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Altered lamin A expression as a possible prognostic biomarker in endometrioid endometrial cancers

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ABSTRACT

Endometrial cancer (EC) is a major cause of mortality for patients worldwide. EC is classified as type I, also called the endometrioid type (EEC), or type II based on histologic properties. Although most cases of low grade EECs do not behave aggressively, in rare instances, even low-grade, well-differentiated EECs can progress in a highly aggressive manner, and the prognosis for recurrent or metastatic EEC remains poor. In this study, we performed a retrospective cohort of several formalin-fixed, paraffin-embedded (FFPE) specimens from patients with EEC to find novel clinical and biological features to help the diagnosis and consequently the treatment EEC. Total RNA and proteins were extracted and analyzed, respectively, by quantitative PCR and western blotting. We found that alteration of lamin A levels is associated with EEC development, thus indicating its possible role as novel potential biomarker. Interestingly, loss of lamin A was consistently associated with lower estrogen receptor (ERs) expression in low grade EEC, whereas in higher grade it was significantly related with E-cadherin mRNA (CDH1) reduced levels. Our data strongly indicate lamin A as a novel predictive biomarker of aggressiveness with a potential for a more systematic integration in clinical practice for individualized therapy in EEC.

Keywords: Endometrioid Endometrial Cancer, Estrogen Receptor, Lamin A, E-Cadherin.

SOMMARIO

Il cancro dell'endometrio è una delle principali cause di mortalità per i pazienti in tutto. E' classificato, in base alle caratteristiche istologiche, in due tipi, il tipo I, chiamato anche tipo endometrioide (EEC), e il tipo II. Sebbene la maggior parte dei casi di basso grado non si comportino in modo aggressivo, in rari casi, anche i tumori dell'endometrio di basso grado e ben differenziato possono progredire in modo molto aggressivo e la prognosi in caso di recidiva o metastasi a distanza rimane infausta.

In questo studio, abbiamo condotto una analisi retrospettiva su diversi tessuti inclusi in paraffina (FFPE) da pazienti con tumore di tipo endometrioide (EEC) allo scopo di identificare nuovi markers per aiutare la diagnosi e di conseguenza il trattamento dei tumori dell'endometrio. In questo studio L'RNA e le proteine estratti sono stati analizzati rispettivamente mediante PCR quantitativa e western blotting.

Abbiamo osservato che alterati livelli di lamin A sono associati allo sviluppo del tumore dell'endometrio, indicando il suo possibile ruolo come potenziale biomarcatore. La diminuzione dei livelli di lamin A è strettamente associata a riduzione dei livelli dei recettori degli estrogeni nei tumori di basso grado, mentre è associata in maniera significativa a riduzione di espressione di mRNA di E-Caderina (CDH1) nei tumori di alto grado.I nostri studi indicano la Lamin A come un nuovo biomarcatore prognostico da utilizzare nella pratica clinica per il trattamento personalizzato del cancro dell'endometrio.

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INTRODUCTION

Endometrial cancer (EC) is a major cause of mortality for patients worldwide. Most EC cases are sporadic, with only 10% considered familiar[1]. In general, patients with EC have a good prognosis since early diagnosis is frequent and the disease has usually not spread beyond the uterus. EC is clinically classified into two groups to assess the risk for metastatic and recurrent disease, type I and type II. Type I, also called the endometrioid type (EEC) because of its histologic similarity to the endometrium, accounts approximately 70–80% of sporadic EC and is characterised by hyperoestrogenic risk factors, low stage and grade, and favourable outcome. By contrast, type II cancers are associated with higher patient age, high stage and grade, non-endometrioid histology, and poor prognosis. However, the clinical and prognostic value of this distinction is suboptimal with substantial phenotypic overlap; about 20% of type I cancers recur with a median survival of 7–12 months, while 50% of type II cancers do not[2]. EC patients require more effective systemic therapy than is presently available to well selected patient populations to increase the likelihood of benefits. In order to improve therapy it is important to understand the processes which inhibit and stimulate cancer progression. Currently, adjuvant and systemic treatment of recurrent and metastatic EC are based on conventional chemotherapy and anti-hormonal treatment.

