Overexpression of the matrix metalloproteinase 11 gene is a potential biomarker for type 1 endometrial cancer

GABRIELA SOFÍA GÓMEZ-MACÍAS[1*], MARÍA LOURDES GARZA-RODRÍGUEZ[2*],
RAQUEL GARZA-GUAJARDO[1*], DANIELA MONSIVÁIS-OVALLE[2*], JESÚS ANCER-RODRÍGUEZ[1*],
HUGO ALBERTO BARRERA-SALDAÑA[2*] and ORALIA BARBOZA-QUINTANA[1*]

Departments of [1]Pathology and Cytopathology, and [2]Biochemistry and Molecular Medicine, School of
Medicine, Autonomous University of Nuevo León, Monterrey, Nuevo León 64460, Mexico

Abstract. Metalloproteinase matrix 11 (MMP11) is a member of the matrix metalloproteinase family, which are able to degrade extracellular matrix components, and may serve a central function in the enhancement of tumor-induced angiogenesis, cell migration, proliferation, apoptosis and connective tissue degradation. In the present study, *MMP11* gene expression was investigated using the reverse transcription-polymerase chain reaction in 68 cases of type I endometrial carcinoma, and all data were analyzed in association with clinical characteristics. Overexpression of *MMP11* was demonstrated in 75%, and sub-expression was demonstrated in 25%, of endometrial cancer cases. Sub-expression cases were associated with good histological parameters, including low histological grade (G1 and G2), early pathological stage, and absence of vascular invasion, metastasis and recurrence. In total, 76.4% of endometrial cancer cases with sub-expression were identified as early stage 1A and B; however, 23.6% of cases were identified as stage 2, with vascular invasion present in 29.4% of cases. On the other hand, cases which demonstrated overexpression with high ranges (>10 times more than control) were associated with adverse histopathological characteristics, including high grade tumor (G3) and vascular invasion. In conclusion, the increased expression of *MMP11* may be used as a prognostic biomarker in patients with type 1 endometrial cancer.

Correspondence to: Dr Gabriela Sofía Gómez-Macías, Department of Pathology and Cytopathology, School of Medicine, Autonomous University of Nuevo León, Av. Madero Y Gonzalitos S/N, Monterrey, Nuevo León 64460, Mexico
E-mail: bpositivo66@hotmail.com

*Contributed equally

Introduction

The extracellular matrix (ECM) is a complex meshwork of proteins and carbohydrate polymers, which are secreted, surrounded and anchored by cells of connective tissues. Modulation of the ECM is important in the development and progression of malignancy. Matrix metalloproteinases (MMPs) and the natural tissue inhibitors of metalloproteinases (TIMPs) act synergistically to regulate ECM turnover. Expression of MMPs and TIMPs is involved in several key aspects of tumor growth, invasion and metastasis (1,2). It is hypothesized that MMPs may be associated with the level of invasion and progression in endometrioid-type carcinoma.

MMP11, a member of the MMP family, is able to degrade ECM components, and may serve functions in angiogenesis, cell migration, proliferation, apoptosis and connective tissue degradation. MMP11 is processed intracellularly and is secreted in its active form, thus MMP11 differs from other MMPs that are expressed as proenzymes and processed to active forms through proteolytic cleavage activated extracellularly. This indicates that MMPs may possess a unique function in tumor development and progression; however, MMP11 is unable to degrade major ECM components (3-7).

Endometrial carcinoma (EC) is the most common malignancy of the female genital tract. It is estimated that there are ~200,000 cases diagnosed worldwide annually, and that ~50,000 women will succumb to the disease. Despite the recognition of several different histological subtypes of EC, these are commonly explained using a dualistic model, which categorizes carcinomas into two major types: Type I and type II. Type I (endometrioid) comprises the vast majority of EC cases, as well as its histological variants due to the histological similarity with normal endometrial glands. Type I present precursor lesions including glandular hyperplasia and are associated with hyperestrogenism, which is defined by high levels of estrogenic hormones. The mutations are located primarily in phosphatase and tensin homolog, β catenin, MutL homolog (MLH)-1 and MLH-6 genes (8,9). Furthermore, patients with type I EC have more favorable prognosis factors

