A functional polymorphism within *NUP210* encoding for nucleoporin GP210 is associated with the risk of endometriosis

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

CAPSULE

Rs354476 polymorphism affects the regulation of *NUP210* by altering the binding with hsa-miR-125b-5p, this provides the rationale for the observed increased risk of endometriosis in carriers of the variant allele.

ABSTRACT

OBJECTIVE:
To investigate whether nucleoporin 210 (GP210, encoded by *NUP210* gene) is involved in endometriosis.

DESIGN:
(i) Immunohistofluorescence analysis for assessing whether GP210 is expressed in endometrial tissues from patients and controls; (ii) genotyping and case-control study for assessing the association between rs354476 within *NUP210* and risk of endometriosis; (iii) *in vitro* luciferase assay for assessing the functional activity of rs354476.

SETTING:
University.

PATIENT(S):
Histologically diagnosed cases (n=175) of endometriosis: minimal or mild (stage I-II) in 48 cases (28%), moderate (Stage III) in 69 cases (39%) and severe (Stage IV) in 58 cases (33%). Controls (n=557) were female blood donors collected at Meyer Hospital of Florence.

INTERVENTION(S):
None.

MAIN OUTCOME MEASURE(S):
GP210 tissue expression; genotype distribution and risk of endometriosis; *in vitro* gene expression measurements.

RESULT(S):
(i) GP210 had positive nuclear immunohistofluorescence staining in endometrial glandular epithelium;(ii) Carriers of the variant allele were associated with increased risks: C/T, OR=1.83, 95%CI=1.04-3.21; T/T, OR=2.55, 95%CI=1.36-4.80; (ii) *in vitro*, luciferase assay showed that rs354476 is a *bona fide* target for hsa-miR-125b-5p.

CONCLUSION(S):
Nucleoporin GP210 is involved in endometriosis. Rs354476 polymorphism affects the regulation of *NUP210* gene expression by altering the binding with hsa-miR-125b-5p, a miRNA already known as playing an important role for endometriosis. This provides the rationale for the observed increased risk of endometriosis in carriers of the variant allele.

Keywords: Polymorphism **/** Endometrium /glycoprotein-210 / *NUP210* /miRNA binding sites

INTRODUCTION

Endometriosis is a common inflammatory disease with endometrium-like tissues ectopically located outside the uterus. This condition is heterogeneous with various manifestations that include superficial and peritoneal lesions, endometriosis cysts in the ovaries (endometrioma, OMA), and nodules (>5 mm) in the deep infiltrating endometriosis (DIE) (1,2). The pathogenesis of endometriosis is unclear and the research supports two main theories. In the "retrograde menstruation", viable endometrial cells invade other tissues (e.g. ovaries) through the fallopian tubes. The Müllerian-remnant theory hypothesizes that DIE represents adenomyosis originated in the retroperitoneum from embryonic rests of the Müllerian duct or extension of adenomyotic nodules arising in the myometrium. There is also an additional "iatrogenic theory" suggesting that a previous pelvic surgery is a risk factor for disseminating endometrial cells outside the uterus (3). The predisposition to this condition has, likely, multifactorial bases (4,5). Several risk factors have been identified, such as an early age at menarche (<12 years) and a short menstrual cycle (<26 days), perhaps contributing to enhance the retrograde menstruation phenomenon. The exposure to polychlorinated biphenyl and dioxin is debated as these agents could play a role through the disruption of circulating hormone levels and/or dysregulation of the immune system. An inverse correlation between the risk of endometriosis and the body mass index was also reported (6). However, pioneer studies observed that there is aggregation of endometriosis cases within families. In fact, the first-degree relatives of affected women showed a risk of about 6-fold higher that the first-degree relatives of unaffected ones, suggesting an important role of the genetic background. This observation was corroborated by studies on twins that reported a hereditability coefficient of about 50% (7). When the first genetic analyses were attempted by carrying out linkage analysis in families, the results showed weak but positive signals within the chromosomal regions 10q26 and 7p13-15, suggesting that the disease is multigenic (8). More recently, genome-wide association studies (GWAS) and meta-analyses helped to unravel part of this genetic complexity. These studies detected increased risk of endometriosis associated with specific alleles of common single nucleotide polymorphisms (SNPs).

