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Identification of long non-coding RNA expression patterns useful for molecular-based classification of type I endometrial cancers

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Abstract. Endometrial cancer is the most frequently diagnosed gynecologic malignant disease. Although several genetic alterations have been associated with the increased risk of endometrial cancer, to date, the diagnosis and prognosis still rely on morphological features of the tumor, such as histological type, grading and invasiveness. As molecular-based classification is desirable for optimal treatment and prognosis of these cancers, we explored the potential of lncRNAs as molecular biomarkers. To this end, we first identified by RNA sequencing (RNA-Seq) a set of lncRNAs differentially expressed in cancer vs. normal endometrial tissues, a result confirmed also by analysis of normal and cancerous endometrium RNA-Seq data from TCGA (The Cancer Genome Atlas). A significant association of a subset of these differentially expressed lncRNAs with tumor grade was then determined in 405 TCGA endometrial cancer profiles. Integrating endometrial cancer-specific expression profiles of long and small non-coding RNAs, a functional association network was then identified. These results describe for the first time a functional 'core' network, comprising small and long RNAs, whose deregulation is associated with endometrial neoplastic transformation, representing a set of cancer biomarkers that can be monitored and targeted for diagnosis, follow-up and therapy of these tumors.

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Abbreviations: EC, endometrial cancer; EEC, endometrioid endometrial cancer; EMT, epithelial-to-mesenchymal transition; FC, fold change; lincRNA, long intergenic non-coding RNA; lncRNA, long non-coding RNA; miRNA, microRNA; ncRNA, non-coding RNA; ceRNA, competing endogenous RNA; NEEC, non-endometrioid endometrial cancer; P-adj, adjusted P-value; piRNA, PIWI-interacting RNA; sncRNA, small non-coding RNA; TCGA, The Cancer Genome Atlas

Key words: endometrial cancer, neoplastic transformation, lncRNAs, molecular signature, functional core regulators

Introduction

Endometrial cancer (EC) is the most common malignancy of the female genital tract, with an estimated incidence of 63,230 new cases in 2018 in the United States (1). With the increasing prevalence of major EC risk factors, such as obesity, diabetes and hypertension, a rising trend has also been recognized in younger women (2). EC is generally classified into two subtypes, defined as type I or endometrioid endometrial cancer (EEC) and type II or non-endometrioid endometrial cancer (NEEC) (3,4). This dualistic classification underlies different patterns of molecular alterations, pathogenesis and clinical outcome (4).

Type I EC, which is the most frequent uterine malignancy (80% of all cases), is an estrogen-dependent lesion, often seen

in conjunction with endometrial hyperplasia, usually developed in peri- and early postmenopausal women (4). Type II EC affects older patients, is not preceded by hyperplasia and comprises more aggressive histologic subtypes, such as papillary serous, clear cell carcinomas and carcinosarcomas (3,5). In general, type I EC has a good prognosis, with a 10-year overall survival rate exceeding 80% (6,7). Surprisingly, despite optimal risk-adapted treatment, a small but substantial number of patients exhibits recurrence and poor survival. In such cases available risk factors or biological markers are not able to reliably predict the poor clinical course. Indeed, it is currently established that a classification only based on morphologic features is inconsistent and that molecular-based classification is desirable for optimal treatment and prognosis of such cancers. Nevertheless, although several genetic alterations have been associated with increased risk of EC (8-10) and mutations in genes such as ATR have been established to be associated with poor clinical outcomes in EEC (11), new molecular markers need to be identified for early diagnosis and treatment purposes.

In 2013, researchers at The Cancer Genome Atlas (TCGA) performed an integrated genomic, transcriptomic and proteomic characterization of 373 endometrial carcinomas by applying array- and sequencing-based technologies, thus drawing a new classification of endometrial cancers according to four main molecular categories according to their genomic features (12). This may guide post-surgical therapy in patients affected with aggressive tumors. Yet, since taking these results into clinical practice is currently cost-prohibitive, molecular surrogates corresponding to each of the subgroups (13), able to successfully classify all patients in the TCGA cohort and to minimize false negatives in the CN-high poor prognosticator group, were identified.

