiomyomas and underwent myomectomy or total hysterectomy. For each patient, the demographics (age and body mass index), symptoms (blood loss at menstruation and dysmenorrhoea), previous treatment for leiomyoma, infertility and previous pregnancies were recorded. The patients affected by leiomyoma that were included in this study did not suffer from other kinds of pathologies and they had not received exogenous hormones in the previous 3 months. In addition, leiomyomas associated with degenerative aspects, such as necrosis, haemorrhage, oedema, calcifications, or all, were excluded from

this study. The local Institutional Review Board approved the research protocol, and every participant provided informed consent. The procedures followed for the collection of samples were in accordance with the Helsinki Declaration of 1975, as revised in 2013. The permission of the Human Investigation Committee of Marche (protocol number 2015 0486, approved on 10 February 2016) was granted.

For Western blotting, leiomyoma and normal myometrium specimens were collected during surgery and immediately stored at −80°C until protein extraction. For morphological investigations, the tissues were fixed in buffered formalin for 24–48 h. The leiomyosarcomas samples used were from women affected by third grade leiomyosarcoma. All patients underwent surgical removal of the tumour by hysterectomy. At the time of diagnosis, the surgical samples of each patient were fixed in formalin and sent to the Institute of Pathological Anatomy of the Università Politecnica delle Marche, where they were grossly examined and sampled after paraffin-embedding for histological evaluation. For the purposes of this investigation, all the histological slides of the patients under study were recovered from the Archives of the Institute of Anatomical Pathology ($n = 5$). Sections stained with haematoxylin and eosin (H&E) were re-examined to identify third grade leiomyosarcoma for morphological investigations.

Cell lines culture

The myometrial (A009 [57M1]) and leiomyoma (A010 [57F9]) cell lines were provided by William H Catherino, MD, PhD (Department of Obstetrics and Gynecology, Uniformed Services University of the Health Sciences, Bethesda, Maryland). The primary myometrial and leiomyoma cells were immortalized following the modified protocol of *Rhim (2003)* using human papillomavirus type 16 as previously described by *Malik et al. (2008)*. Cells were cultured in fresh Dulbecco Modified Eagle Medium (DMEM-F12) (Corning, New York, USA) supplemented with 10% fetal bovine serum (Euroclone, Milan, Italy), 1% antibiotic (penicillin–streptomycin (EuroClone, Milan, Italy), 1% fungizone (amphotericin B) (Euroclone, Milan, Italy) and 1% glutamine (Gibco) (Life Technologies, Carlsbad, CA, USA) at 37°C in 95% air 5% CO^2.

Leiomyosarcoma cell lines were purchased from ATCC (Manassas, Virginia, USA). The cells were cultured in fresh DMEM low glucose (Euroclone, Milan, Italy) supplemented with 10% FBS (Euroclone, Milan, Italy), 1% antibiotic (penicillin–streptomycin) (Euroclone, Milan, Italy) at 37°C in 95% air 5% CO^2.

Immunohistochemistry

Paraffin sections were deparaffinized and rehydrated using xylene and a graded series of ethyl alcohol. To break the methylene bridges and expose the antigenic sites to allow the antibodies to bind, the section was immersed in a container of 1 mM EDTA buffer, pH 8, and kept in a water bath at 100°C for 20 min.

To inhibit endogenous peroxidase activity, sections were incubated for 60 min with 3% hydrogen peroxide in methanol and washed in phosphate-buffered saline (PBS). To block non-specific background, the sections were incubated for 20 min at room temperature with normal horse serum for hypusinated eIF5A, fibronectin, diluted 1:75. Sections were then incubated overnight at 4°C with rabbit polyclonal anti-hypusine (ABS1064) (Merck-Millipore Burlington, Massachusetts, USA), known to detect only hypusinated eIF5A, diluted 1:200 and with mouse polyclonal anti-fibronectin (Sigma-Aldrich, St Louis, MO, USA) diluted 1:600. For negative control, the section was incubated with non-immune serum instead of the primary antibody.

After washing in PBS, the sections were incubated with biotinylated anti-rabbit immunoglobulin G (IgG) made in horse diluted 1:200 (Vector Laboratories, Burlingame, CA, USA) for hypusinated eIF5A and with biotinylated anti-mouse IgG made in horse diluted 1:200 (Vector Laboratories, Burlingame, CA, USA) for 30 min. The peroxidase ABC method (Vector Laboratories, Burlingame, CA, USA) was carried out for 1 h at room temperature using 3,3′-diaminobenzidine hydrochlorides (Dako, Agilent Technologies, Santa Clara, CA, USA) as chromogen. Sections were counterstained in Mayer's haematoxylin, dehydrated, and mounted with Eukitt solution (Orsatec GmbH) (Industriepark Werk Bobingen GmbH & Co. KG, Bobingen, Germany).

N1-guanyl-1,7-diamineheptane (GC-7) treatment

Myometrial, leiomyoma and leiomyosarcoma cells were treated directly in growth medium without other vehicle with N1-guanyl-1,7-diamineheptane (GC-7) at different concentrations: 10 nM, 50 nM or 100 nM (Merk-Millipore) compared with untreated control and incubated for 48 h at 37°C in 95% air 5% CO_2. The same treatment was used for investigated cell proliferation assay and western blotting.