It is hypothesized that the associations could be driven by the genotyped SNP, or by others in elevated linkage disequilibrium (LD), because they could affect the function of the gene to which they belong. These studies are very helpful to spot specific genes and can contribute to generate hypotheses on the etiology of the disease. Thus, a role of sex steroid hormones was suggested by positive associations between the risk of endometriosis and intronic variants within *ESR1* (rs1971256), *FSHB* (rs74485684), and *GREB1* (rs11674184) genes. The regulation of embryogenesis and cell fate was hypothesized given the positive signals found upstream *HOXA10* (rs12700667), or within *WNT4* (rs12037376), *FN1* (rs1250241), and *ID4* (rs760794). Other signals were also suggestive for a role of inflammatory cytokines (*IL1A*, rs10167914), cell cycle regulators (*CDKN2B-AS1*, rs1537377; *ETAA1*, rs6546324) or angiogenesis (*KDR*, rs1903068) (9-11).

Overall, these genetic polymorphisms explain only a fraction (up to 5.19%) of the total variance, clearly indicating that more studies need to be undertaken. Recent evidences suggested that single nucleotide polymorphisms (SNPs) within microRNA (miRNA) binding sites (miRSNPs) could play a role. These SNPs weaken or strengthen the binding between mRNAs and their targeting miRNAs (12) affecting protein translation or mRNA half-life. Thus, their effects on gene expression could provide a rational base for the observed associations between specific miRSNPs genotypes and the risk of developing various human diseases, including (but not limited to) cardiovascular diseases (13,14), cancer (15-21), and, as said, endometriosis. MiRSNPs associated with endometriosis include: (i) rs14647 within *WHSC1* (22), (ii) rs3813486, rs1127473, rs3211066 within *SLC22A23* (22), (iii) rs7201 within *MMP2* (23), (iv) rs334348 within *TGFBR1* (24) and (v) rs1434536 within *BMPR1B* (25), affecting the binding sites of hsa-miR-99b, hsa-miR-125a-3p, hsa-miR-520g, hsa-miR-628–5p, and hsa-miR-125b-5p, respectively. In particular, this latter miRNA was found repeatedly involved in endometriosis. A previous study showed that hsa-miR-125b-5p was up-regulated (up to 10-fold) in serum of women affected by endometriosis as compared to healthy women, suggesting that it could be an independent diagnostic biomarker (26,27). This miRNA was also associated with endometrial receptivity in hyper-stimulated women undergoing to *in vitro* fertilization and embryo transfer (28).

Moreover, it was involved in endometrial dysfunctions as well as cancer (28-32). Furthermore, at least 19 out of the over 900 predicted targets for hsa-miR-125b-5p (Targetscan, freely available at the URL: http://www.targetscan.org) were involved in endometriosis, as reported in scientific literature (see supplementary table 1). All these findings support the notion that hsa-miR-125b-5p is one of the most important miRNAs involved in endometriosis and they strongly suggest that the identification of its targets could help in elucidating the pathogenic mechanisms. In this context, the study of miRSNPs involving hsa-miR-125b-5p could constitute a useful strategy to this aim. A previous work carried out by our group showed that the expression of a fluorescent reporter gene chimerized with the 3'-UTR (untranslated region) of *NUP210* bearing the T-rs354476 was reduced when compared to the C-rs354476 counterpart. Moreover, *in silico* predictions suggested that the polymorphism could be a target site for hsa-miR-125b-5p. In summary, rs354476 within the 3'-UTR of *NUP210* is strong candidate miRSNP for hsa-miR-125b-5p suggesting that *NUP210* should be further investigated in the context of endometriosis (15). *NUP210* maps at 3p25.1 and encodes the nucleoporin GP210 (alias the nuclear pore glycoprotein-210). Nucleoporins are involved in the structural organization of the so-called "nucleolar channel system" (NCS). Described since 1961 (33), the NCS consists of several layers of tubular membrane cisternae within the nuclei of cells constituting the normal endometrium glandular epithelium (EGE) (34). This organelle appears in the mid-secretory phase of the menstrual cycle when the endometrium is receptive to implantation of the fertilized egg (35,36) and it can be observed prematurely when women undergo to ovarian hyper-stimulation (37). Its absence or its delayed development characterized several cases of unexplained primary infertility (38-40) and it is considered as a major hallmark of the postovulatory endometrium (41). Thus, it is not surprising to observe that nucleoporins were variably involved in several gynecological disorders. For example, endometrium of women affected by recurrent miscarriages showed over-expression of *NUP210* in a specific time-window as compared to healthy controls (42). In summary, because rs354476 is a putative miRSNP of nucleoporin GP210 for miR-125b-5p, it could represent an ideal candidate to be studied in relation to endometriosis. Thus, in the present work, we verified and showed for the first

time that GP210 is expressed in the normal EGE. Then, we explored the association of rs354476-*NUP210* with the risk of endometriosis and we assayed whether rs354476 could be a *bona fide* miRSNP for miR-125b-5p.