The Encyclopedia of DNA Elements (ENCODE) project estimates that almost 62-75% of the DNA is transcribed into RNA, but only 2% of the transcriptome is finally translated into proteins (14). However, this non-coding part of the genome plays many key roles in several biological processes and diseases such as in cancers (15). Thus, the landscape of non-coding RNAs (ncRNAs) is increasing day by day and has emerged as a major source of biomarkers (16,17), being considered as priority transcripts to be monitored for functional significance in both pathogenesis and progression of ECs. Based on their size, ncRNAs are divided in two main categories: Small ncRNAs [sncRNAs (<200 nt)] and long ncRNAs [lncRNAs (>200 nt)]. Moreover, they are connected to each other, with some lncRNAs acting on post-transcriptional regulation through the modulation of certain sncRNA subgroups (18).

Among sncRNAs, four major functional groups have been identified in mammals: microRNAs (miRNAs), PIWI-interacting-RNAs (piRNAs), small nucleolar-RNAs (snoRNAs) and endogenous small interfering-RNAs (endo-siRNAs) (19). sncRNAs can exert large-scale and diverse effects on cellular processes by regulating gene expression, protein translation and genomic organization. In particular, lncRNAs may contain also miRNA-binding sites and can compete with miRNAs for interaction with their target mRNAs and thereby block their effects on these mRNAs.

The classification of lncRNAs is more complex than that of sncRNAs, as they can be divided on the basis of their size, association with annotated protein-coding genes, or with other DNA elements of known function (18,20). Thus, lncRNAs represent a heterogeneous group of RNAs, which have been identified as important molecules in several biological processes, performing tumor suppression and oncogenic functions in various types of cancer (18).

Using small RNA sequencing and microarrays, we previously determined significant differences in sncRNA expression patterns between normal, hyperplastic and neoplastic endometrium of patients affected with EEC (21). This led to the definition of a sncRNA signature (129 miRNAs, 2 of which were not previously described, 10 piRNAs and 3 snoRNAs) recapitulating neoplastic transformation.

In the present study, we aimed to analyze the role of lncRNAs in EEC and to examine a possible connection with the sncRNAs previously observed. To this aim, we first performed lncRNA profiling in a set of EEC samples and paired normal tissues, to identify lncRNA molecules more likely to be associated with the cancerous state. These were also confirmed in a set of 20 paired cancer-normal profiles deposited in the TCGA database. The 80 more highly differentially expressed were found to be significantly associated with different tumor grade among 405 EEC RNA profiles in TCGA. Finally, we identified a small/long RNA functional core whose deregulation was predicted to be strongly associated with endometrial neoplastic transformation. Indeed, computational analysis identified, among the most affected functional pathways, focal adhesion, MAPK and Wnt signaling, confirming previously published data (21).

Materials and methods

Patients and tissue collection. Six patients, who underwent type A radical hysterectomy with or without pelvic lymphadenectomy for EC, were enrolled in the present study at the University of Salerno. Samples were collected from December 2013 to November 2014 in the Division of Gynecology and Obstetrics, 'SS. Giovanni di Dio e Ruggi d'Aragona' University Hospital of Salerno (Italy). Average age of patients was 64 (range, 51-80 years). The study protocol received institutional review board (IRB) approval before the beginning of the study, in accordance with the code of ethics of the Declaration of Helsinki, and informed consent was obtained from all patients.

From each patient a sample of neoplastic tissue and corresponding normal endometrium were collected, washed and stored at -80˚C until analysis. The histology of each EC was classified according to World Health Organization criteria, whereas surgical staging followed FIGO (International Federation of Gynaecology and Obstetrics) standards.

RNA isolation and quality controls. Tissue specimen were disrupted and homogenized using TissueLyser LT (Qiagen, Hilden, Germany). Total RNA was extracted with miRVana RNA Isolation kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. Before use, the RNA concentration in each sample was assayed with a NanoDrop ND-2000c (Thermo Fisher Scientific, Inc.) and

its quality was assessed with the Agilent 2100 Bioanalyzer using Agilent RNA 6000 Nano kit (Agilent Technologies, Inc., Santa Clara, CA, USA).

RNA sequencing and data analysis. RNA-Seq libraries were prepared as previously described (22). Briefly, 1 μg of total RNA was used in a library preparation according to the Illumina TruSeq Stranded Total RNA Sample Preparation protocol (Illumina, Inc., San Diego, CA, USA). Each library was sequenced on HiSeq 2500 (Illumina) at a concentration of 8 pM for 200 cycles plus 7 additional cycles for index sequencing in the paired-mode (2x100 base pair).