compared with type II EC (8-12). Several risk factors have been previously associated with type I EC including obesity and the use of estrogen therapies, which are associated with an imbalance between estrogen and progesterone. Combination therapy of oral contraceptives, which are associated with predominant states of progesterone, decreases the risk of EC (9,13-17). Other risk factors include nulliparity, early menarche and late menopause (9). A previous study investigating the expression of MMP1 and MMP2 in EC demonstrated that these biomarkers are associated with poor survival (18). Type II EC are high-grade carcinomas that cannot be graduated and exhibit a poor prognosis; however, these were not included in the present study owing to their behavior, spreading through the serous, having a high stage at the time of presentation and a poor prognostic.

In the present study, the expression levels of *MMP11* were measured using the reverse transcription-polymerase chain reaction (RT-PCR) in 68 cases of type I EC and associated with clinical pathological parameters including histological grade (G1-G3), vascular invasion [verified with D240 immunohistochemical (IHC) staining and cluster of differentiation (CD)31], pathological tumor stage (pT1a/1b/2/3), disease recurrence, and mortality. The present study verified expression levels of hormone receptors (estrogen and progesterone receptors) and cell proliferation index using IHC staining, and associated gene expression and pathological parameters with overall prognosis. To the best of our knowledge, there are no previous studies, which have investigated gene association in patients with type I EC. The expression of MMP11 at the protein level using IHC was not performed due to limited sample availability.

Materials and methods

Ethical approval and consent to participate. The ethics committee of the University Hospital of the Autonomous University of Nuevo León (Monterrey, Mexico) approved the present study. The requirement for consent to participate was not applicable.

Samples and histopathological analysis. In total, 68 cases of type I EC were obtained from the archives of the Pathology Department of the University Hospital of the Autonomous University of Nuevo León during a 5-year period (January 2009 to December 2014). In total, 21 cases (30.8%) had endometrial tissue adjacent to the tumor, which was included as an internal control for the present study. In total, 20 control cases of proliferative and secretory endometrium were included. EC cases were assessed to verify type, histological grade (G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated) and pT stage (pT1a, pT1b, pT2 and pT3a) according to the College of American Pathologists and the International Federation of Gynecology and Obstetrics. In each case, associated clinical pathological data were obtained and analyzed.

IHC analysis. Fixation took place in 10% neutral buffered formalin (NBF). The most representative tumor areas were selected and 3-μm-thick sections were produced. The sections were deparaffinized through 3 10 min washes in xylene (98.3%)

at room temperature, rehydrated in a descending alcohol series (100, 95, 70 and 50% ethanol, and finally PBS for 5 min) followed by microwave epitope retrieval [750 W for 7.5 min and 500 W for 5 min (x4) in citrate buffer (pH 6); Thermo Fisher Scientific, Inc., Waltham, MA, USA]. Endogenous peroxidase activity was blocked by incubation with 1% hydrogen peroxide in methanol for 30 min before incubation for 1 h (room temperature) with the polyclonal antibodies against estrogen receptor α (ERα; clone 1D5 M704), progesterone receptor (PR; clone PgR 636), Ki67 (clone A047), D240 (clone D2-40) and CD31 (clone JC70A). The sections were stained according to the standard avidin-biotin method from the manufacturer, for 30 min at room temperature (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) and counterstained with Harris hematoxylin (100%). All antibodies were purchased from Dako; Agilent Technologies, Inc. Tissue microarray slides were evaluated in a standard light microscope (magnification, x100) for immunohistochemical staining.

IHC expression. Positive and negative controls for each marker were included on each slide. Positivity indicated that >10% of cells demonstrated nuclear positivity for ER and PR, and an index of high proliferation was considered when >5% of cells demonstrated nuclear positivity for Ki67. Furthermore, D240 and CD31 staining was used to confirm the angiolymphatic invasion observed histopathologically. Although no gold standard for identifying angiolymphatic invasion exists, the presence of tumor cells within a vascular space, red blood cells surrounding the tumor cells, identification of endothelial lining of the space, a presence of an elastic lamina surrounding the tumor and tumor cells attached to the vascular wall may be beneficial data for the identification of vascular invasion in the histological sections stained with hematoxylin and eosin (H&E). The immunohistochemical stains for CD31 and D2-40 were used to assist in the detection of angiolymphatic invasion (Table I).