The cellular action of estrogens is mediated trough the estrogen receptors (ERs) that belong to the nuclear steroid receptor superfamily. Two distinct ERs, defined as ER-α and ER-β, have been identified. In the human uterus, ER-α is the predominant subtype[3-5]. Expression of ERs has been correlated with stage, histologic grade and survival[6-7]. Loss of ERs has been significantly associated with aggressive phenotype and poor survival in EC patients. In particular, early stage, well differentiated ECs usually retain ERs expression, whereas advanced stage, poorly differentiated tumours often lack one or both receptors. Recently, it has also been observed an association between lack of ER-α and epithelial-mesenchymal transition (EMT)[8].

EMT enables epithelial cells to acquire a like mesenchymal potential with increase motility and ability to extravasate and circulate. The process of EMT is associated with the progressive redistribution or downregulation of the apical and basolateral epithelial cell-specific tight and adherens junction proteins such as E-cadherin and cytokeratin, and novel expression of mesenchymal molecules such as vimentin and N-cadherin. Importantly, some promising studies showed that targeting EMT markers might be an interesting and successful tool in future cancer therapy[9-11]. In EC, aberrant expression of major EMT markers have been identified in metastatic disease and associated with adverse prognosis, such as such as lower expressions of E-cadherin and alpha-catenin, and overexpressions of N-cadherin, beta-catenin, vimentin, and matrix metalloproteinases, thus indicating the prognostic impact of EMT status[12].

Numerous studies suggest that reduced or absent lamin A expression is a common feature of a variety of different cancers, including small cell lung cancer (SCLC), skin basal cell and squamous cell carcinoma, testicular germ cell tumour, prostatic carcinoma, leukemia and lymphomas[13-18]. Expression and function of lamin A are involved in regulation of gene expression in health and disease through interplay with cell cycle progression, DNA replication, signal transduction pathways, transcription factors, chromatin-associated proteins and tissue homeostasis and the reduction in its expression frequently correlates with proliferative capacity and differentiation state. Lamin A is a type V intermediate filament (IF) protein encoded by the LMNA gene and a major nuclear architectural protein important for maintaining nuclear membrane inner structure integrity and function[19].Disruption of one or more of these functions due to lamin mutations cause a group of inherited diseases affecting various tissues and organs or causing accelerated ageing[20-24].

In this study, we analysed several EEC tissues to find novel clinical and biological features to help the diagnosis and consequently the treatment of early EEC. We observed a large decrease in the levels of lamin A mRNA (LMNA) and protein levels in EEC as compared with benign tissues. Moreover, LMNA loss further increased in higher grade EEC tissues. Interestingly, clustering of the mRNA expression of ERs and LMNA indicated an association between low expression of LMNA and loss of ERs in low grade EECs, thus suggesting a potential role of lamin A in EC invasiveness and aggressiveness in less aggressive ECs. In grade 3 EECs, generally expressing very low levels of ERs and lamin A, these correlation did not occur. Several papers support the hypothesis that E-Cadherin expression patterns in high-grade EC are associated with more aggressive characters and poor prognosis to ECs[25-27]. We observed a

significant correlation between decreased LMNA expression with lower E-cadherin mRNA (CDH1) levels in high grade ECs. Altogether, our findings strongly support the potential role of lamin A status in EC aggressiveness and its role as prognostic biomarker in association with ER status or with CDH1 expression in low grade or high grade EECs, respectively. Moreover, our results indicate that evaluation of LMNA expression related with ERs status may be used as predictive biomarker in low grade EECs.

MATERIALS AND METHODS

Patient cohort

A retrospective cohort of formalin-fixed, paraffin-embedded (FFPE) specimens from patients with endometriod endometrial cancer (EEC, n=80) and normal tissue specimens (NE, n=13) from patients who underwent a hysterectomy to treat other benign disease were collected. According with the histologic grade, we analysed 31 grade 1 (G1), 14 grade 2 (G2) and 35 grade 3 (G3) samples. Biopsies were sampled for primary tumors in hysterectomy specimens.

RNA extraction and RT-PCR

Total RNA derived from FFPE tissues was extracted using the PureLink™ FFPE Total RNA Isolation Kit (Invitrogen) following the manufacturer's instructions and reverse-transcribed using PrimeScript RT reagent kit (Takara). The quality of the total RNA was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington DE, USA). Quantitative PCR (qPCR) was performed using SYBR Select (Applied Biosystems) on an ABI Prism 7500 apparatus (Applied Biosystems). mRNA expression was normalized for 18S rRNA levels. Relative mRNA expression was calculated using the comparative Ct method ($2^{-\Delta\Delta Ct}$).