Western Blotting

Proteins were extracted by TRIzol® reagent (Invitrogen ThermoFisher, Waltham, MA, USA) following the manufacturer's instructions. The collected purified proteins were quantified by Bradford assay (Sigma-Aldrich, St Louis, MO, USA) so that an equal amount of proteins was loaded on a 4–12% NuPAGE gel (Invitrogen, ThermoFisher, Waltham, MA, USA). The separation of proteins was carried out under reducing conditions by SDS-PAGE. Proteins were transferred to a 0.2-μm nitrocellulose membrane by a X-cell II apparatus (Invitrogen ThermoFischer, Waltham, MA, USA) according to the manufacturer's instruction. The nitrocellulose membrane transferred proteins were detected by Ponceau S solution (Sigma-Aldrich, St Louis, MO, USA). The membrane was blocked for 1 h at room temperature with 5% (weight/volume) non-fat milk powder, which had been dissolved in Tris-buffered saline with Tween 20 (TBST) (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20). After this, the membrane was incubated overnight at 4°C with the hypusinated eIF5A polyclonal rabbit primary antibody diluted 1:2000 (Merk-Millipore, Burlington, MA, USA), with fibronectin monoclonal mouse primary antibody diluted 1:30000 (Sigma-Aldrich, St Louis, MO, USA) and with ß-actin monoclonal mouse primary antibody diluted 1:1000 (Sigma-Aldrich, St Louis, MO, USA) or 1:3000 dilutions for monoclonal mouse tubulin (Sigma-Aldrich, St Louis, MO, USA). The next day, the membrane was washed four times in TBST and subsequently incubated for 2 h in 5% (weight/volume) non-fat milk powder in TBST and incubated with 1:5000 peroxidase-conjugated made in donkey secondary antibody antirabbit IgG (Jackson ImmunoResearch Laboratories, Baltimore, Pennsylvania, USA) and incubated with 1:1000 horseradish peroxidase linked antimouse IgG (Abcam, Cambridge, UK). Unbound secondary antibody was removed by washing in TBS-Twee. Membranes were then treated with Clarity Western ECL substrate (BioRad, Hercules, California, USA) and imaged

