Deregulated expression of Elastin Microfibril Interfacer 2 (EMILIN2) in gastric cancer affects tumor growth and angiogenesis

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https://doi.org/10.1016/j.mbplus.2020.100029

Abstract

Gastric cancer is a frequent human tumor and often a lethal disease. Targeted therapy for gastric carcinomas is far behind vis-à-vis other solid tumors, primarily because of the paucity of cancer-driving mutations that could be efficiently and specifically targeted by current therapy. Thus, there is a need to discover actionable pathways/proteins and new diagnostic and prognostic biomarkers. In this study, we explored the role of the extracellular matrix glycoprotein EMILIN2, Elastin Microfibril Interfacer 2, in a cohort of gastric cancer patients. We discovered that EMILIN2 expression was consistently suppressed in gastric cancer and high expression levels of this glycoprotein were linked to abnormal vascular density. Furthermore, we found that EMILIN2 had a dual effect on gastric carcinoma cells: on one hand, it decreased tumor cell proliferation by triggering apoptosis, and on the other hand, it evoked the production of a number of cytokines involved in angiogenesis and inflammation, such as IL-8. Collectively, our findings posit EMILIN2 as an important onco-regulator exerting pleiotropic effects on the gastric cancer microenvironment.

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Introduction

Gastric cancer (GC) is a relative common disease worldwide and the third leading cause of cancer-related deaths [1]. Most of GC patients are diagnosed with locally advanced or metastatic disease followed by surgical resection, when feasible. High response rates have been reported with a combination of docetaxel, cisplatin, and 5-fluorouracil (5-FU), despite the high toxicity of this chemotherapeutic approach, and among other drugs the use of oxaliplatin is also an alternate choice [2–5]. Unfortunately, the life expectancy of the patients affected by advanced or metastatic GC is very poor, with ~10% for 5-year relative survival rate and the median overall survival of only 1 year [6]. Thus, the development of new targeted therapies and/or new predictive biomarkers for GC patients is a major goal for fighting this grave disease.

Recent mutational analyses have prompted the employment of targeted drugs in patients displaying hyperactivation of epidermal growth factor receptor (EGFR), mTOR or c-Met. Unfortunately, clinical trials have shown that the GC patients do not benefit from
these types of treatment [7–13]. On the contrary, a combination of conventional chemotherapy and the humanized monoclonal antibody trastuzumab targeting the human epidermal growth factor receptor 2 (HER2) improved the progression free survival (PFS) and overall survival. Nonetheless only a small percentage of GC patients carry this type of mutation [14–17]. More recently, much attention has been directed toward understanding the tumor microenvironment as a possible mean for the development of new therapies or predictive biomarkers [18–23]. One emerging promising target is the immune microenvironment, where the extracellular milieu is an important regulator [24–36]. The use of the immune checkpoint programmed cell death-1 (PD-1) blocking agents, the blockage of its ligands (PD-L1 or B7-H1), as well as the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), have shown remarkable therapeutic efficacy against several solid tumors [39], and may also represent a significant advance in GC treatment [40].

Another important target is angiogenesis, the development of new blood vessels from pre-existing vasculature. Angiogenesis is switched on during cancer development since vascularization is required to support tumor cell growth through the supply of oxygen and nutrients [41,42]. Anti-angiogenic therapy has been approved for the treatment of a number of tumors and its efficacy has also been explored in GC [43,44]. However, despite slight survival benefits observed for instance with the use of ramucirumab, a VEGFR-2 antibody [45,46], anti-angiogenic therapy has provided suboptimal results. This could be due to the fact that this process is regulated by a plethora of cytokines and growth factors, receptors and also by extracellular matrix (ECM) constituents [47–56]. Thus, it is mandatory to better characterize the newly-formed blood vessel together with the microenvironmental structures supporting the tumor proper.