RNA-Seq data analysis was performed as described in the study by Tarallo *et al* (23). In details, fastQ underwent to quality control using FastQC tool [http://www.bioinformatics.babraham.ac.uk/projects/fastqc/]. The mapping of paired-end reads was performed on reference genome assembly hg19 using STAR (version 2.5.2b) (24). The quantification of lncRNAs expressed for each sample was performed using HTSeq-Count algorithm (25), while differential expression analysis was performed with DESeq2 (26). Only lncRNAs showing a cut-off of |fold change| ≥1.5 and adjusted P-value (P-adj) ≤0.05 were considered for further analysis.

TCGA raw data, corresponding to either 20 tumor and paired normal or 405 EEC tissues, were analyzed as described above and compared to the in-house investigated samples.

Bioinformatic analyses. Pearson correlation between in-house differentially expressed lncRNAs and paired tumor-normal TCGA data was computed using R software (https://www.r-project.org/).

Non-negative matrix factorization (NMF) was computed considering the normalized expression values in endometrial cancer data taken from TCGA, considering the list of in-house differentially expressed lncRNAs showing |fold change| ≥3 and P-adj ≤0.05 using the R package 'nmf' available in CRAN (27).

Kaplan-Meier survival analysis and Cox proportional-hazard regression was generated using MedCalc 18.5 software (https://www.medcalc.org/). lncRNA and mRNA targets of the selected miRNAs were computed using miRWalk 2.0 (28). The miRNA, lncRNA and mRNA associated competing endogenous RNA (ceRNA) network was designed as described by the study of Wang *et al* (29). Gene Ontology analysis was performed using Co-LncRNA tool (30).

Results

Long non-coding RNA profiling in EEC tissues. lncRNA expression profiling was performed by next-generation sequencing in samples of type I endometrial cancer (EEC), to evaluate their possible deregulation during carcinogenesis.

To this aim, six patients were selected out of a larger cohort following initial characterization according to defined clinicopathological parameters (Table I). For each patient, two endometrial biopsies were obtained from pathological and adjacent normal tissue (normal tissues indicated as: 3N, 4N, 14N, 15N, 18N and 19N; tumor tissue samples indicated as: 3T, 4T, 14T, 15T, 18T and 19T). Normal tissues were collected as far as possible from the area presenting a cancerous lesion, to reduce the possibility of cross-contamination.

Table I. Clinicopathological features of the tissue samples analyzed from 6 patients affected with endometrioid endometrial cancer.

Characteristics	N	Patient nos.
Age (years)		
>55	5	3, 14, 15, 18, 19
≤55	1	4
Tissue categories		
Normal	6	3N, 4N, 14N, 15N, 18N, 19N
Endometrial cancer	6	3T, 4T, 14T, 15T, 18T, 19T
Stage (FIGO)		
I-II	5	3T, 4T, 14T, 15T, 18T
III-IV	1	19T
Histological grade		
G1	3	4T, 14T, 15T
G2	1	3T
G3	2	18T, 19T
Lymph node metastasis		
Positive	0	
Negative	5	3T, 4T, 15T, 18T, 19T,
Nx	1	14T
Invasion		
T1 and T2	5	3T, 4T, 14T, 15T, 18T
T3 and T4	1	19T

More than 200 million sequences were obtained by RNA-Seq analysis for the 12 samples sequenced, that after filtering out low quality reads and trimming the adaptors led to approximately 20 million reads/sample.

The obtained reads were aligned against the human genome reference (hg19), paying particular care in lncRNA identification. lncRNA molecules included in the human genome are classified in seven categories [long intergenic non-coding RNA (lincRNA), antisense, sense_overlapping, processed_transcript, sense_intronic, bidirectional lncRNA and miRNA] according to GENCODE gene annotation, the largest manually curated catalog of human lncRNAs (31). More than 24,000 RNA molecules, considering both coding and non-coding ones, were identified in total in the investigated samples and the percentage of lncRNAs belonging to the aforementioned categories is reported in Fig. 1A. In particular, most of the lncRNAs expressed within the investigated samples belonged to two main categories: lincRNAs (41%) and antisense transcripts (37%).