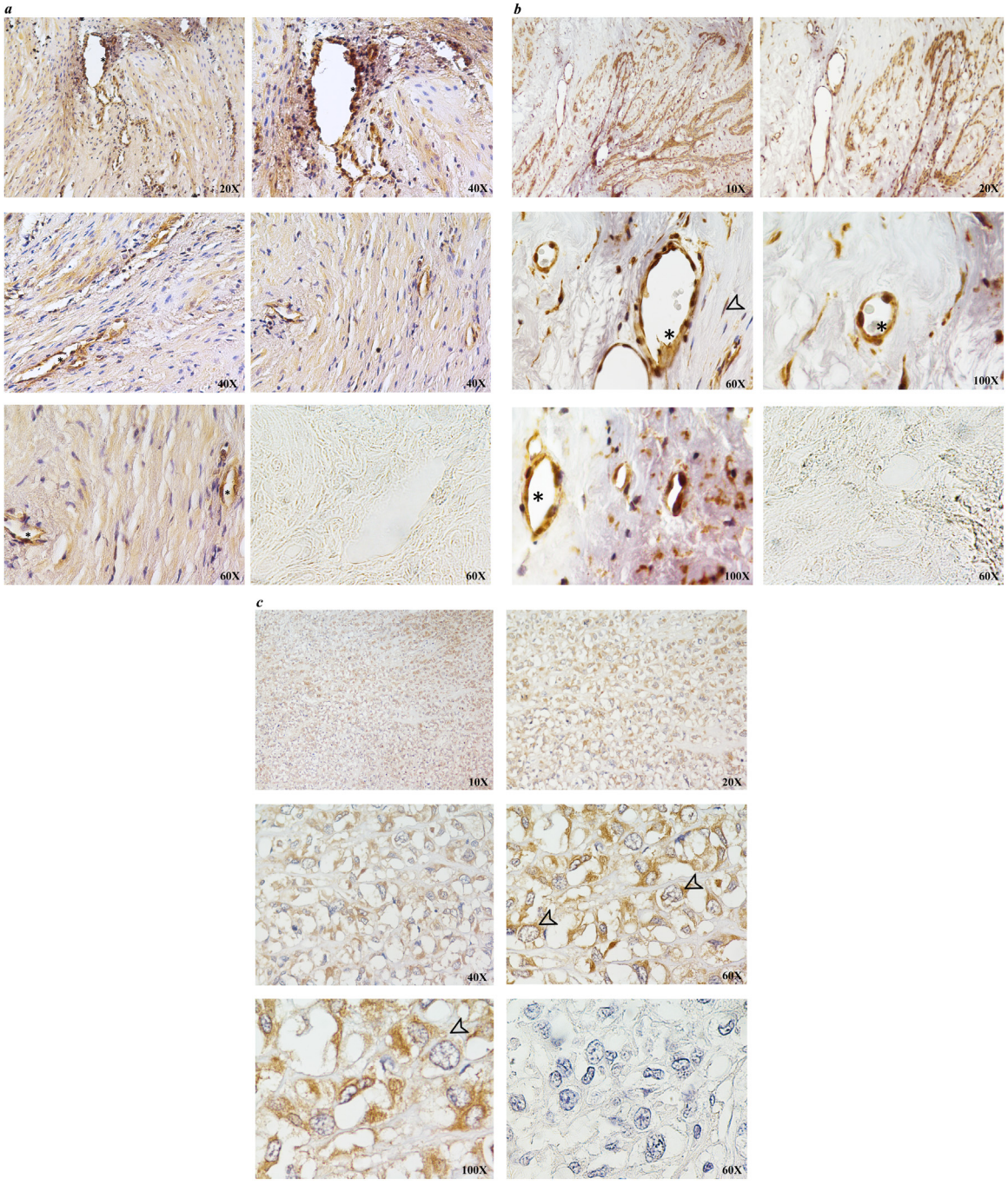

FIGURE 1 Images of immunohistochemical staining in human uterine tissues. (A) Myometrial tissue microscopic observation of negative controls (magnification 60X, bottom right) and hypusinated form of the eukaryotic translation initiation factor 5A (eIF5A). Endothelial cells (*) shows the hypusinated eIF5A (magnifications 20X, 40X and 60X); (B) leiomyoma tissue microscopic observation of negative controls (magnification 60X, bottom right) and hypusinated eIF5A. Endothelial cells (*) and some smooth muscle cells (arrowheads) shows expression of hypusinated eIF5A (magnifications 10X, 20X, 60X and 100X); (C) leiomyosarcoma tissue microscopic observation of negative controls (magnification 60X, bottom right) and hypusinated eIF5A. The expression of the hypusinated eIF5A in human leiomyosarcoma tissue is localized in bizzare cells (arrowheads), with strong staining (magnifications 10X, 20X, 40X, 60X and 100X). Counterstaining with Mayer's haematoxylin (blue). Representative images shown.

with a Bio-Rad ChemiDoc MP system. Finally, the Image J 1.49n software (National Institute of Health, (http://imagej, nih.gov/ij) was used to measure protein levels corresponding to the bands on the membrane. HCT-116 extract was used as a positive control for the protein detection (*Martella et al., 2020*).

Cell proliferation assay

Myometrial, leiomyoma and leiomyosarcoma cells were seeded in 96-well plates at initial densities of 1×10^3 cells per well in total volume of 0.5 ml DMEM/F12 supplemented with 10% FBS for myometrial and leiomyoma cells and DMEM low glucose supplemented with 10% FBS for leiomyosarcoma cells. Cells

were treated with GC-7; 10 nM, 50 nM or 100 nM. Cell proliferation was determined by MTT (3- [4,5-dimethylthiazol−2-yl]-2,5-diphenyltetrazolium bromide) assay. This assay is based on the reduction of the tetrazolium salt by intracellular dehydrogenases of viable living cells, leading to the formation of purple formazan crystals. At the end of treatment (2 days), cells were washed with PBS and incubated with MTT solution at 0.5 mg/ml for 3 h at 37°C. The medium was then removed, and the crystals were dissolved in isopropyl alcohol acidified with hydrochloric acid. In the final step, absorbance was measured at 570 nm using Multiskan GO microplate reader (Thermo Scientific, Waltham, MA, USA).

Statistical analyses

GraphPad Prism version 8.2.1 for OS X (GraphPad, San Diego, CA) was used for statistical analyses. The data were analysed using non-parametric Kruskal−Wallis one−way analysis of variance, followed by post-hoc Dunn test for multiple comparisons. Results are expressed as significant when $P < 0.05$.

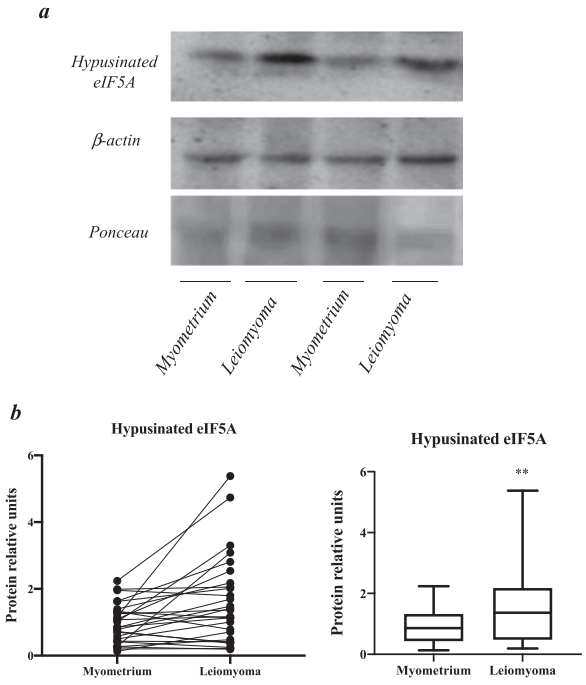

FIGURE 2 Hypusinated form of the eukaryotic translation initiation factor 5A (eIF5A) expression in human myometrial and leiomyoma tissues. (A) Representative Western blotting analysis showing the levels of hypusinated eIF5A (45 kDa) in individual patient-matched myometrium and leiomyoma tissues ($n = 27$). β-actin (42 kDa) and Ponceau (44 kDa) used as loading controls; (B) quantification of hypusinated eIF5A in myometrium and leiomyoma tissues, normalized to β-actin. Results are represented as line graph and linking paired specimens and as box-and-whisker plots ($n = 27$). Box-and-whisker plots represent median, interquartile range and range. Differences detected by Kruskal−Wallis ($P = 0.0046$).