MATERIALS AND METHODS

Patients

The study was carried out in consecutive women who attended the endoscopic surgical services of the Obstetrics and Gynaecology Unit of the San Raffaele Scientific Institute and of the Department of Obstetrics and Gynaecology of the University of Siena between December 2009 and December 2010. All the patients underwent complete pre-surgery clinical examination before the diagnostic-operative laparoscopy. Indications to laparoscopy included chronic pelvic pain, infertility, ovarian cysts and myomas. All patients were regularly menstruating, without desire of pregnancy. None was taking medications except sporadic nonsteroidal anti-inflammatory drugs. Patients with dysfunctional uterine bleeding, endometrial pathology or autoimmune disease were excluded from the study. Three physicians highly skilled in the evaluation and treatment of endometriosis staged all patients according to the revised American Society for Reproductive Medicine classification (43). Endometriosis was documented in 175 women. Stage of the disease was found to be minimal or mild (stage I-II) in 48 cases (28%), moderate (Stage III) in 69 cases (39%) and severe (Stage IV) in 58 cases (33%). Laparoscopies were performed by experienced surgical teams and diagnoses were always confirmed by standard histological examination of biopsies from representative endometriotic lesions. The endometrial specimens presented here were withdrawn from consecutive women presenting at the clinical practice in the period December 2009 and December 2010. They were classified as proliferative or secretory according to the last menstrual period and confirmed by ultrasound scans by employing a transvaginal probe at 4.5 to 7.0 MHz, and by histological examination. Diagnosis of superficial peritoneal lesions of endometriosis was based on direct visualization when endometriotic implants presented as typical lesions; diagnosis of ovarian

endometrioma was defined by the occurrence of at least one ovarian cyst lined by endometriotic tissue; diagnosis of deep infiltrating endometriosis was defined when lesions infiltrated to a depth of at least 5 mm, beneath the peritoneal surface, in at least one of the subsequent sites: *a)* the bladder muscularis propria; *b)* the anterior rectovaginal pouch, posterior vaginal fornix or retroperitoneal area between the anterior rectovaginal pouch and the posterior vaginal fornix; *c)* the uterosacral ligament; *d)* the bowel muscularis propria (44).

Immunohistofluorescence

For immunohistofluorescence localizations of GP210 protein we analyzed: healthy endometrial tissues from 4 volunteers (2 women were in proliferative, 2 in secretory phase), OMA lesions from 4 patients plus their endometrial eutopic counterparts (2 patients were in the proliferative phase, 2 in secretory phase), and DIE lesions from 4 patients with their eutopic endometrial counterparts (2 patients were in proliferative phase and 2 in secretory phase).

Tissues were fixed in 10% neutral buffered formalin at room temperature (RT) for 24 h., paraffin-embedded, sectioned and mounted on Superfrost Plus microscope slides (Fisher Scientific, Ottawa, ON, Canada). After deparaffinization with xylene and dehydration with ethanol, sections were treated with PT Module buffer 2 (Thermo Scientific) - EDTA, pH 8. Samples were then incubated in 5% BSA (Bovine serum albumin) in PBS (phosphate buffered saline) for 30 min and finally incubated overnight at 4°C with the rabbit anti-NUP210 polyclonal antibody (Novus Biologicals) (1:50 in PBS-1% BSA) or polyclonal rabbit IgG (Novus Biologicals) as negative control. Glass slides were then washed in PBS and incubated for 1 h at RT with FITC- labeled goat anti-rabbit IgG antibody (1:100) (SIGMA). After washing in PBS, the sections were stained with 6-diamino-2-phenylindole (DAPI) for nuclei counterstaining, in mounting medium (Santa Cruz Biotechnology) and then observed with a Leica DMB 6000 microscope (Leica, Wetzlar, Germany). TIFF Images were captured with a CFTR 6500 digital camera (Leica, Wetzlar, Germany) and the levels of the intensities were quantified on the green channel with the software ImageJ 1.52 (Rasband, W.S., ImageJ, U. S. National Institutes of

Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2018.). The level of the background in our samples was evaluated by using the same isotype antibody at the same protein concentration as the primary antibody followed by the secondary antibody, as usual. The background was subtracted and the net fluorescence was expressed as arbitrary units.

Case-control association study

The polymorphism rs354476 was evaluated in a case-control association study. Controls were female blood donors collected at Meyer Hospital of Florence, with a minimal age of 18 years whose details were published previously (45). According to the Helsinki declaration, both healthy and affected volunteers gave their written informed consent to participate in the study and the study protocol was cleared by the local Ethical Committee (Azienda Ospedaliero-Universitaria Pisana, Comitato Etico Area Vasta Nord-Ovest, CEAVNO; Azienda Ospedaliera Universitaria Senese, Comitato Etico Area Vasta Sud_Est, CEASVE).