Then, since deregulation of lncRNAs has been associated with a broad range of physiological defects in multiple diseases, including cancer, to investigate whether lncRNA expression patterns may change during endometrial carcinogenesis, differential expression analysis was performed (tumor vs. normal) and a pool of lncRNAs specifically deregulated in cancerous tissues was identified.

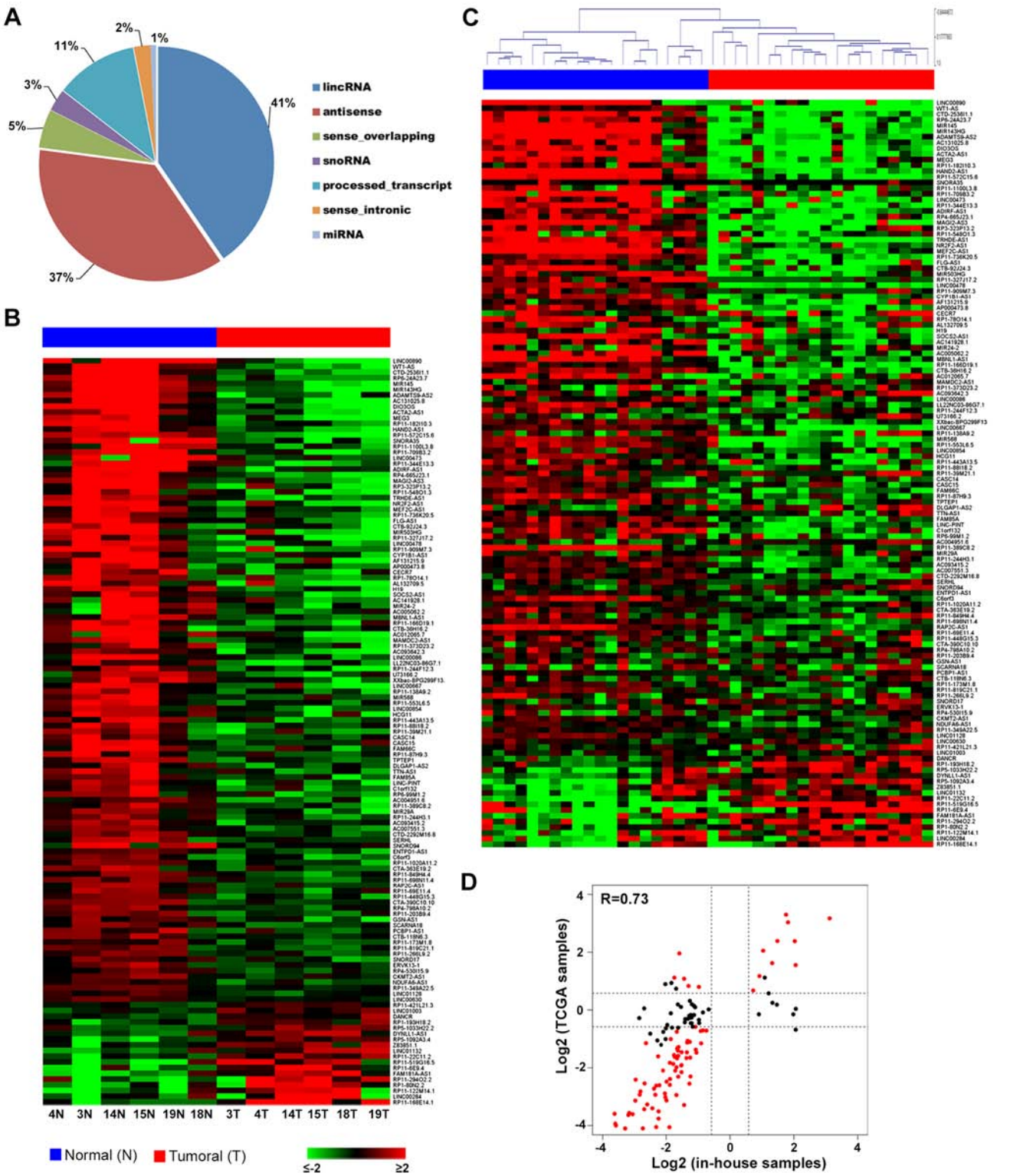

Figure 1. lncRNA profiling by RNA-Seq. (A) Pie-chart showing lncRNA classification according to GENCODE. (B) Heatmap showing 131 lncRNAs differentially expressed (|FC| ≥1.5 and P-adj ≤0.05) in EEC vs. paired normal tissues in in-house profiled samples. (C) Heatmap showing relative expression of the 131 lncRNAs previously identified among TCGA RNA-seq profiles of EEC and paired normal tissues. In both heatmaps the expression value of each lncRNA has been log2 transformed and centered on the median value. Expression values lower or higher than the median are shown in green or red, respectively. (D) Plot showing the correlation between in-house generated and TGCA deposited lncRNA profiles. Red and black dots indicate lncRNAs with P-adj ≤0.05 and P-adj >0.05 respectively, associated to corresponding FC. EEC, endometrioid endometrial cancer; lncRNA, long non-coding RNA; TCGA, The Cancer Genome Atlas; P-adj, adjusted P-value; FC, fold change.

In particular, 131 lncRNAs (Fig. 1B) differentially expressed between EEC samples and the corresponding normal endometrium were found considering |fold change (FC)| ≥1.5 and P-adj ≤0.05, suggesting their involvement in endometrial carcinogenesis. The full list of statistically significant differentially expressed RNAs is available at ArrayExpress (E-MTAB-7041).