RESULTS

Levels and localization of hypusinated eIF5A in myometrium, leiomyoma and leiomyosarcoma tissues

The immunohistochemical staining shows the levels and the localization of hypusinated eIF5A in myometrium, leiomyoma and leiomyosarcoma tissues. The hypusinated form of eIF5A was present in endothelial cells and in the cytoplasm of smooth muscle cells (FIGURE 1A−FIGURE 1C). The intensity of the staining seemed to be high in leiomyosarcoma (FIGURE 1C), intermediate in leiomyoma (FIGURE 1B) and low in myometrial tissues (FIGURE 1A).

The levels of hypusinated eIF5A were also quantified by Western blotting. Protein extracts of myometrium and leiomyoma of 27 patients were analysed. As leiomyosarcoma is a rare cancer, it was not possible to find protein extracts from human samples. The results presented in FIGURE 2 showed that the levels of hypusinated eIF5A were significantly higher in leiomyoma compared with the adjacent myometrium ($P = 0.0046$) (FIGURE 2B).

Characterization of hypusinated eIF5A in myometrium, leiomyoma and leiomyosarcoma cell lines

Western blot analysis conducted on protein extracts from myometrium, leiomyoma and leiomyosarcoma cell lines demonstrated that hypusinated form of eIF5A is present in all the analysed cell lines (FIGURE 3). This result allowed us to proceed further in our investigation by carrying out functional in-vitro studies.

The detection of HCT-16 extracts, used as a positive control, is presented in the **Supplementary Figure**.

The effect of inhibition of eIF5A hypusination on proliferation and fibrotic marker expression expression in myometrium, leiomyoma and leiomyosarcoma cell lines

To evaluate the effect of inhibition of eIF5A hypusination on myometrium, leiomyoma and leiomyosarcoma, cell lines

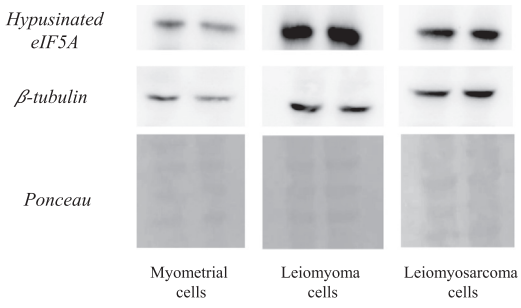

FIGURE 3 Protein expression in leiomyosarcoma, myometrial and leiomyoma cell lines. Representative gel showing the levels of hypusinated form of the eukaryotic translation initiation factor 5A (eIF5A) (45kDa) in myometrial, leiomyosarcoma and leiomyosarcoma cell lines (replicates $n = 2$). β-tubulin (50 kDa) and Ponceau (40 kDa) used as loading controls.

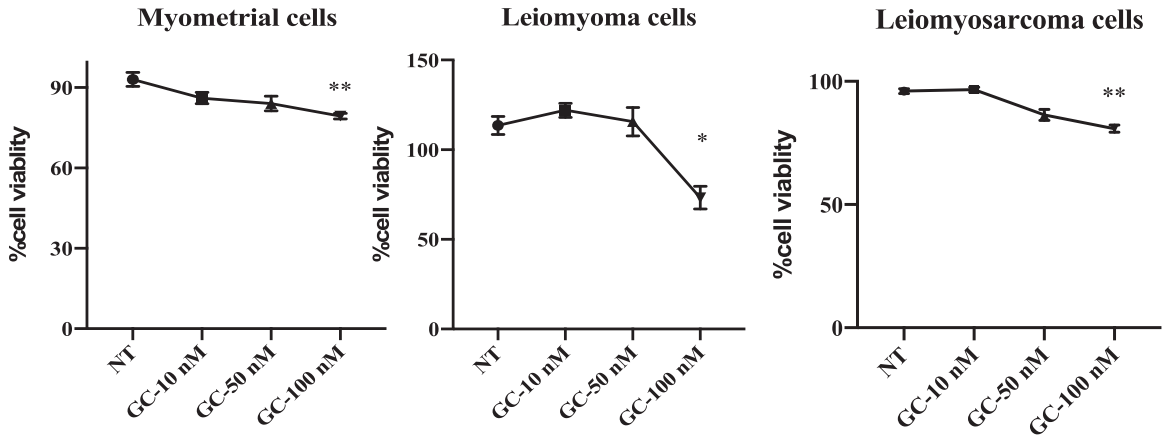

FIGURE 4 Representative cell viability assay graph after N1-guanyl-1,7-diaminoheptane (GC-7) (10 nM, 50 nM, 100 nM) treatments in myometrial, leiomyoma and leiomyosarcoma cell lines. The MTT cell viability assay carried out in myometrial, leiomyoma and leiomyosarcoma cell lines after the treatment with different doses of GC-7 10 nM ($P = 0.0429$), 50 nM ($P = 0.0030$), 100 nM ($P = 0.0044$) for 48 h. NT, no treatment control. Data are expressed as mean ± SD ($n = 6$); differences tested by Kruskal–Wallis test.