RNA extraction and quantification. Macrodissections of the most representative areas of EC tumor cases were performed and total RNA was obtained using an All Prep DNA/RNA formalin-fixed paraffin-embedded kit (Qiagen, Inc., Valencia, CA, USA), according to the manufacturer's protocol. RNA was quantified and qualified using a NanoDrop 200 spectrophotometer (Thermo Fisher Scientific, Inc.).

Expression levels of MMP11 using RT-PCR. To detect expression levels of *MMP11* in endometrial tissues, samples were analyzed using RT-PCR. For relative quantification, RT was performed using SuperScript® VILO™ cDNA Master Mix (Thermo Fisher Scientific, Inc.) in the Verity Thermal Cycler (Applied Biosystems; Thermo Fisher Scientific, Inc.) using 2 μg total RNA for cDNA synthesis for 10 min at 25°C followed by an enzyme inactivation step of 5 min at 85°C.

Expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method and normalized to the internal reference gene β-actin (19). RT-PCRs were performed using 2X TaqMan Universal Master Mix with uracil-N-glycosylase (Applied Biosystems; Thermo Fisher Scientific, Inc.) in the StepOnePlus™ RT-PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with 250 ng cDNA using thermocycling conditions outlined by the manufacturer's protocol, using TaqMan probes (40 cycles

Table I. Clinical and pathological parameters of cases (n=68).

Variable	Frequency	Percentage
Grade		
G1	3	4.4
G2	57	83.8
G3	8	11.7
Vascular invasion[a]		
Present	43	63.2
Absent	25	36.7
pT stage		
pT1a	38	55.8
pT1b	19	27.9
pT2	4	5.7
pT3a	7	10.2
Lymph node dissection		
Performed	37	54.4[b]
Not performed	31	45.5
Adjuvant therapy		
Administered	43	63.2
Not administered	25	36.7
Recurrence		
Present	5	7.3
Absent	63	92.6
Mortality	3	4.4

[a]Corroborated with CD31 and D240 stains; [b]3 of these patients demonstrated lymph node metastasis. pT, pathological tumor.

of 15 sec at 95°C with an extension at 60°C for 1 min). To detect fluorescent signals, the pre-developed TaqMan Gene Expression assay Hs00968295_m1 for MMP11 was used, and Hs99999903_m1 for β-actin was utilized as an internal control (forward, 5'-GTGGGCCGCTCTAGGCACCAA-3', reverse, 3'-CTCTTTGATGTCACGCACGATTTC-5', belong to Applied Biosystems; Thermo Fisher Scientific, Inc.). RT-PCR was performed in independent replicates. Two biological replicates for each sample were used for RT-PCR analysis and three technical replicates were analyzed for each biological replicate. A value of >1 was considered as overexpression, and <1 was considered as sub-expression, according to the standard value on control secretory endometrium (the endometrial cycle has two secretory and proliferative phases, which were used as controls). To analyze the gene expression stability, geNORM v3.4 software (Genome Biology, London, UK) was used.

Statistical analysis. An analysis of the possible association between patterns of MMP11 expression and clinical pathological variables including age, histological grade, pathological stage, vascular invasion, recurrence and mortality, as well as expression levels of hormone receptors (ER/PR) and Ki67, were performed using the χ^2 test. P<0.05 was considered to indicate a statistically significant difference. Receiver operating characteristic analysis was used to validate the overexpression

of *MMP11* at a specificity of 0.49 with a confidence interval of 0.33-0.64, and sensibility of 0.75 with a confidence interval of 0.65-0.85.