DNA extraction and genotyping

The genomic DNA was isolated from peripheral blood using the Puregene Blood Kit (Gentra Systems, Inc., Minneapolis, MN). The quantitation was performed with the use of Qubit fluorimeter (Life Technologies Inc., Grand Island, New York, USA), (Quant-iT™ dsDNA HS Assay Kit, Life Technologies, USA). Thirteen blood samples from patients did not yield a DNA of quality for genotyping (<0.1ng/µL), thus the genotypes are available for 162 patients and 557 controls. Genotyping of rs354476 was carried out using Pre-design TaqMan® SNP Genotyping Assays, assay id#: C___8755527_10 (Life Technologies Inc., Grand Island, New York, USA). The genotyping of fifteen samples (2%) was repeated blindly as quality control. The results showed complete concordance between replicates.

In silico predictions

According to our previous work, hsa-miR-125b-5p had a differential binding with *NUP210*-3'-UTR according to the genotype of rs354476, whereas the closely related hsa-miR-125a-5p (belonging to the same family) could bind to NUP210-3'-UTR but it is not affected by the SNP (10). The prediction

was repeated, here, by using "RNA-cofold" (freely available at the URL: http://rna.tbi.univie.ac.at/cgi-bin/RNAcofold.cgi) (46) to assess the binding free energies between *NUP210*-mRNA-C-allele or *NUP210*-mRNA-T-allele and either hsa-miR-125b-5p or hsa-miR-125a-5p.

Vectors employed and in vitro assays

We cloned the 3'-UTR regions of *NUP210* carrying alternatively, C-rs354476 or T-rs354476, in the vector pmiRGLO. Thus, the luciferase from Photinus *pyralis* (Firefly luciferase), with the reporter gene Fluc was chimerized with the *NUP210*-3'-UTR. The vector express Renilla *reniformis* (Renilla luciferase), Rluc reporter as internal standard. Cells were transiently transfected with pmiRGLO chimeric construct, and with the synthetic hsa-miR-125b-5p, or alternatively with the hsa-miR-125a-5p, or (QIAGEN Spa, Italy) or with the "All Stars Negative Control siRNA", both used as negative controls. Each experimental point was repeated six times and each experiment was repeated three times. Forty-eight hours following transfection, cells were lysed and the activity of Fluc and Rluc was measured, using dual-luciferase reporter assay kit and a luminometer (Berthold Technologies, Germany). For both reporter genes, the ratio of the luminescence values was calculated as Fluc /Rluc luminescence ratio (FRLR) in cells treated with the miRNA of interest (FRLR–miRNA) or treated with the NC (FRLR-NC). The final measure of relative activity was expressed with the parameter R-NORM, calculated as the ratio FRLR-miRNA/FRLR-NC, and this parameter was used for the statistical analyses. All the technical details of cloning and luminescence measurements are reported in supplementary materials.

Statistical analyses

The statistical power of the case-control association study was calculated with the tool "PS—power and sample size calculation," (freely available at the URL:http://biostat.mc.vanderbilt.edu/PowerSampleSize) (47) and it showed that the sample size of

cases and controls was large enough to provide a power of 80% to detect an Odd Ratios (OR) of 1.86 and 92% to detect an OR of 2.04 in a dominant model (with Type-I error=0.01).

To verify whether genotypes followed the Hardy and Weinberg equilibrium (HWE), we applied the chi-square test (1 degree of freedom) on the genotype counts of whole sample set. We used a multivariate logistic regression analysis (MLR) to examine the associations between the genotypes and the risk of cancer. The association analyses were based on the estimation of the ORs and their 95% confidence intervals (CIs). In MLR, the ORs were adjusted for covariates as linear variables (OR_{adj}). Each genotype category was compared using the common homozygotes as reference category and the P-value of the association (P_{ass}) was calculated separately for heterozygotes and homozygotes, in an additive model. Moreover, the most likely mode of inheritance was evaluated by performing an extended MAX test (48), which is based on multiplicity-adjusted P-values for the Cochran-Armitage trend test (CATT) statistics (49) of the dominant, additive, and recessive models. When the MAX test showed that the dominant or the recessive model was significant, then we performed the MLR analysis for that model. R-NORM was calculated by averaging the results obtained from three independent experiments, and each experiment was carried out using twelve independent replicates. R-NORM values were compared between genotypes using the multifactor analysis of variance (MANOVA). All the statistical tests were two-tailed and carried out using Statgraphics Centurion software (StatPoint Inc., USA).