were treated with the specific DHPS inhibitor GC-7 at different concentrations (10 nM, 50 nM or 100 nM) for 48 h. After treatment, the effect of inhibition of eIF5A hypusination on cell proliferation was evaluated by MTT. The results obtained show that the treatment with 100 nM GC-7 significantly reduced cell proliferation in myometrium, leiomyoma and leiomyosarcoma cell lines ($P = 0.0429$; $P = 0$, 0030; $P = 0.0044$, respectively) (FIGURE 4).

Furthermore, the effect of inhibition of eIF5A hypusination on the expression of the fibrotic marker fibronectin in myometrium, leiomyoma and leiomyosarcoma cell lines was evaluated. The treatment with GC-7 significantly reduced the hypusination of eIF5A in both leiomyoma and myometrium cell lines at concentrations of 50 nM and 100 nM ($P = 0.0030$; $P = 0.0077$; $P = 0,0028$; $P = 0,0005$) (FIGURE 5A and FIGURE 5B), and also in leiomyosarcoma at 100 nM ($P = 0,0409$) (FIGURE 5C). The results showed no significant difference in expression of fibronectin after treatment with GC-7 in myometrial cells (FIGURE 6A). In contrast, the expression of fibronectin was significantly reduced by the treatment with GC-7 in leiomyoma cell line at 50 nM and 100 nM ($P = 0,0030$; $P = 0,0006$), and in leiomyosarcoma at 100 nM ($P = 0,0288$) (FIGURE 6B and FIGURE 6C).

Relationship between fibronectin expression and hypusination of eIF5A in leiomyosarcoma tissue

In the light of the results obtained on fibronectin expression in leiomyosarcoma cells, we hypothesized that this protein is involved in leiomyosarcoma progression, as reported in other cancers. It is known that fibronectin is involved in tumoural

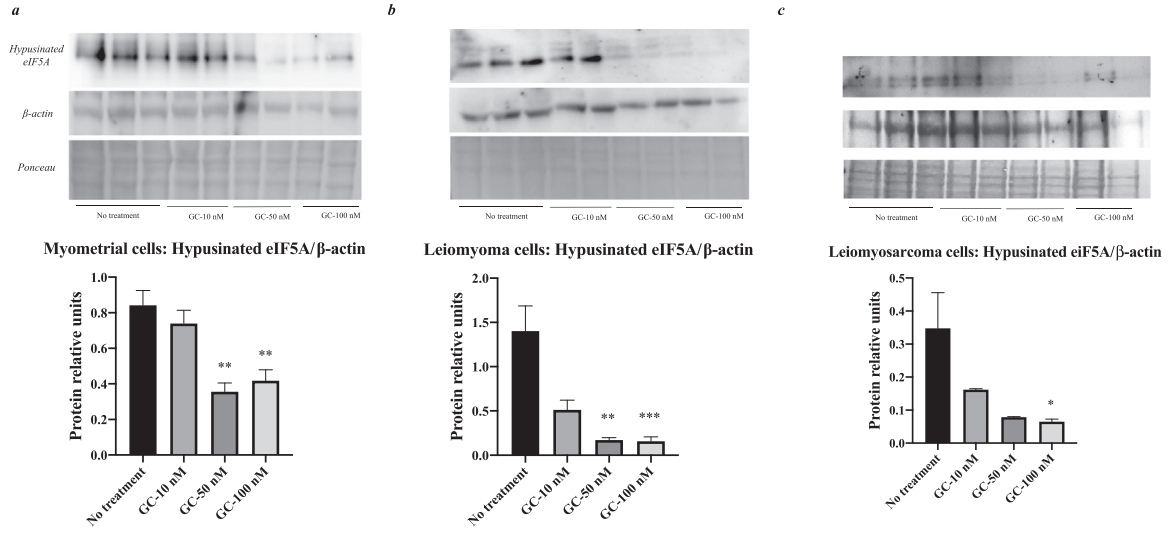

FIGURE 5 Effect of N1-guanyl-1,7-diaminoheptane (GC-7) on hypusinated form of the eukaryotic translation initiation factor 5A (eIF5A) in myometrial, leiomyoma and leiomyosarcoma cells. Western blotting, representative gel (upper panel), and densitometric analysis (lower panel) for hypusinated eIF5A (45 kDa) in (A) myometrial cells ($P > 0.9999$; $P = 0.0030$; $P = 0.0077$); (B) leiomyoma cells ($P = 0.6941$; $P = 0.0028$; $P = 0.0005$); (C) leiomyosarcoma cells ($P > 0.9999$; $P = 0.2867$; $P = 0.0409$). GC-7 treatment for 48 h at 10 nM, 50 nM, 100 nM (each $n = 6$), Untreated controls ($n = 9$). Densitometry shown as mean + SD. Differences tested by Kruskal–Wallis test. β-actin (42 kDa) and ponceau (40 kDa) were used as loading controls.