Results

Clinical-pathological parameters. The mean age of patients with EC was 55 years (range, 33-82 years). Histological grades for type I EC included G2 (83.8%) followed by G3 (11.7%) and G1 (4.4%). Angiolymphatic (vascular) invasion was present in 63.2% of cases (Table I). Invasion in histological H&E sections was investigated and analyzed for association with CD31 and D240 staining. No false positives or false negatives were observed (Table I). The majority of patients with angiolymphatic invasion demonstrated overexpression (70.6%), and presented G2 staging in 87% of cases and stages pT1b, 2 and 3a in >90% of cases. With regard to the pathological stage, the majority of cases were localized (stage 1A and B; 83.8%), no patient presented at stage IV. Patients underwent pelvic lymph node dissection in 54% of cases and 8% demonstrated lymph node metastases. In total, 63% of patients with EC received adjuvant therapy (radiotherapy and chemotherapy) and disease recurrence occurred in 7% of cases, all of which were localized at the vaginal vault level. Within the first 2 years of surgery, 3 patients who had presented at advanced-stage at the time of diagnosis had succumbed to the disease. Clinical pathological data are presented in Table I.

Expression levels of MMP11. MMP11 was overexpressed in 75% (n=51), and sub-expressed in 25% (n=17) of EC cases. Levels of overexpression ranged between 1.1- and 600-fold (Fig. 1). Cases with sub-expression were primarily associated with G1 (well-differentiated) followed by G2 (moderately differentiated); however, all G3 (poorly differentiated) cases demonstrated overexpression, and the remainder G2 cases were overexpressed. In 76.4% of cases with sub-expression, early pT stages (1A and B) were demonstrated, and 23.6% presented at stage 2. The remaining early-stage cases (23.6%), 76.4% of stage 2 cases and all stage 3 cases were overexpressed. In total, 29.4% of cases with sub-expression demonstrated vascular invasion. Internal controls with endometrium adjacent to the tumor demonstrated overexpression in 66.6% with expression levels ranging between 1.1- and 3.5-fold, and 33.4% with sub-expression of the gene *MMP11* (Fig. 1).

Statistical analysis. No statistically significant association between *MMP11* expression and age, nuclear grade, adjuvant therapy, recurrence or mortality was identified. However, pT pathological stage and vascular invasion demonstrated a statistically significant association with gene expression (P=0.03 and 0.01, respectively; Table II).