Primers

LMNA fw	GGACAATCTGGTCACCCGC
LMNA rv	TGGCAGGTCCCAGATTACATG
ESR1 fw	TACTGACCAACCTGGCAGACAG
ESR1 rv	TGGACCTGATCATGGAGGGT
ESR2 fw	AGTTGGCCGACAAGGAGTTG
ESR2 rv	CGCACTTGGTCGAACAGG
CDH1 fw	CCCACCACGTACAAGGGTC
CDH1 rv	ATGCCATCGTTGTTCACTGGA
18S rRNA fw	CCTGGATACCGCAGCTAGGA
18S rRNA rv	GCGGCGCAATACGAATGCCCC

Immunoblotting

The paraffin from thin sections of FFPE specimens was melted at 72°C for 20 minutes using heat in the presence of a specially designed Melting Buffer contained in the PureLink™ FFPE Isolation Kit used for RNA extraction (Invitrogen). Tissues were then separated from the melted paraffin by centrifugation. Proteins were extracted in a high pH lysis buffer (20 mM Tris HCl pH 9.0, 0.2 M Glycine, 2% (w/v) SDS). The samples were first incubated on ice for 5 min, and mixed by vortexing, then boiled at 100°C for 20 min followed by an 1 hour incubation at 80° C for 2 hours. After extraction, any remaining unsolubilized material was pelleted at 14000 × g for 20 minutes, and protein concentration of total protein extracted was determined by the BCA Protein Assay (Pierce Chemicals Co., Rockford, IL, USA). The Pierce BCA Protein Assay is a detergent compatible formulation and the protein standards were prepared using the same lysis buffer as the samples. Proteins were resolved by SDS-PAGE and electrotransferred to nitrocellulose. Each membrane was blocked with 5% non-fat dry milk in Tris buffered saline-Tween-20 (TBST) for 1 hour at room temperature and subsequently incubated with primary antibody for 16 hours at 4˚C. The following antibodies were used: anti-Lamin A (Santa Cruz), and anti-β actin (Sigma-Aldrich). Immunoreactivity was detected by sequential incubation with HRP-conjugated secondary antibody.

Statistical analysis

Data were reported as mean and standard deviation. Differences were considered statistically significant when $P \leq 0.05$. Student T test was performed for the comparison of results from qRT-PCR (*P<0.05, **P<0.01, ***P<0.001).

RESULTS

Lamin A protein and mRNA altered expression levels are associated with EEC aggressiveness.

A retrospective study was performed in a cohort of FFPE specimens from patients with EEC and of benign (NE) specimens from patients who underwent a hysterectomy to treat other benign disease (n=13). According with the histologic grade, we analysed 31 grade 1 (G1), 14 grade 2 (G2), 35 grade 3 (G3) EC tissues. Biopsies were sampled for primary tumors in hysterectomy specimens. Histologic are represented in **Table 1**.

To assess the possible involvement of lamin A in EEC, its protein expression levels were assessed by western blotting using an anti-lamin

Table 1.
Clinicopathological features of 80 EECs. RT= adjuvant radioteraphy; CHT=adjuvant chemoteraphy. BMI= body mass index; MI= myometrial infiltration.

Table I *Clinicopathological features*	Total EECs	G1 EECs	G2 EECs	G3 EEC
No of cases	80	31	14	35
Age	Median 63 years Range 42-88	Median 58 years Range 42-88	Median 66 years Range 47-88	Median 66 years Range 43-84
MI >50%	36/80 (45,0%)	8/31 (25,8%)	4/14 (28,5%)	24/35 (68,5%)
BMI>30	26/80 (32,5%)	5/31 (16,1%)	5/14 (35,7%)	16/35 (45,7%)
Limph node metastasis	5/80 (6,25%)	0/31 (0%)	0/14 (0%)	5/35 (14,3%)
Clinical stage (FIG.UREO 2008)				
I and II	52/80 (65,0%)	31/31 (100%)	6/14 (42,8%)	15/35 (42,8%)
III and IV	28/80 (35,0%)	0/31 (0%)	8/14 (57,1%)	20/35 (57,1%)
RT	27/80 (33,7%)	3/31 (0,9%)	2/14 (14,2%)	22/35 (62,8%)
CHT	11/80 (13,7%)	0/31 (0%)	4/14 (28,4%)	7/35 (20,0%)

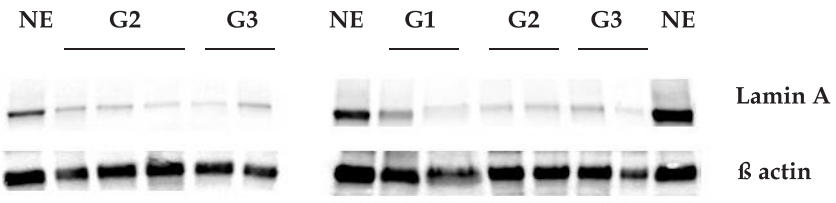

Figure 1A.
Representative immunoblottings of proteins extracted from benign (NE), G1, G2 and G3 EEC FFPE tissues with anti-Lamin A antibody. Anti-β actin was used as loading control.