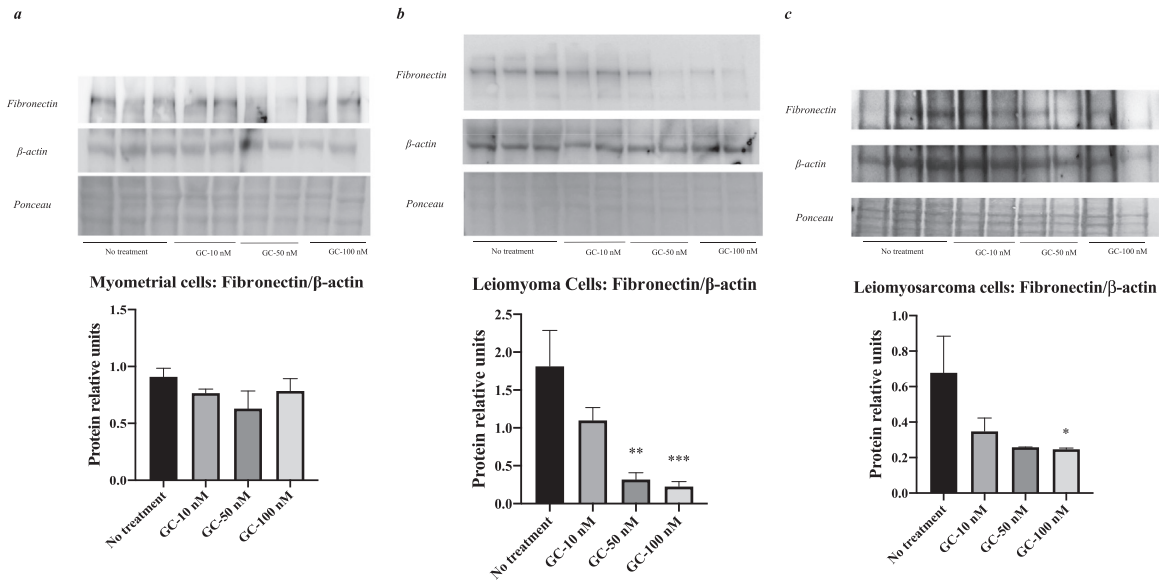

FIGURE 6 Effect of N1-guanyl-1,7-diaminoheptane (GC-7) on fibronectin expression in myometrial, leiomyoma and leiomyosarcoma cells. Western blotting, representative gel (upper panel), and densitometric analysis (lower panel) for fibronectin (240 kDa) in (A) myometrial cells ($P > 0.9999$; $P = 0.0625$; $P = 0.6960$); (B) leiomyoma cells ($P = 0.9772$; $P = 0.0030$; $P = 0,0006$); (C) leiomyosarcoma cells ($P > 0.9999$; $P = 0.3071$; $P = 0.0288$). GC-7 treatment for 48 h at 10 nM, 50 nM, 100 nM (each $n = 6$), Untreated controls ($n = 9$). Densitometry shown as mean + SD. Differences tested by Kruskal–Wallis test. β-actin (42 kDa) and ponceau (40 kDa) were used as loading controls.

progression; however, to the best of our knowledge, it has never been explored in leiomyosarcoma. Interestingly, we found that fibronectin is highly expressed in leiomyosarcoma tissue especially in the malignant area (FIGURE 7A). In the marginal area of tumours, the healthy tissue showed absent or low expression of fibronectin (FIGURE 7B), whereas its expression rises in the invasive area and reachs its maximum expression in the malignant aggressive part of the tumour (central area of the tumour) (FIGURE 7A and FIGURE 7C). Finally, the fibronectin stain also persisted in the necrotic area (FIGURE 7A). To explain the biological significance of the effect of hypusinated eIF5A on fibronectin expression in leiomyosarcoma, their relationship was explored. Comparing immunohistochemical staining, similar localization of fibronectin and hypusinated eIF5A that appeared highly represented in the bizarre cells of leiomyosarcoma was observed (FIGURE 7C). These cells are either round or polygonal with eosinophilic and clear cytoplasm and have a high mitotic capacity four or more mitoses/10 high-power fields (FIGURE 7C). On the contrary, in the marginal area, i.e. where the smooth muscle tissue meets the normal tissue, the two antibodies specific for fibronectin and hypusinated eIF5A had different behaviour: the hypusinated eIF5A was highly represented in the sarcoma cells, whereas the fibronectin was less expressed in the cells on the border (FIGURE 7B).

DISCUSSION

In the present study, we demonstrated the occurrence of the hypusinated form of translation factor eIF5A in myometrium, leiomyoma and leiomyosarcoma. In addition to the constant presence of hypusinated eIF5A in endothelial cells, we observed an increasing trend of the hypusinated form of the translation factor from normal myometrium to neoplastic benign leiomyoma up to neoplastic malignant leiomyosarcoma tissues. We confirmed the higher levels in leiomyoma compared with myometrium by Western blotting. On the other hand, leiomyosarcoma is rare, and it was not possible to find protein extracts to perform Western blotting for this kind of tissue. Anyhow, the expression of hypusinated eIF5A detected by immunohistochemistry was very strong in leiomyosarcoma tissue. The hypusinated eIF5A pattern data suggested that it is involved in leiomyoma and leiomyosarcoma pathogenesis. Therefore, we evaluated the in-vitro effect of hypusinated eIF5A on cell proliferation and fibronectin expression in myometrial, leiomyoma and leiomyosarcoma cell lines.