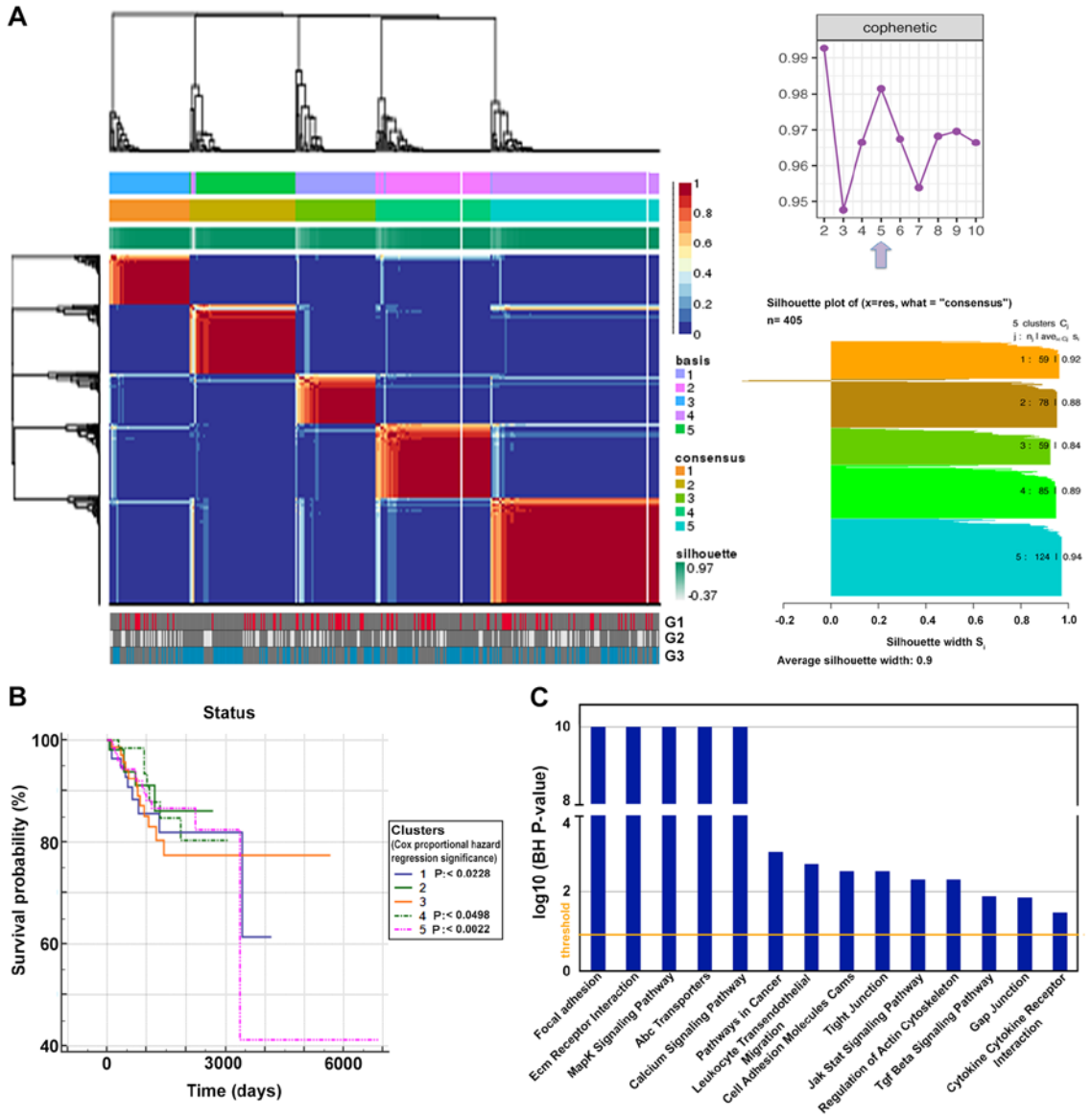

Figure 2. lncRNAs as markers of endometrial carcinogenesis. (A) Unsupervised clustering (non-negative matrix factorization), using 80 differentially expressed lncRNAs in own dataset compared to TCGA sample (|FC| ≥3 and P-adj ≤0.05), depicting five clusters. In the lower panel, sample stratification according to tumor grade is shown; in the upper right panel a plot shows the change of cophenetic coefficient at rank 2 to 10, the arrow displays the optimal number of subgroups, In the lower right panel, silhouette plots of the 5 consensus groups, the number of members and average silhouette width are shown. (B) Kaplan-Meier curves showing the overall survival trends among the five clusters according to transcriptional subtypes (log-rank test for trend P-value=0.5890). (C) Functional pathways mainly affected after lncRNA-mRNA co-expression analysis performed with the Co-LncRNA tool (BH P-value ≤0.05). lncRNAs, long non-coding RNAs; TCGA, The Cancer Genome Atlas; P-adj, adjusted P-value; FC, fold change.

To identify the most relevant lncRNAs in the development of EEC, we performed genome-wide analysis by comparing our data with TCGA molecular RNA-Seq profiles of 20 patients for which data from primary EEC tumors and the corresponding normal endometrial tissues were available. By analyzing the expression of the 131 lncRNAs identified in our patients to be deregulated (|FC| ≥1.5 and P-adj ≤0.05), it emerged that most of them had the same trend in the TCGA dataset, discriminating between cancer and normal tissues (Fig. 1C). In particular, comparing their expression levels, we observed a 0.73 correlation between the two datasets (Fig. 1D). It is also interesting to observe that the lncRNAs having an opposite trend between the two sets of samples were mostly those showing little changes among our samples and being not statistically significant in the TCGA group.

To confirm the consistency of our results, we investigated in particular the behavior of 80 lncRNAs whose expression levels were strongly modulated in tumor samples (|FC| ≥3 and P-adj ≤0.05) among the molecular RNA-Seq profiles of 405 primary EEC tumors deposited in TCGA database.

Thus, we performed integrative analysis in order to identify molecularly distinct tumor EEC subgroups associated with specific clinico-biological features. Unsupervised hierarchical clustering revealed five clusters of lncRNAs that correlated with specific clinical parameters. In particular, the most statistically relevant and discriminating factor resulted in histological grading (P<0.01 by Chi-square test) (Fig. 2A).

Moreover, these five clusters hold statistically different weight on survival (P=0.5890), identifying five subgroups of EECs with slightly different trends in overall survival,