A antibody. Results showed a large reduction of lamin A protein levels in EC compared with benign tissues (**Figure 1A**). To investigate if lamin A down-modulation occurred also at mRNA level, we performed qRT-PCR analysis. Results displayed that LMNA levels in EECs were significantly lower than those in corresponding non-cancerous tissues (**Figure 1B**), indicating the involvement of an altered modulation at transcriptional levels of lamin A expression in EECs. Interestingly, decreased LMNA expression correlated with histological differentiation significantly, thus suggesting a potential role of lamin A as predictive marker of EC aggressiveness (**Figure 1B** and **Table 2**). Lamin A levels were very similar in G2 and G3 , whereas a significant reduction was observed in G2-G3 compared with G1 samples, thus suggesting that lamin A loss maybe an early event in EC.

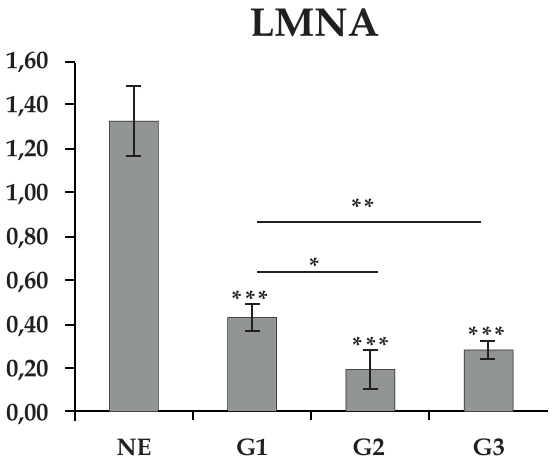

Figure 1B.
*Average expression of LMNA mRNA expression examined by qRT-PCR±SD in EEC tissues. mRNA expression was normalized for 18S rRNA levels. The error bars indicate the standard error. Statistical significance: *P<0.05, **P<0.01, ***P<0.001. The error bars indicate the standard error.*

Low levels of lamin A are associated with myometrial invasion

In our cohort of tissues, clinicopathologic features indicated that 25,8%, 28,5% and 68,5% of G1, G2 and G3 EECs analyzed displayed myometrial infiltration >50%, respectively (**Table 1**). Very interestingly, all G1 and G2 samples from tumors with myometrial invasion > 50% showed low levels of LMNA, thus suggesting a possible role of lamin A in tumor invasion prevalently in early stage of EECs.

Lamin A mRNA levels are associated with ER loss in low grade EECs

Expression of ERs has been correlated with EEC stage, histologic grade and survival. It has been shown that high levels of ERs directly correlate with better tumor differentiation and less myometrial invasion. In particular, loss of ERs has been significantly associated with aggressive phenotype and poor survival in EEC patients[28]. It is worth to note that the ER-α is predominant subtype in the human ERs and that a significant correlation between ER-α protein and ESR-1 mRNA expression has been previously estimated by microarray and qPCR analysis[29]. Therefore, we firstly evaluated mRNA expression levels of ER-α (ESR1) in our cohort of samples. Our data demonstrate that loss of ESR1 expression correlates with an aggressive clinopathologic phenotype, confirming data in literature (**Figure 2A** and **Table 2**). To explore potential biologic role of lamin A in process contributing the aggressive phenotype of ECs, we focused our attention on transcriptional differences between EECs expressing (ERs positive) and not expressing (ERs negative) both ER-α and ER-β, and LMNA mRNA levels. Clustering of the mRNA expression of ERs and LMNA indicated a significant association

Table 2.
Clustering of LMNA, ERs, and CDH1 expression levels. EEC histological grade in relation to levels of ERs, LMNA and CDH1 expression. Lamin A, ESR1, ESR2, and CDH1 mRNA was examined by qRT-PCR. Cut off=ECC over benign samples ≤ 0,5