First, we confirmed the presence of hypusinated eIF5A in all three cell types by Western blotting; therefore, we treated the cells with GC-7 to inhibit the hypusination of the translation factor using different concentrations, 10 nM, 50 nM and 100 nM, as previously reported (*Shi et al., 1996*). Our results showed a significant reduction of cell proliferation in all cell types, after treatment with concentration 100 nM of GC-7, suggesting that hypusinated eIF5A is involved in cell proliferation.

These findings are in line with research reporting the role of hypusinated eIF5A in invasion and metastasis. In fact, it has been found that the over-expression of eIF5A favours the epithelial–mesenchymal transition and leads to metastases in non-small cell lung cancer (*Xu et al., 2014*), bladder cancer (*Wei et al., 2014*), hepatocellular carcinoma (*Tang et al., 2010*) and in oesophageal squamous cell carcinoma (*Li et al., 2014*). Furthermore, xenograft studies have shown that eIF5A overexpression increases metastases (*Wei et al., 2014*). Other studies have shown that eIF5A is one of the first four upregulated genes in colon cancer (*Nakanishi and Cleveland, 2016*). *He et al. (2011)* demonstrated an important correlation between high levels of eIF5A and poor prognosis even in patients with gastric cancer and high levels of eIF5A related to advanced clinical stage also in non-lung cancer cells. The same results had already been obtained in ovarian cancer by *Yang et al. (2009)*.

Considering the centrale role of ECM matrix in leiomyoma pathogenesis (*Leppert et al., 2006*; *Islam et al., 2013*; *2018*;