An increasing body of evidences highlights the crosstalk between angiogenesis and the immune response [57,58], and, in this context, the secreted glycoprotein Elastin Microfibril Interfacer 2 (EMILIN2) may play a prominent role. EMILIN2 [59] belongs to the EDEN protein family [60–62] and, like Multimerin-2, another member of this protein family, is involved in angiogenesis [49–52,63–67]. EMILIN2 modulates different signaling pathways affecting the activation of receptors on the tumor cell surface [68–71]. Interestingly, we have recently shown that EMILIN2 triggers the expression of IL-8 in endothelial cells and fibroblasts [72]. Thus, since IL-8 affects both angiogenesis [73–75] and inflammation [76], the levels of EMILIN2 expression may significantly impact on different processes involved in GC onset and progression. In this study, we assessed the direct and indirect effects of EMILIN2 in GC cell viability and angiogenesis. Our results posit EMILIN2 as key molecule for the development of new biomarkers for GC patient-tailored treatments.

## Results

**EMILIN2 is often lost in gastric cancer and its expression associates with that of CD31**

We have previously shown abnormal expression of EMILIN2 in a relatively small cohort of GC patients [64]. To corroborate these initial observations, we analyzed the expression of EMILIN2 in the GC and adjacent gastric mucosa from 19 patients with gastric carcinomas (Table 1). We found intense EMILIN2 staining in the lamina propria adjacent to the gastric glands (Fig. 1A). Although there was a non-uniform expression among the patients, we found a significant decrease of EMILIN2 expression in GC patients ($P < 0.001$, Fig. 1B). Interestingly, EMILIN2 expression levels were variable in the gastric mucosa of GC patients, despite detectable in all the samples analyzed (Figs. 1B and 2A).

Next, we verified if the expression of EMILIN2 would associate with the clinical GC stage (I–II or III–IV), histotype (diffuse or intestinal) or anatomical location (cardia/fundus, body or antrum) of the tumor. We found no significant correlation with any of these parameters (Fig. 1C–E).

Since we have recently demonstrated that EMILIN2 triggers angiogenesis by evoking IL-8 expression in endothelial cells and fibroblasts [52], we assessed if the vascularization of the gastric samples changed in relation to the expression of EMILIN2. We used an antibody specifically recognizing the cell surface glycoprotein CD31, an established endothelial cell marker. In all the gastric

| Patients | Number of cases | Percentage |
|----------|----------------|------------|
| General information | | |
| All | 25 | 100 |
| Males | 14 | 56 |
| Females | 11 | 44 |
| Staging | | |
| I–II | 9 | 36 |
| III–IV | 16 | 64 |
| Histologic type (Lauren classification) | | |
| Diffuse | 13 | 52 |
| Intestinal | 12 | 48 |
| Site | | |
| Body | 14 | 56 |
| Antrum | 8 | 32 |
| Fundus | 2 | 8 |
| Cardia | 1 | 4 |
mucosa samples we found an abundant and regular vascularization (Fig. 2A, B). Notably, EMILIN2 expression correlated with that of CD31 ($P = 0.0116$, Fig. 2B). In contrast, in most tumor tissues EMILIN2 expression was lost (Figs. 1B and 3A) and also in this case the levels of expression associated with the extent of tumor vascularization (Fig. 3B). These results suggest that EMILIN2 could be a good prognostic indicator of GC development as increased neovascularization is a sign of a more aggressive phenotype [77].

EMILIN2 directly affects the viability of gastric carcinoma cells

Having established a correlation between EMILIN2 expression and GC, we hypothesized that EMILIN2 could directly impact the behavior of GC
cells. To this end, we employed the GC cell lines AGS originally isolated from an adenocarcinoma of the stomach [78] and MKN74, and verified if they expressed EMILIN2. Unlike the colon cancer cell line Caco-2, no EMILIN2 mRNA was detected in these cells (Fig. 4A). Accordingly, we found no protein expression by either immunoblotting (Fig. 4B) or immunofluorescence (Fig. 4C) analyses. However, we found moderate expression of EMILIN1 in both the gastric tumor cell lines.

Next, we determined whether EMILIN2 could act as an adhesive substrate for AGS and MKN74 cells. To perform these studies, we used recombinant EMILIN2 protein purified in our laboratory (Fig. S1). We employed recombinant fibronectin, collagen type I and IV, and laminin as positive controls. Notably, AGS and MKN74 cells did not adhere to EMILIN2 (Fig. 4D). Overlapping results were obtained with the CAFCA adhesion assay (Fig. 4E). A plausible explanation for this finding is that both AGS and MKN74 cells do not express the α4β1 and α9β1, the putative integrins engaged by EMILIN2, as assessed by FACS analyses (Fig. S2A, B).