Table 2	ERs neg	LMNA<0,5	CDH1<0,5	LMNA<0,5 ERs neg	LMNA<0,5 ERs pos	CDH1<0,5 LMNA<0,5	CDH1>0,5 LMNA<0,5
G1	6/31 (19,3%)	23/31 (74,2%)	15/31 (48,4%)	6/6 (100%)	17/25 (68,0%)	14/23 (60,8%)	9/23 (39,1%)
G2	3/14 (21,4%)	12/14 (85,7%)	9/14 (64,3%)	3/3 (100%)	9/11 (81,8%)	8/12 (66,6%)	4/12 (33,3%)
G3	25/35 (71,4%)	31/35 (88,5%)	27/35 (77,1%)	23/25 (92,0%)	8/10 (80,0%)	26/31 (83,9%)	5/31 (16,1%)

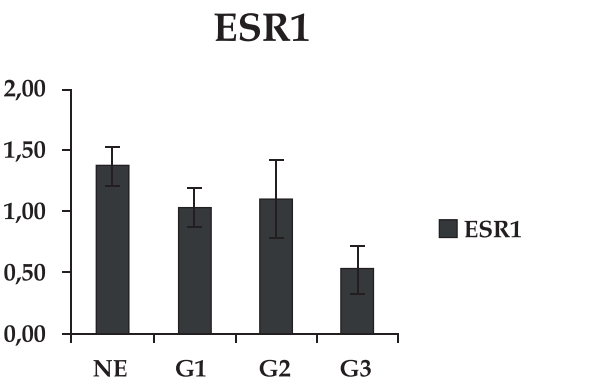

Figure 2A.
Average expression of ESR1 mRNA examined by qRT-PCR±SD in EECs (G1, G2, and G3) and benign FFPE tissues (NE). mRNA expression was normalized for 18S rRNA levels.

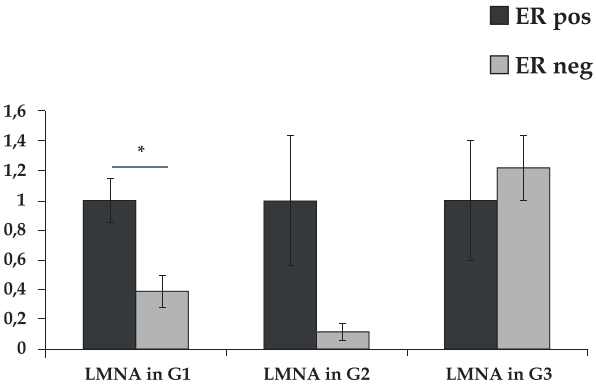

Figure 2B.
*LMNA expression in ERs negative compared with ERs positive (fold over control) EEC tissues. Statistical significance: *P<0.05. The error bars indicate the standard error.*

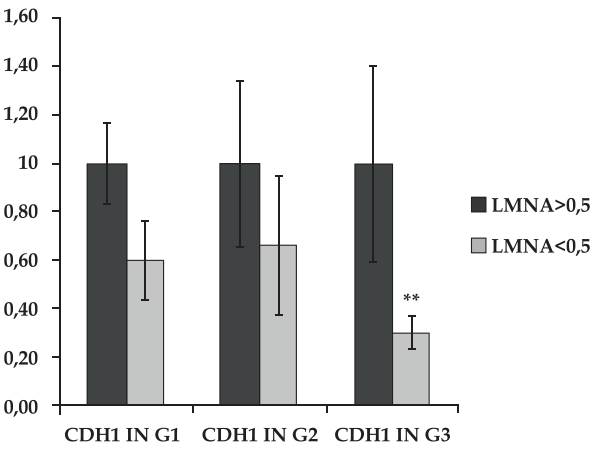

Figure 3.
*CDH1 expression and LMNA status. Average of CDH1 mRNA expression examined by qRT-PCR±SD in EEC tissues expressing low levels of LMNA compared with positive LMNA tissues- The error bars indicate the standard error. Cut off=ECC over benign samples ≤ 0,5. Statistical significance: **P<0.01. The error bars indicate the standard error.*

between lack of ERs expression, decreased LMNA expression (fold over control < 0,5) and higher histologic differentiation grade (**Figure 2B and Table 2**). Very interestingly, all ERs negative G1 and G2 EEC samples expressed concomitantly low LMNA levels (**Table 2**). Analysis performed in G3 EEC specimens, generally expressing very low levels of ERs and LMNA, displayed a different behavior since no differences in LMNA expression levels were detected in ERs positive compared with ERs negative tissues (**Figure 2B**), thus indicating that LMNA down-modulation associated with ERs loss may be an early event in EC transformation.