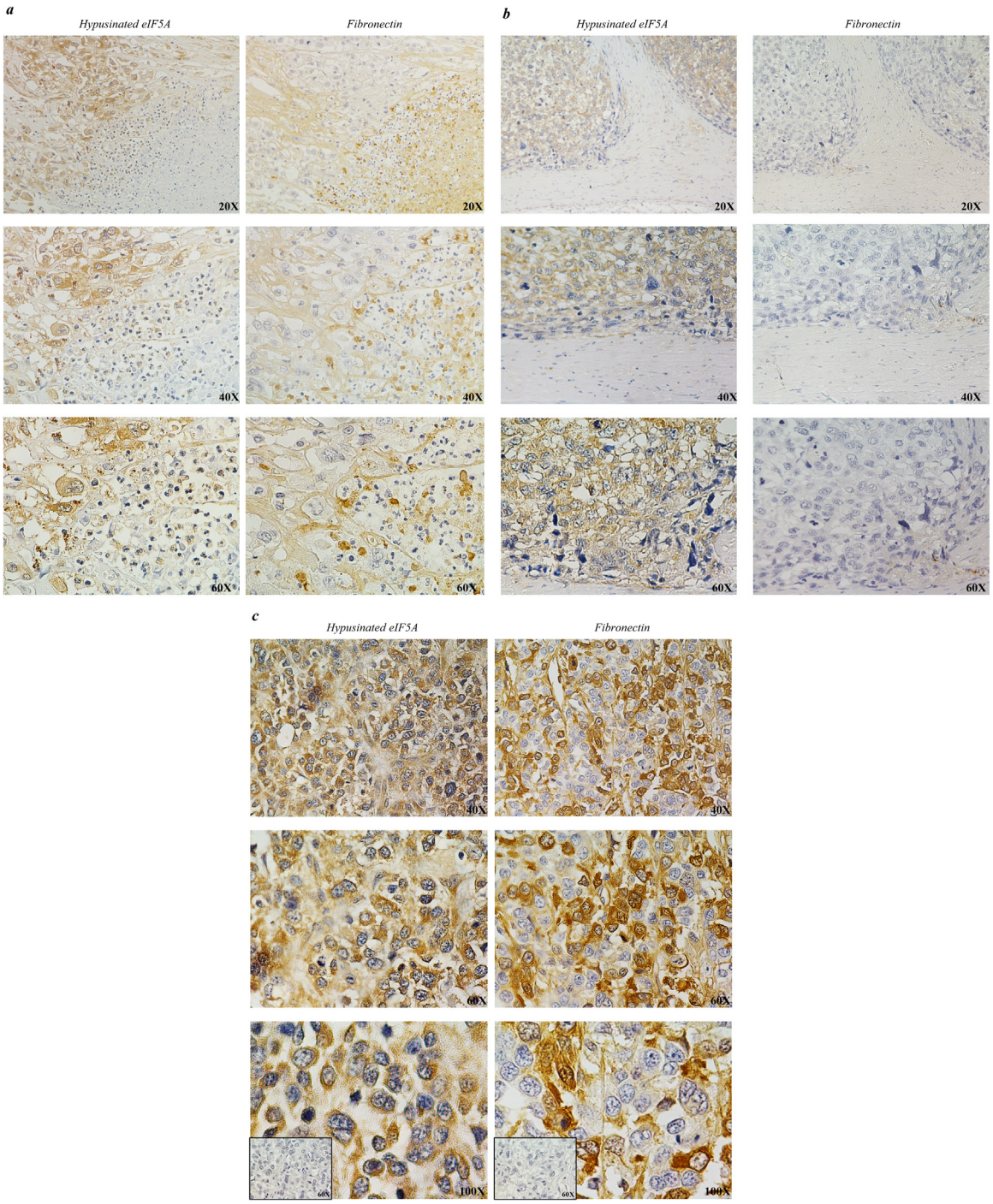

FIGURE 7 Representative images of immunohistochemical staining of Hypusinated form of the eukaryotic translation initiation factor 5A (eIF5A) and fibronectin in human leiomyosarcoma tissue. (A) Aggressive and necrotic leiomyosarcoma tissue. Stain was present in the cancer cells for both antibodies, and fibronectin staining in the necrotic area; (B) marginal area of leiomyosarcoma tumour and healthy tissue. The marginal pathological area showed the presence of hypusinated eIF5A, adjacent healthy tissue showed low expression for both antibodies; (C) aggressive leiomyosarcoma tumour tissue. Both antibodies were strongly present in the sarcoma cells. Counterstaining with Mayer's haematoxylin (blue). Magnifications 20X, oil immersion 60X and 100X. Bottom left, negative controls of both antibodies.

Giuliani et al., 2019), in the present study we also evaluated the in-vitro effect of eIF5A hypusination on fibronectin expression. We found that, although the levels of eIF5A hypusination significantly decreased with 50 nM or 100 nM of GC-7 in both myometrium and leiomyoma cell lines, the expression of fibronectin significantly decreased only in leiomyoma cells after the treatment with both 50 nM and 100 nM concentrations of GC-7, and not in myometrium cell lines. Therefore, the hypothesis that hypusinated eIF5A may be involved in leiomyoma pathogenesis is plausible.

We also found that GC-7 treatment also reduces fibronectin expression in leiomyosarcoma cells. This finding is extremely meaningful because fibronectin is also involved in tumourigenesis and metastasis.

From the primary tumour mass, some cells migrate away and invade the surrounding stroma, giving origin to the metastasis process. Efficient cell migration and invasion is possible only if there is adhesive interaction of cells with the ECM scaffold and proteolytic degradation on ECM (*Itagaki et al., 2020*). Dynamic variation of ECM can bring different pathological condition, including cancer progression (*Pearce et al., 2018*) In cancer, the fibronectin fibres acquire a specific anisotropic orientation, extend outwards in a many different directions, and seem to guide cells outside of the original neoplasm. Although cancer tissues seem to be highly disorganized, the alignment of fibronectin has a pathophysiological significant order; in fact, it directs the invasion of cancer cells in a centrifugal way away from the origin of malignancy (*Erdogan et al., 2017*). It has emerged that cancer-associated fibroblasts (CAF), one of the most abundant cell types in the tumour microenvironment endowed with the ability to promote tumour growth, carry out their action through binding to $\alpha v\beta 3$-integrin, promoting the organization of fibronectin in this way (*Olumi et al., 1999*; *Orimo et al., 2005*; *Lin et al., 2019*). Under physiological conditions, fibroblasts are able to maintain homeostasis of the ECM (*Kalluri and Zeisberg, 2006*). In contrast, CAF secrete high levels of ECM proteins, such as fibronectin, type I and type II collagen, and express oncofetal isoforms of fibronectin (*Schor et al., 2003*; *Clarke et al., 2016*' *Gopal et al., 2017*). Moreover, CAF can alter the architecture and physical properties of the ECM, influencing cell migration, invasion and growth (*Jolly et al., 2016*; *Kaukonen et al., 2016*). In fact, CAF generate tracks that cancer cells follow through force-mediated matrix remodelling and deforming collagen I matrices (*Gaggioli et al., 2007*; *Attieh et al., 2017*).

Despite reports of fibronectin in different cancer tissues (*Olumi et al., 1999*; *Orimo et al., 2005*; *Lin et al., 2019*), no information is available on leiomyosarcoma. Therefore, in the present study we evaluated the expression of fibronectin in leiomyosarcoma. The presence of fibronectin in the central part of the tumour suggests that it could participate in the formation and progression of leiomyosarcoma, promoting the migration and invasion of cancer cells. Interestingly, in this part of the tumour, we found a high expression of fibronectin that correlates with high levels of hypusinated eIF5A. Hypusinated eIF5A could also, therefore, be involved in the pathogenesis of the leiomyosarcoma through regulation of fibronectin expression.

In conclusion, our results demonstrated the presence of hypusinated eIF5A in myometrial, leiomyoma and leiomyosarcoma tissues and its effect on cell proliferation and fibronectin expression. In particular, we suggest that eIF5A has fibrotic effect in leiomyoma and cancer progression activity in leiomyosarcoma. Hypusinated eIF5A, therefore, seems to be involved in leiomyoma and leiomyosarcoma pathogenesis, and may be a possible new therapeutic target for these uterine pathologies.

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DATA AVAILABILITY

The data that has been used is confidential.

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