To further dissect the role of the molecule in gastric cancer cell behavior, we challenged AGS and MKN74 cells with EMILIN2.

Notably, EMILIN2 significantly impaired the proliferation of AGS and MKN74 cells (P < 0.001, Fig. 5A) and evoked apoptosis as detected by TUNEL assays (P < 0.01 and P < 0.03, respectively, Fig. 5B). These results indicate that EMILIN2 can suppress cancer growth, in accordance with our previous findings of increased apoptosis in sarcoma cells [70,71].
EMILIN2 influences the expression of angiogenic cytokines by GC cells

Given the striking association between EMILIN2 expression and the vascularization pattern, we hypothesized that EMILIN2 could elicit the expression of pro-angiogenic factors also in tumor cells. To verify this hypothesis, we challenged AGS cells with EMILIN2 and determined by qPCR the expression of several molecules involved in angiogenesis. EMILIN2 did not affect the expression of MCP-1, TIMP-1 and angiogenin, and slightly despite significantly reduced the expression of CXCL-16 and thrombospondin1 (TSP1) and increased that of insulin growth factor-binding protein 2 (IGFBP2) (Fig. S3A). Notably, EMILIN2 strongly elicited the expression of serine protease inhibitor1 (SERPINE1), vascular endothelial growth factor A (VEGFA) (Fig. 5C). Interestingly, the highest increase in mRNA was that of IL-8 (Fig. 5C), a cytokine involved also in the recruitment of inflammatory cells [79]. EMILIN2-challenged MKN74 cells displayed a strong increase of IL-8 expression, and a slight increase of SERPINE1 and VEGFA, despite the difference was not significant (Fig. 5C, Fig. S3B). This result not only confirms our previous findings pinpointing EMILIN2 as an important regulator of IL-8 expression [72], but also demonstrates that it can be triggered in tumor cells.

Collectively, our findings demonstrate that EMILIN2 is a key component of the gastric parenchyma and tumor microenvironment, and that its loss may profoundly affect the outcome of GC patients acting at different levels of gastric cancer development and progression.
Fig. 4. EMILIN2 does not represent an adhesion substrate for AGS and MKN74 gastric cancer cells. A, Evaluation of EMILIN2 expression by AGS and MKN74 cells according to the qPCR analyses. The colorectal cancer cell line Caco-2 was used as positive control. GAPDH expression was used for normalization. B, Top panel: WB analysis of the EMILIN2 levels in CM from AGS, MKN74 and Caco-2 cells. Bottom panel, Ponceau stained membrane showing protein loading. C, IF analysis of the expression of EMILIN2, left, fibronectin, middle and EMILIN-1, right in AGS, MKN74 and Caco-2 cells; extracellular matrix molecules are shown in green and nuclei were stained with TO-PRO® 3 (blue). Scale bar = 50 μm. D, Evaluation of the adhesion of AGS and MKN74 cells to fibronectin (FN), collagen type I (Coll I), collagen type IV (Coll IV), Laminin, EMILIN2 or BSA as negative control, assessed by means of xCELLigence instrument. E, Cell adhesion (CAFC assay) of AGS and MKN74 cells to fibronectin (FN), collagen type I (Coll I), collagen type IV (Coll IV), Laminin, EMILIN2 or BSA as negative control. Graphs represent the mean ± SD; P values were obtained using the one-way ANOVA test.
Discussion

In this study, we analyzed the expression of the ECM glycoprotein EMILIN2 in the gastric mucosa and gastric carcinomas from a cohort of well documented patients with GC. We found that EMILIN2 is expressed primarily and uniformly in the lamina propria of the gastric mucosa, although the expression levels are variable among patients. Based on the fact that EMILIN2 can have a dual effect on gastric cancer, i.e., it can have direct effects on tumor cells as well impinging on microenvironmental cues, we hypothesize that the expression of this important secreted glycoprotein could affect not