RESULTS

According to previous results, *NUP210*- 3'-UTR is a strong candidate target for hsa-miR-125b-5p. Here, we evaluated further whether the gene could be a *bona fide* target for this miRNA by analyzing the data within the TCGA database (freely available at the URL: https://portal.gdc.cancer.gov/). We found that increased expression of hsa-miR-125b-5p correlates with reduced levels of *NUP210* mRNA in a series of 281 specimens from corpus uteri. This negative correlation (depicted in Supplemental figure 1) is highly statistically significant (P=0.004) and it shows that the inter-individual variations of hsa-miR-125b-5p explain, alone, almost the 3% (r^2=2.58) of the total inter-

individual variability of *NUP210* mRNA. This result is in agreement with the fact that miRNAs could act as negative regulators of gene expression also by leading to reduced levels of their targeting mRNAs. Thus, the hypothesis that *NUP210* is a target for hsa-miR-125b-5p has been further corroborated by this analysis.

Then, we proceeded by assessing whether GP210 is actually expressed within EGE. We evaluated, by immunohistofluorescence staining, the expression of GP210 in healthy and eutopic endometrium as well as in ectopic lesions. Representative pictures are reported in figure 1 and the corresponding fluorescence values (for the green channel) measured in the glandular epithelium and in the stroma are graphed in supplemental figure 2. In the proliferative phase of the healthy endometrium, GP210 staining was markedly present in the EGE (Fig. 1A). The signal was positive within the cytoplasm of epithelial cells, mostly on the luminal side of the glands. Here we could measure an average of 112.5 units of fluorescence (standard error of the average, SE=6.39). Stromal cells, surrounding the glands, presented a poor expression, limited to small areas or spots around the nuclei where we measured an average of 39 units (SE=5.12; $P<0.001$). In the secretory phase (Fig. 1B) the signal showed the same distribution but with an overall reduced intensity. In the cytoplasm of EGE cells the reduction was statistically significant (average=57.3 units, SE=4.01; $P<0.001$), whereas in the stroma the reduction was of a lesser extent (av=24.96 units, SE=9.35; P=0.08). Proliferative endometrium from patients suffering of endometriosis did not show specific differences as compared to EGE from healthy women. In fact, the pattern and the intensities of GP210 staining were remarkably similar in both the secretory (Fig. 1C) and proliferative (Fig. 1D) phases with non-statistically significant differences when compared to their healthy counterparts (in the cytoplasm of EGE cells: av=92.6 units, SE=5.6 and av=55.8 units, SE=20.7, respectively; in the stroma: av=39.5 units, SE=3.16, and av=31.8 units, SE=10.4, respectively). When compared to healthy endometrium, OMA (Fig. 1E), showed a less organized EGE with a slightly reduced GP210 cytoplasmatic fluorescence (av=76 units, SE=14.1; $P<0.001$), and similar intensity in the stroma (av=37.2 units, SE=11.2; P=0.09). The most obvious difference with the eutopic endometrium was found for the peritoneal DIE, where poorly organized

EGE structures could be detected but lacking any cytoplasmic expression of GP210. The immunostaining of GP210 was low in the stroma (av=10.3 units, SE=2.83) (Fig. 1F).

In silico predictions and our previous results suggested that rs354476, within the NUP210-3'-UTR, could affect the binding site for the hsa-miR-125b-5p. Here we replicated the calculations using RNA-cofold and confirmed that this latter miRNA had higher binding free energy for T (-10.84 kcal/mol) as compared to C (-10.03 kcal/mol) allele ($\Delta\Delta G$=0.81 kcal/mol). On the same time, hsa-miR125a-5p is predicted to bind the *NUP210*-3'-UTR but it shows similar binding free energies for both alleles (-13.32 kcal/mol for C- vs -13.27 kcal/mol for T-allele), thus its use as internal control for the *in vitro* experiments is warranted.

The miRSNP rs354476 was then evaluated in relation to the risk to develop endometriosis in a case-control association study. In the present study cases were slightly younger than controls (32.5±5.46 given as group average ± standard deviation, and 46.14±12.08, respectively), the genotype frequencies among controls followed the HWE, and the allele frequencies were in agreement with those described for the same population, Tuscans (i.e. TSI, Tuscany) within dbSNP (freely available at the URL: http://www.ncbi.nlm.nih.gov/SNP/ and in the database of 1000Genomes at the URL:http://www.internationalgenome.org/). Following MLR, the heterozygotes (C/T) and homozygotes (T/T) showed a statistically significant association with the risk of endometriosis (ORadj=1.83, 95% CI=1.04-3.21; P=0.035 and ORadj=2.55, 95% CI=1.36-4.80; P=0.0036, respectively). Demography and results are reported in table 1. When looking at the different clinical stages, increased risks were observed in all grading groups for TT genotype, with a highly statistical significance for patients at stage IV (table 2). In this stage almost all volunteers (50 out of 54) were carriers of the T-allele and their risk was particularly increased (dominant model; ORadj = 6.58, 95% CI=1.94-22.30; P=0.002). No differences in the average age groups were noticed according with the staging (table 2).