Loss of lamin A is associated with E-cadherin status in high grade ECs

Several papers support the hypothesis that E-Cadherin expression patterns in high-grade EC are associated with more aggressive characters and poor prognosis to ECs[30, 36-38]. Analysis of CDH1 mRNA status in our cohort of ECCs confirmed these studies. In fact, as shown in **Table 2**, we found that 48,4%, 64,3%, and 77,1% displayed low levels of CDH1 levels (fold over control < 0,5) in G1, G2 and G3 EECs, respectively, thus indicating that an increase number of cases with higher grade generally display reduced expression levels of CDH1 compared with lower grade ECs. To assess the possible correlation between CDH1 mRNA levels and LMNA expression in our cohort of EC samples, we compared CDH1 levels in samples expressing low LMNA levels (fold over control < 0,5) with those expressing higher LMNA levels (fold over control > 0,5). Interestingly, we observed a significant decrease of CDH1 levels in tissues expressing low levels of LMNA compared to those expressing higher levels only in G3 EEC tissues (**Figure 3**). These evidences suggest an association between decreased lamin A expression and low levels of CDH1 in high grade EECs, thus further indicating a possible involvement of lamin A in tumor differentiation and aggressiveness and suggesting its role in EMT.

DISCUSSION

Although three quarters of ECs are confined to the uterus and treated at an early stage, 15%–20% recur after primary surgery with limited effect of systemic therapies in metastatic disease[2,31-34]. Thus, one important clinical challenge is to accurately predict risk of recurrence within this good prognosis patient subgroup in order to well selected patient populations for more extensive surgery and adjuvant therapy. The aim of our

study is to identify novel biomarkers with a potential for a more systematic integration in clinical practice for individualized therapy in EC.

We focused our attention on the expression of lamin A, a nuclear protein involved in cell differentiation and cancer development. The expression of lamin A is often reduced or absent in cells that are highly proliferative, including various human malignancies such as colon cancer, cervical cancer, lung cancer, prostate cancer, gastric cancer, ovarian cancer and leukemia and lymphoma[13,18]. Assessment of lamin A protein and mRNA levels in our cohort of FFPE tissues displayed a large and significant decrease of its expression compared with benign samples. Moreover, we observed a significant correlation between lamin A loss and advanced stage disease and a correlation with increased myomerial infiltration. It is worth to note that we observed a significant reduction of LMNA mRNA levels in G1 compared with higher grades, whereas no differences in its expression levels were detectable between G2 and G3 EECs, thus suggesting that alteration of LMNA expression maybe an early event in EC. The identification of patients with poor prognosis among the presumed low-risk endometrioid G1 and G2 cases represents a particular therapeutic challenge. Subgroup analyses of prognostic factors among patients with endometrioid histology have confirmed a prognostic value of ERs expression in curettage specimens in retrospective studies[6-8]. In fact, patients with ERs negative EEC are more often diagnosed with higher grade and advanced stage disease[7]. Thus, we clustered mRNA expression of LMNA and ERs. Our data indicated a significant association between low LMNA expression and lack of ERs in G1 and G2 EECs, suggesting that lamin A may represent a novel prognostic biomarker in low grade EC. Alterations in E-cadherin expression have been linked to decreased cell–cell adhesion, metastatic potential, tumor dedifferentiation, and deep myometrial invasion in endometrial and other carcinomas. The hallmarks of EMT in cancer cells include changed cell morphology and increased metastatic capabilities in cell migration and invasion[35]. A recent meta-analysis indicated that EC patients with reduced expression of E-cadherin may have a poorer prognosis than those with normal or higher expression of E-cadherin in high grade ECs[36] and that down-regulation of E-cadherin plays a major role in EMT and associates with myometrial invasion, histologic grade and metastasis[37,38]. In this study, we observed that a significant decrease of CDH1 mRNA was associated with LMNA loss in G3 tumors, suggesting the possible involvement of altered LMNA expression in EMT in high grade EEC.

Our data strongly indicate lamin A as a novel putative biomarker in EC. We hypothesizes that lamin A down-modulation in association with ERs status or CDH1 levels in low grade or high grade tumors, respectively, may represents a predictive marker of aggressiveness in EECs. Our findings also support the concept that divergent molecular pathways are involved in different histological grade of ECs.

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