Fig. 5. EMILIN2 impacts on gastric cancer cell viability and on the expression of key angiogenic cytokines. A, Graph indicating the viability of AGS and MKN74 cells challenged or not (CTRL) with EMILIN2, as assessed by MTT assays. B, Representative images and quantification of the percentage of apoptotic AGS and MKN74 cells challenged or not (CTRL) with EMILIN2, as assessed by TUNEL assays. Scale bar = 100 μm. C, Real-time PCR evaluation of the expression of SERPINE1, VEGFA and IL-8 by AGS (top graphs) and MKN74 (bottom graphs) cells challenged or not (CTRL) with EMILIN2. Graphs represent the mean ± SD; \( P \) values were obtained using the paired Student's \( t \)-test.
only tumor onset but also tumor progression. Indeed, our findings show a strong correlation between EMILIN2 expression and blood vessel density, highlighting the prominent role of EMILIN2 in GC vascularization.

We demonstrate that EMILIN2, despite not representing a substrate for GC cell adhesion, directly affects their viability. It is likely that the lower proliferation rate of AGS and MKN74 cells observed in the presence of EMILIN2 is due to the increased percentage of apoptotic cells observed under treatment with the soluble protein. In fact, in previous studies carried out using another cell model, we demonstrated that EMILIN2 can trigger tumor cell apoptosis by activating the extrinsic apoptotic pathway receptors DR4 and DR5 [71]. AGS cells express these receptors [80], whose activity could be increased in EMILIN2-rich microenvironments. In addition, since like other GC cells AGS can acquire resistance to the action of TRAIL [80], it is possible that EMILIN2 may resensitize resistant GC cells to the action of this cytokine. Accordingly, high EMILIN2 levels could negatively affect tumor cell growth and could represent a favorable prognostic marker for GC patients.

We further report for the first time that the direct action of EMILIN2 on GC cells could be the consequence of an indirect impact on the tumor microenvironment. Our data point to EMILIN2 playing a role in the crosstalk among cancer, endothelial and inflammatory cells. This concept is supported and corroborated by an increase in cytokines in the gastric adenocarcinoma cells exposed to EMILIN2. Several of these cytokines are involved in tumor angiogenesis. For example, we find a marked increase in VEGFA, a master regulator of angiogenesis [81]. Interestingly, Multimerin2, another member of the same protein family, also affects the VEGFA availability, however this occurs through the sequestration of the cytokine rather than affecting the mRNA levels [65,66], a further indication of the complex regulatory functions of ECM milieu. We also find an increase in SERPINE1, the inhibition of which by miR30-c was recently demonstrated to suppress tumor growth [82]. Despite the mechanisms by which EMILIN2 affects SERPINE1 expression need to be elucidated, it is possible that it may act through the regulation of miR30-c expression. EMILIN2 did not significantly affect the expression of VEGFA and SERPINE1 in MKN74 cells, suggesting that its action may depend on the peculiar gene mutational profile of the different tumor cells. Also, IGFBP2 plays an important role in angiogenesis [83] and induces the up-regulation of VEGFA [84]. Despite EMILIN2 only slightly increased IGFBP2 expression in AGS cells, it is possible that the increased levels of IGFBP2 elicited by EMILIN2 are sufficient to drive also the expression of VEGFA.

We previously discovered that EMILIN2 induces IL-8 expression in endothelial cells and fibroblasts present in the microenvironment [72]. In this study, we expand this knowledge and demonstrate that EMILIN2 stimulates IL-8 production also in gastric adenocarcinoma cells. Interestingly, IL-8 is produced by different cell lines including monocytes [85], and we have unpublished evidences that EMILIN2 is expressed also by this cell type. Thus, EMILIN2 may also regulate the expression of IL-8 by inflammatory cells recruited to the tumor microenvironment.

Increased IL-8 expression was recently demonstrated to occur following the down-regulation of protein tyrosine phosphatase receptor delta (PTPRD) and to associate to increased angiogenesis and metastasis in GC [86]. Despite EMILIN2 triggers IL-8 expression through the activation of the EGF/EGFR1 signaling pathway, it cannot be excluded that it may also play a role in this context.