To further understand the functional activity of the C/T variation we performed *in vitro* experiments by employing chimeric vectors co-transfected with miRNAs. We tested hsa-miR-125b-5p for its

differential binding with T or C alleles and used hsa-miR-125a-5p as reference miRNA not having such an allelic-discriminating activity. Moreover, a random sequence miRNA, the known "All Stars Negative Control siRNA", was also employed as further negative control. Thus, we measured the normalized expression level (R-NORM) of the gene reporter in a dual luciferase assay in HCT116 cells following co-transfections with miRNAs and chimerized vectors. The results are represented in figure 2. The activity is expressed as percent compared to the results obtained after the co-transfection of miRNA having the random sequence (referred as 100%). We found that, as expected, hsa-miR-125b-5p and hsa-miR-125a-5p caused a reduced expression of the reporter gene (MANOVA, $P=3.7x10^{-3}$ and $P=2x10^{-4}$, respectively). However, according to the *in silico* predictions, the two alleles responded differently to the different miRNAs (MANOVA with two-way interaction, P=0.0173). In fact, following the co-transfection with hsa-miR-125b-5p, the fluorescence of the C-allele was similar (97.3%) to that of the miRNA having random sequence, whereas that measured for T-allele had a statistically significant reduction of almost 20%. On the contrary, following the co-transfection with hsa-miR-125a-5p, the fluorescence of the reporter gene chimerized with the 3'-UTR carrying the C-allele was reduced of a similar extent (about 17%) of that measured for the T-allele (about 16%).

DISCUSSION

Present study strongly suggested that *NUP210* mRNA is a *bona fide* target for hsa-miR-125b-5p, a key-miRNA involved in endometriosis, and that rs354476 affects their binding behaving as an actual miRSNP. These hypotheses are supported by previously published findings and by the inverse correlation between the expression of *NUP210* mRNA and hsa-miR-125b-5p in the TCGA dataset. A further support was provided by our *in vitro* assay carried out with the use of the synthetic miRNAs and chimeric constructs. This study also showed that GP210 is expressed within EGE and this result is corroborated also by those reported within "The Human Protein Atlas Portal" (available at the URL: http:// www.proteinatlas.org). Healthy EGE and eutopic endometriosis did not show particular

differences each other, whereas a reduced staining was noticed in the OMA and a lack of staining was found in the peritoneal DIE. In the stroma the expression was always very poor.

In agreement with the hypotheses of a role for hsa-miR-125b and *NUP210* in endometriosis, the case-control association study showed that the miRSNP rs354476 is associated with increased risks, particularly strong for the more sever forms. We are aware that the association study could present some limitations. For example, cases and controls did not match for age. However, healthy controls were older than cases, and endometriosis is often underdiagnosed (with a 6.7-year mean latency from onset of symptoms to definitive diagnosis) (50). Thus, this fact reassured about potential occult endometrioses among controls at the corresponding age of patients. In any case, the results were adjusted for this covariate. Another limitation could have been represented by the sample size, however the statistical power of the study was adequate (80%) to detect OR>1.86.

On the other hand, the study presents also several strength points. Firstly, a positive association was found in a genetic locus close to *NUP210* following a large meta-analysis of previous GWASs, suggesting that this genomic region could be a hot spot for endometriosis susceptibility. Moreover, a previous GWAS reported that rs6806012, a SNP in very close LD with rs354476, was associated with the risk of endometriosis, confirming present findings (51). In addition, the allele frequencies measured in this study are corroborated by data from genomic databases for Tuscans. In fact, the series of controls analyzed here shows a T-allele frequency similar to that found in 1000Genomes for this population. Endometriosis patients show a higher allele frequency, therefore if we pooled together Tuscans and present controls the statistical difference versus endometriosis would be even stronger. Furthermore, the epidemiologic data are reinforced by the *in vitro* assay: the co-transfection of hsa-miR-125b-5p caused a reduction of the expression of the reporter gene chimerized with rs354476 T-allele, whereas, the C-allele was not affected (the expression of the reporter gene was similar to that of the negative control). On the other hand, the co-transfection with hsa-miR-125a-5p was able to reduce the expression of the reporter gene of a similar extent for both alleles. Thus, putting together previous and present results, we have reasons to believe that the association is unlikely to be