IHC expression. The ERα IHC stain was positive in 80.8% of cases, and associated with histological grades 1 and 2 (92.7%). The PR IHQ stain was expressed in 76.4% of cases; increased proportions of PR were associated with low histological grades (92.4%) and early pathological stages (90.5%). The rate of cell proliferation determined using immunohistochemical staining with Ki67 was increased in 45 (66%) of cases and decreased in 23 (44%) of cases; 95.6% of the latter demonstrated low nuclear
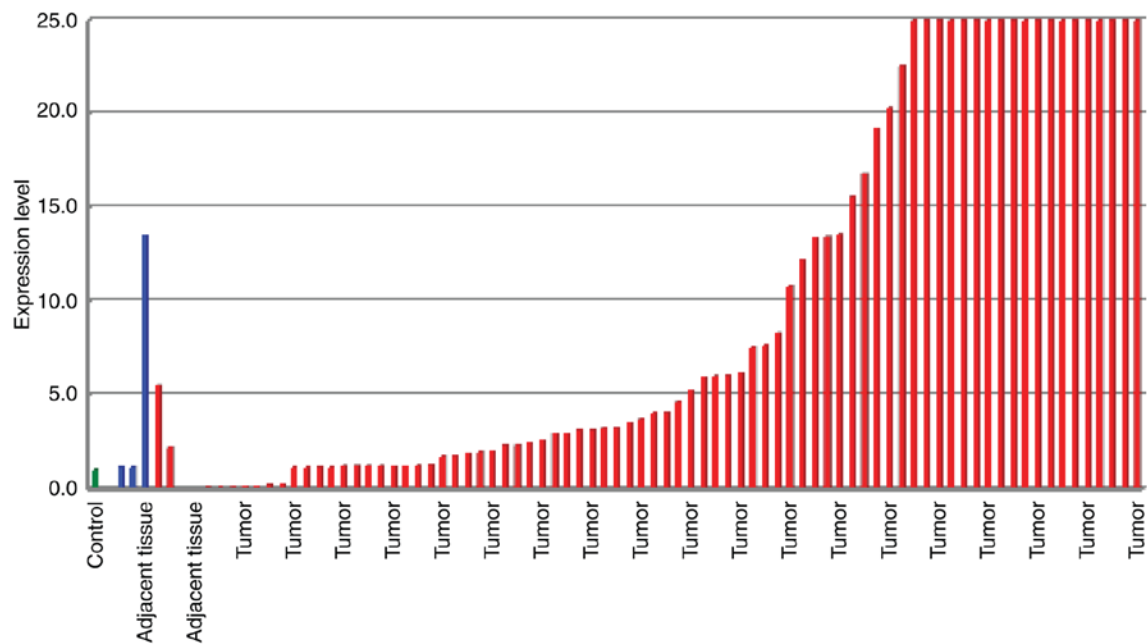
Figure 1. Expression levels of *MMP11* gene in EC tumors and adjacent tissues. Values >1 were considered as overexpression, ranging between 1.1- and 600-fold. MMP, matrix metalloproteinase.
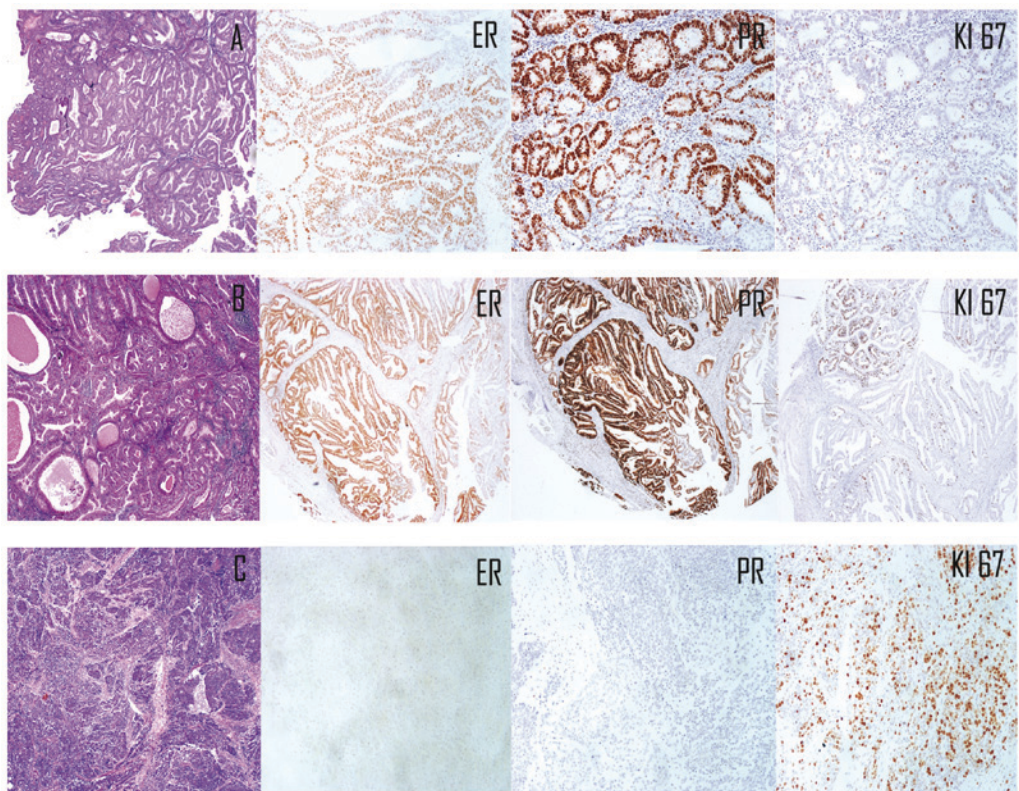

Figure 2. Immunohistochemical staining of EC using H&E, ER, PR and Ki67 staining. (A) Well-differentiated endometrioid carcinoma (magnification, x100). (B) Moderately differentiated endometrioid carcinoma (magnification, x100). (C) Poorly differentiated endometrioid adenocarcinoma (magnification, x100). H&E, hematoxylin and eosin; EC, endometrial carcinoma; ER, estrogen receptor; PR, progesterone receptor.

grades (1 and 2), and all cases presented at stages 1A and A (Fig. 2). Hormone receptors were not statistically significant factors (P=0.25 and 0.20 for ER and PR, respectively); however, the cell proliferation index of Ki67 was significant (P=0.04; Table II).