In addition, IL-8 is also produced by mast cells, whose density is increased in GC and correlates with the tumor vascularization, the number of metastatic lymph nodes and the patients’ survival [87]. As to whether EMILIN2 plays a function also in this context needs further investigation. Other important cellular components of the tumor microenvironment are cancer-associated fibroblasts (CAFs) which express high levels of IL-8 [88]. Since EMILIN2 affects IL-8 production by fibroblast it would be interesting to determine if this mechanism can also play a role in this context of CAFs or if this mechanism is only regulated by the interaction with cancer cells.

Thus, the multiple effects that EMILIN2 exerts in the tumor microenvironment are complex and the outcome is a balance between positive and negative regulators of tumor growth. It must be also pointed out that the intratumoral vessels developed in the presence of EMILIN2 are particularly inefficient and leaky [72]. This may negatively impact on tumor growth as well as drug delivery and, consequently, efficacy of the treatments.

In this perspective EMILIN2 may represent a key molecule regulating the interplay between angiogenesis and inflammation which represents a major field of investigation [57]. Taken together these results highlight the prominent role of EMILIN2 in GC and suggest that its loss associates with multiple changes involving both cancer cells and the tumor microenvironment.

Materials and methods

Patients

For this study, 19 patients with locally advanced gastric cancer were consecutively enrolled. The methodologies conformed to the standards set by the Declaration of Helsinki. This study was approved by the Institutional Board of the CRO-IRCCS,
National Cancer Institute of Aviano (PN), Italy (IRB no. CRO-2014-03). The clinical evaluation is shown in Table 1. The patients underwent neoadjuvant multiregimen chemotherapy (oxaliplatin, capecitabine and taxane) for an average of 3 months followed by surgical resection according to standard guidelines. Laboratory and pathological results were collected from the Cancer center database.

Cells and antibodies

The AGS, MNK74, 293-EBNA (E293), and Caco-2 cell lines were obtained from ATCC (Manassas, VA, USA), maintained at 37°C under a humidified atmosphere containing 5% CO2 and verified to be free of mycoplasma contamination using the MycoAlert™ Mycoplasma Detection kit (LONZA). AGS cells were cultured in F-12 medium (Gibco, Milan, Italy) containing 10% fetal bovine serum (FBS; Gibco, Milan, Italy); for 293-EBNA cells 250 μg/ml G418 were added. The MNK74 cell line was cultured in F-12 medium (Gibco, Milan, Italy) with 10% fetal bovine serum (FBS; Gibco, Milan, Italy). Caco-2 and 293-EBNA cells were cultured in Dulbecco’s modified eagle’s medium (DMEM) with 10% fetal bovine serum (FBS; Gibco, Milan, Italy). The polyclonal anti-EMILIN2 antibody was produced in our laboratories as previously described [70,89]. The monoclonal anti-human CD31 antibody was from Invitrogen (Milan, Italy). The mouse anti-human antibody was produced in our laboratories as previously described [70,89]. The monoclonal anti-human CD31 antibody was from Invitrogen (Milan, Italy), the anti-β-actin antibody from Cell Signaling (Danvers, MA, USA). The secondary antibodies conjugated with Alexa Fluor 488, 546 and TO-PRO-3 were from Invitrogen (Milan, Italy). HRP-conjugated secondary antibodies were from Amersham (Milan, Italy).

Immunofluorescence

For immunofluorescence analyses on tumor samples, serial cryostatic sections (7 μm) were collected on positively charged slides (BDH Superfrost Plus), air dried at room temperature (RT) and fixed with PFA for 15 min. After washing with phosphate-buffered saline (PBS), the slides were incubated with 0.5% Triton X-100 in PBS for 5 min at RT, saturated with 1% BSA, 10% normal goat serum (DAKO) in PBS for 1 h at RT, and stained ON at 4°C with the appropriate antibodies. Next, the samples were washed with PBS and incubated with the appropriate secondary antibodies and TO-PRO3 to stain the nuclei for 1 h at RT. After washing with PBS, the slides were mounted in Mowiol containing 2.5% (w/v) of 1,4-diazabicyclo-(2,2,2)-octane (DABCO). Images were acquired with a Leica TCS SP8 Confocal system (Leica Microsystems Heidelberg, Mannheim, Germany), using the Leica Confocal Software (LCS). Fluorescence intensity and quantification was evaluated by means of the Volocity software (PerkinElmer Inc., Waltham, MA, USA).