a chance finding. In summary, we suggest that miRSNP rs354476 could affect the regulation of *NUP210* expression, thereby constituting a small but meaningful risk factor for this complex disease. Mechanistically, one could speculate that the hsa-miR-125b-5p has a poor affinity for allele C-rs354476 causing increased expression of GP210. On the contrary, hsa-miR-125b-5p has a higher affinity towards the corresponding T-allele, leading to a reduced expression of GP210. If this hypothesis were correct it would mean that GP210 is not only a marker of healthy EGE but, more importantly, it could be causally involved in the maintenance of its healthy status. Specific studies on the role of nucleoporins in the endometriosis are lacking. However, several works reported a relationship between endometrial carcinoma and *NUP88*. An increased expression of this protein was associated with pre-malignant endometrial lesions, early-stage endometrial cancer, and myometrial invasion of endometrial carcinoma (52,53). Nucleoporins have been widely documented in the context of cell differentiation and embryogenesis. Already in 2010, a role for *NUP98* in the embryonic development of zebrafish was showed (54). In 2012, *NUP210* was clearly identified to play a key-role in determining the fate cell during embryogenesis (55). Moreover, it was showed that the expression of nucleoporins is localized to specific embryonic anatomical structures and showed specific pattern of expression during specific embryogenesis stages in Xenopus *laevis* (56) and in mouse (57). Furthermore, a specific role of *NUP153* in the early stages of embryogenesis was showed in rabbit (58) and in cow (59). In particular, a role in the development of primordial germ cells was showed for *NUP50* (60). Finally, nucleoporins are gaining increasing attention also for their role in the cellular physiology of reproductive system in humans (61).

Thus, present data seem to fit better with the Müllerian-remnant theory suggesting that endometriosis could be generated by slight defects in the regulations of the cell fate in women, during the embryogenesis. More studies are warranted to deep the analysis on nucleoporins in the context of endometriosis.

ACKNOWLEDGEMENTS

This work was in part sustained by AIRC (Associazione Italiana Ricerca Cancro, investigator grant year 2008) and by the Istituto Toscano Tumori, grant system 2010, and Istituto Toscano Tumori (Italy) (grant n. I56D15000010002).

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LEGENDS

Figure 1. Representative images of immunohistofluorescence localization of GP210 (green) in healthy (A,B) and in eutopic endometrium from endometriotic patients (C,D), in OMA (E) and in DIE (F) ectopic lesions. Nuclei were counterstained with DAPI (blue). Bar represents 25μm.

Figure 2. R-NORM of NCV compared to cells treated with a control miRNA (posed at 100%). Each bar shows the average and the SD of six independent replicates. Results are graphed according to the 3'-UTR genotype of the chimeric vector. Statistical analysis was performed with a multifactor analysis of variance model. Asterisk denotes a statistically significant difference between groups (P-value<0.05). The HCT116 cell line showed a decreased expression of the reporter gene following the treatment with both miR-125a and miR-125b-5p, in particular in presence of allele T.

Supplemental figure 1. Regression analysis between the expression of hsa-miR-125b-5p and *NUP210* mRNA according to the TCGA dataset (available at the URL: https://portal.gdc.cancer.gov/) following total RNA sequencing (with a next-generation sequencing approach) and expressed as FPKM, (i.e. Fragments PerKilobase Million). The analysis is performed on 281 samples from corpus uteri.

Supplemental figure 2. Graphic showing the measurements obtained with ImageJ on the TIFF images from the immunohistofluorescence analysis. White bars are the

values (in arbitrary units on the green channel) taken from endometrial glandular epithelium (EGE), dotted bars are the values taken from the surrounding stroma. The samples analyzed are reported in the barchart and, in the order, represent: healthy endometrium in the proliferative phase (n=2), healthy endometrium in the secretory phase (n=2), eutopic endometrium in the proliferative phase from patients with OMA (n=2) or DIE (n=2), eutopic endometrium in the secretory phase from patients with OMA (n=2) or DIE (n=2), and the corresponding lesions OMA (n=4), and DIE (n=4).

Table 1. Case-control association study for rs354476. The difference of age means was compared with the student's *t*-test. The adjusted ORs and their 95% confidence intervals (CI) were calculated with multiple logistic regression (MLR) analysis.