Discussion

The expression of *MMP* in EC has been previously studied; among these types 1, 2, 7 and 9 demonstrated overexpression and were associated with a poor prognosis (19-28). MMP14 has

Table II. Clinical and pathological parameters, hormone receptors and Ki67 associated with matrix metalloproteinase 11 expression.

Variable	P-value
Age	0.12
Nuclear grade	0.13
pT pathological stage	0.03
Metastatic lymph nodes	0.21
Angiolymphatic invasion	0.01
Adjuvant therapy	0.20
Recurrence	0.43
Mortality owing to disease	0.75
α-Estrogen receptors	0.25
Progesterone receptors	0.20
Ki67	0.04

pT, pathological tumor.

been associated with increased myometrial and lymph node invasion; however, the study was limited to a small number of cases (26). Previous studies regarding carcinomas within the biliary tract, oral cavity, thyroid and colon demonstrated an association between *MMP11* overexpression and a poorer prognosis (27-29). In the present study, overexpression of *MMP11* ranging between 1.1- and 600-fold normal values was demonstrated and was identified to be associated with adverse histopathological characteristics including high nuclear grade, advanced stages, angiolymphatic invasion, recurrence and mortality; in contrast, cases with sub-expression which represented the total number of cases with well-differentiated and moderately differentiated histological grades presented at an early stage in the disease process.

In the present study, the majority of the cases (83.8%) were represented by a moderately differentiated histological grade (G2), and were diagnosed in the early stages pT1a and pT1b (83.7%), which coincides with results reported in previous literature (30-33). This is a limitation of the present study because of the low percentage of carcinomas that are well and poorly differentiated, as well as a low number of late stages.

Furthermore, previous studies investigating cancer cell lines associated with hormones, including breast, ovarian and prostate carcinoma, demonstrated an association between overexpression of MMPs and increased proliferation and invasion of carcinogenic cells (34). In the present study, the expression of hormone receptors (ERα and PR) was evaluated in cases with type I EC (endometrioid and variants). Cases with an increased percentage of positivity of hormone receptors (ER and PR; >70%) were associated with fewer adverse histopathological features when compared with cases that demonstrated negativity or sub-expression of these receptors.

Previous studies have attempted to identify MMP11 as a potential predictive tumor biomarker in patients with gastric carcinoma; results demonstrated a significant increase in the serum levels in these patients, thus proposing it as a biomarker for diagnosing certain types of carcinomas. The results of the present study suggest that EC may be included, once data are validated, as the results in the present study are in agreement with those of a previous study in terms of progression and prognosis (35).

However, to the best of our knowledge, there have been no previous studies, which associated the cell proliferation index with the expression of MMPs. In the present study, the rate of proliferation was determined using Ki67 staining, in which an increased rate of proliferation was identified to be associated with adverse histopathological parameters, increased levels of *MMP11* expression (P=0.04), vascular invasion and pathological staging. This supports the hypothesis that MMPs are associated with the level of invasion and progression in endometrioid-type carcinoma.

Despite previous evidence demonstrating that *MMP11* overexpression is a potential biomarker in this type of neoplasia, a key limitation to the present study is the relative lack of IHC analysis. It is recommended that future studies increase the number of cases and perform MMP11 protein IHC analysis in tissue microarrays to corroborate *MMP11* overexpression as a biomarker.

To conclude, the increased *MMP11* expression in type I EC is associated with a poor prognosis. Overexpression may be used as a prognostic biomarker in patients with type I EC; however, studies with a larger sample size are required to support this hypothesis.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GSGM, MLGR and DMO were responsible for the conception and design of the study, and the acquisition, analysis and interpretation of data. RGG, JAR, OBQ and HABS made substantial contributions to conception and design and acquisition of data. They were involved in revising it critically for intellectual content and final approval of the version to be published.

Ethics approval and consent to participate

The present study was approved by the ethics committee of the University Hospital of the Autonomous University of Nuevo León (approval no. AP 14-001).

Consent for publication

Not applicable for this manuscript.

Completing interests

The authors declare that they have no competing interests.

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