FACS analyses

Cell were detached with 5 mM EDTA in PBS and stained with the following antibodies: isotype control APC, anti-α1 PE, anti-α3 APC and anti-αVβ3 PE (Miltenyi Biotec); isotype control PE, anti-α, PE and anti-β3 PE (Biolegend). Cells were analyzed using FACS LSR Fortessa (BD Biosciences) and data were analyzed using DIVA software (BD Biosciences).

Centrifugal assay for fluorescence-based cell adhesion (CAFCA)

The quantitative cell adhesion assay used in this study is based on centrifugation and has been extensively described [92]. Six-well strips of flexible polystyrene substrates (bottom units), were coated with the different substrates (10 μg/ml). AGS cells were labeled with the vital fluorochrome calcein AM (Invitrogen) for 15 min at 37°C and then aliquoted into the bottom miniplates (50,000/well). The miniplates were centrifuged at 200 ×g for 5 min at 37°C to synchronize the contact of the cells with the substrate, incubated for 20 min at 37°C and subsequently mounted together with a similar miniplate (top unit) to create communicating chamber for a reverse centrifugation at 50 ×g. The relative number of cells bound to the substrate (i.e. remaining in the wells of the bottom miniplates) was estimated by top/bottom fluorescence detection in a computer-interfaced Infinite 1000 PRO microplate reader (Tecan Italia Srl).

RT-qPCR

Upon treatment with 5 μg/ml of recombinant EMILIN2 or PBS for 24 h, or, in alternative, with conditioned media from mock or EMILIN2-transfected E293 cells (EMILIN2 concentration ~5 μg/ml), total RNA was isolated from the cell lines with Trizol and reverse transcribed using AMV-RT (Promega, Milan, Italy). Semi-quantitative endpoint reactions were performed with GoTaq DNA polymerase (Promega, Milan, Italy) and Real-time PCRs with SYBR™ SYBR® Green Supermix (Bio-Rad, Milan, Italy). The oligonucleotide sequences were: MCP-1 forward: `CAGAAGTGAGGGTTCAGGATTCC`; reverse: `ATTCTTTGGTTGGAGTGAG`; TIMP-1 forward: `CCTGCACGTGTCGCCAC`; reverse: `TCTGGTTACCTTGGTGGTGC`; Angiogenin forward: `AGTCAATTTTTCTCGTGGATAC`; reverse: `AACAAGGATTCCAGTGACTGTC`; CXCL-16 forward: `CCATGAGTGTCGAATTT`; reverse: `GGGGGCTGTCGGTAAA`; TSP1 forward: `CTCCCCTATGCT`
ATCACAG', reverse: 'AGGAACCTGGGCATTGGAG'; VEGFA forward: 'AGTCAGACAACG'; reverse: 'TTCCCTTCTCTGGAACGTGATT'; SERPINE1 forward: 'CCAGCTGAAACAGGAGGAG'; reverse: 'CTCCTGTACAGATGCCGGA'; IGFBP2 forward: 'ACATCCAACATCACCATG'; reverse: 'TTCCCTTTCCTCGAACTGATTT'; SERPINE1 forward: 'CCAGCTGACAACAGGAGGAG'; reverse: 'CTCCTTGTACAGATGCCGGA'; IGFBP2 forward: 'ACATCCCCAACTGTGACAAG'; reverse: 'ATCAGCTTCCCGGTGTTG'; IL-8 forward: 'CATTGACCAAGGAAATCGGC'; reverse: 'CACAAGATAGTTACAGCCATACC'; GAPDH forward: 'GAGAGACCCTCACTGCTG'; reverse: 'GATGGTACATGACAAGGTGC'.

Cell transfection and immunoblotting

E293 cells were transfected using FuGene6 reagent (Promega, Milan, Italy). Cells transfected with the pCEP-Pu-EMILIN2 or the empty vector were then selected with 250 μg/ml G418 and 0.5 μg/ml puromycin. Confluent cells were then incubated in serum-free medium for 48 h and the conditioned medium collected. For Western immunoblotting, cells were lysed in cold RIPA buffer (150 mMNaCl, 10 mM Tris, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 5 mM EDTA) containing a protease inhibitor cocktail (Roche Diagnaostics S.p.a., Milan, Italy). Proteins were resolved in 4% to 20% Criterion Precast Gels (Bio-Rad, Milan, Italy) and transferred onto Hybond-ECL nitrocellulose membranes, blocked with 5% dry milk in TBS-T buffer, probed with the appropriate antibodies, and developed using enhanced chemiluminescence (Amersham, Milan, Italy) or the Odyssey Infrared Imaging System (Li-COR Biosciences, Lincoln, USA).