	Controls	**Cases**	**OR[a] (95% CI)**	***P*-value**
Age (years)				
Mean ±SD	46.14±12.08.0	32.53±5.46		$<10^{-4}$
Allele frequency rs354476-T	0.482 (537/1114)	0.514 (180/350)	**1.34 (1.05-1.72)**	**0.0198**
Genotypes				
C/C	148	30	Ref	
C/T	281	84	**1.83 (1.04-3.21)**	**0.035**
T/T	128	48	**2.55 (1.36-4.80)**	**0.0036**
Best model	Additive	**P-trend=0.019**		

[a] adjusted for covariates

Table 2. Characteristics of patients affected by endometriosis and association analysis (with MLR) between genotypes of rs354476 and risk of endometriosis, stratified for grade of the disease.

	Controls	**Stages I-II**			**Stage III**			**StageIV**		
		Age: 32.5±5.9			Age:32.0±5.2			Age:33.1±5.5		
Genotypes	**n**	**n**	**OR[a] (95% CI)**	***P*-value**	**n**	**OR[a] (95% CI)**	***P*-value**	**n**	**OR[a] (95% CI)**	***P*-value**
C/C	148	8	Ref		18	Ref		4	Ref	
C/T	281	23	2.30 (0.86-6.13)	0.10	26	0.80(0.38-1.66)	0.55	36	**6.86(1.99-23.64)**	**0.002**
T/T	128	12	2.91 (0.98-8.66)	0.06	22	1.93 (0.88-4.21)	0.10	14	**5.90(1.55-22.49)**	**0.009**
Total	557	43			66			54		

[a]adjusted for age

Supplementary material

Vectors employed and in vitro assays

We PCR-amplified both the common and variant 3'-UTR regions of *NUP210* carrying the rs354476. The amplification was done using two primers having a sequence of six bases to their 5' ends, recognized by the restriction enzyme SacI -->gagctc (forward primer: GAGCTCGCCGCGTGAAGGTTCCCGGA) and XhoI -->ctcgag (reverse primer: CTCGAGAAAAAATCACATAGAACTTTATTAA). The PCR product (of 1456 base pairs) was cloned in the pUC57 vector. Successively, the plasmid was cleaved with SacI and XhoI and the insert was cloned downstream a vector, the pmiRGLO vector (Promega, USA), containing the luciferases from Photinus *pyralis* (Firefly luciferase, Fluc) and from Renilla*reniformis* (Renilla luciferase, Rluc), as reporter gene. The pmiRGLO vector, carrying both Fluc and Rluc, was chimerized placing the 3'-UTRs of interest at the 3'-end of the Fluc reporter gene (being Rluc the internal reference). HCT116 cell lines were transfected with a pmiRGLO chimeric vector (NUP chimeric vector, NCV) having the elements in the following order: 1) Fluc; 2) NUP210-3'-UTR; 3) Rluc. HCT116 were plated at a density of about $4x10^4$ cells per well in a 24-well plate and incubated overnight at 5% CO_2, 37°C in a humidified incubator. Cells were transiently transfected with 1.5µl of Attractene® Transfection Reagent (QIAGEN Spa, Italy), with 0.4µg of pmiRGLO chimeric construct, and with 5 nM of the synthetichsa-miR-125b-5p, or alternatively with 5 nMof hsa-miR-125a-5p, or (QIAGEN Spa, Italy) or with the "All Stars Negative Control siRNA", both used as negative controls. Each experimental point was repeated six times and each experiment was repeated three times. Forty-eight hours following transfection, cells were lysed with 100 µl of 1x Passive Lysis Buffer (PLB) (dual-luciferase reporter assay kit, Promega, USA) after washing with PBS. Supernatants were used for the measure of activity of Fluc and Rluc, using dual-luciferase reporter assay kit and a luminometer (Berthold Technologies, Germany). The Fluc reporter was measured by adding Luciferase Assay Reagent II (LARII). After the measurement of Fluc, the reaction was quenched and Rluc reaction was

simultaneously initiated by adding Stop &Glo Reagent (dual-luciferase reporter assay kit, Promega,USA). For both reporter genes, the measurements of the luminescence values of non-transfected cells (background) were subtracted from the values measured in cells transfected with the pmiRGLO vector (the net luminescence value, NLV). The Fluc NLV was divided by the Rluc NLV (i.e. the Rluc NLV was used as reference). Then, the Fluc NLV/Rluc NLV luminescence ratio (FRLR) was calculated in cells treated with the miRNA of interest (FRLR–miRNA) or treated with the NC (FRLR-NC). The final measure of relative activity was expressed with the parameter R-NORM, calculated as the ratio FRLR-miRNA/FRLR-NC, and this parameter was used for the statistical analyses.