Impedance measurement

To quantitatively monitor cell behavior in real-time, we adopted the Real-Time Cell Analyzer dual plate instrument xCELLigence (Roche) which measures the electrical impedance caused by cell attachment and proliferation and expressed as the cell index, an arbitrary measurement defined as (Rn – Rb)/15, in which Rb is the background impedance of the well measured with medium alone, and Rn is the impedance of the well measured at any time (t) in the presence of cells. Thus, the cell index is a reflection of overall cell number, attachment quality, and cell morphology that change as a function of time. The Real-Time Cell Analyzer dual plate instrument was placed in a humidified incubator maintained at 37 °C 5% CO2. For adhesion experiments, the E-plates 96 were precoated over night at 4 °C with the indicated molecules (10 μg/ml), and cells were then seeded at 50,000 cells/well in FCS-free medium. Cells were monitored once every 5 min for 2 h. Data analysis was performed using Real-Time Cell Analyzer software (version 1.2) supplied with the instrument.

MTT and TUNEL assay

10⁴ AGS and MKN74 cells were plated in 96 well plates, let adhere and incubated with 5 μg/ml of recombinant EMILIN2 or PBS for 24 h. In alternative, cells were treated with conditioned media from mock or EMILIN-2-transfected E293 cells (EMILIN2 concentration ~5 μg/ml). Next, cells were incubated for 3 h with 5 mg/ml MTT and absorbance detected at 560 nm. The results were reported as % of cell proliferation. The apoptotic rate was determined using the In Situ Cell Death Detection Kit, Fluorescein (Merck KGaA, Darmstadt, Germany).

Statistical analyses

Statistical analyses were performed with the SigmaPlot software and the values represent the mean ± SD. The statistical significance of the differences was determined by the two-sided Student’s t-test for the comparisons between two groups. For all the evaluations reported in the manuscript the investigators were blinded. All the measurements were included for the statistical analyses and differences were considered statistically significant when P ≤ 0.05.

Author contributions

EA, AF, AC and EPo analysis of data, conducting and designing experiments, data interpretation, writing and editing of the manuscript. RC, MF, SM obtaining clinical samples. EPI conducting and designing experiments, editing of the manuscript. RP, AF conducting experiments. RD obtaining cryostat sections. MM and PS experimental supervision, data interpretation, editing of the manuscript. RVI critical revision and editing of the manuscript; MM writing of the manuscript.

Ethical approval

The collection of human materials was approved by the appropriate Institutional Board of the CRO-IRCCS of Aviano, Italy (IRB no. CRO-2014-03).

Declaration of competing interest

The authors declare no competing interest in the design or interpretation of the experiments, or in the writing of the manuscript.
Acknowledgements

We thank the Italian Association of Cancer Research (AIRC) (grant# IG-23643 to MM) and the Ministry of Health, Italy (grant# RF-2018-12365425 to MM and grant# RF-2016-02361525 to PS and RC) for funding this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mbreplus.2020.100029.

Received 21 December 2019; Received in revised form 13 February 2020; Accepted 13 February 2020
Available online 19 February 2020

Keywords:
Tumor microenvironment;
Extracellular matrix;
Angiogenesis;
Inflammation;
Gastric cancer

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Abbreviations used:
CAFCA, Centrifugal Assay for Fluorescence-based Cell Adhesion; CD31, cluster of differentiation 31 also known as PECAM-1; ECM, extracellular matrix; EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor receptor 2; EMILIN 2, Elastin Microfibril Interface 2; 5-FU, 5-fluorouracil; GC, gastric cancer; IGFBP2, insulin growth factor-binding protein 2; PFS, progression free survival; Serpin 1, serine protease inhibitor 1; VEGFA, vascular endothelial growth factor A.

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