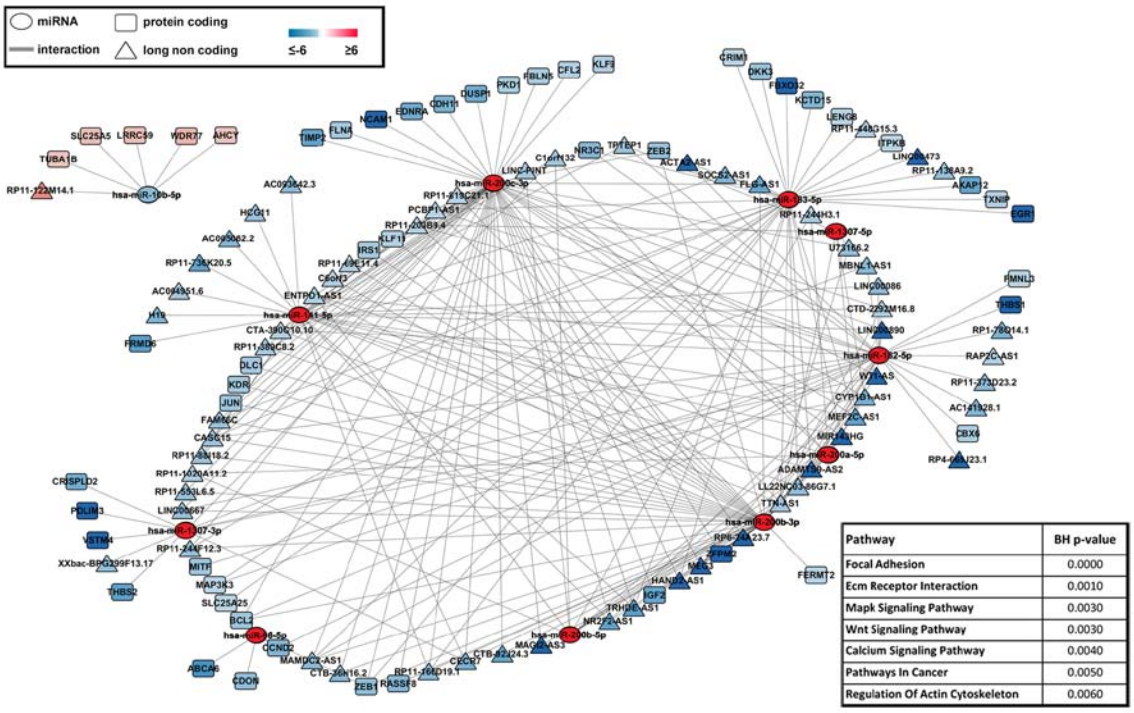

Figure 3. ceRNA network. lncRNAs and mRNAs showing inverse correlation with previously identified miRNAs are shown. The functional pathways influenced by the predicted long/small RNA combination are listed in the table. ceRNA, competing endogenous RNA; lncRNAs, long non-coding RNAs; mRNAs, messenger RNAs; miRNAs, microRNAs.

as shown in the Kaplan-Meier survival curve of Fig. 2B. Additionally, we analyzed the survival of patients separately in each of the five groups using clinical stage and grade as covariates by applying Cox proportional-hazards regression model. In this way, we found that clusters 1 and 4 were significantly associated with survival in patients at stage 3 (P<0.0228 and P<0.0498, respectively), while survival was strongly associated in patients belonging to cluster 5 classified in stage 4 (P<0.0022). All these patients showed a significantly worse survival.

mRNA-lncRNA co-expression and ceRNA network construction. To better investigate the functional significance of our observations, we correlated the 80 most deregulated lncRNAs with mRNA profiles obtained in the tumor tissues analyzed here. This led to the identification of a set of pathways specifically affected by mRNA-lncRNA co-expression, including MAPK, JAK/Stat and TGF-β signaling (Fig. 2C) that was found also to be affected by sncRNAs in our previous study (21).

Indeed, by integrating these data with previous results from studies of sncRNAs in EEC and considering the possible interplay between miRNAs-mRNAs-lncRNAs, a ceRNA network was obtained, revealing miRNAs specifically targeting either mRNAs or lncRNAs differentially expressed in EEC. To this aim, the list of 129 miRNAs representing a signature of endometrial neoplastic transformation was filtered applying P-value correction (Bonferroni correction), leading to 11 more significant miRNAs. An integrative network was then drawn to show miRNA-mRNA-lncRNA correlations (Fig. 3). In details, the analysis was focused on miRNAs, thus, showing mRNA and lncRNA targets having a negative correlation with respect to miRNA expression. In this context, the most significantly affected functional pathways were found, among others, focal adhesion, ECM receptor interaction, MAPK and Wnt signaling (Fig. 3), suggesting that specific small/long transcripts combination may be directly involved in EEC onset and progression and thus being key markers to be monitored in diagnosis and follow-up of the disease.

Discussion

Molecular classification of ECs represents an objective that clinicians are trying to achieve in order to provide independent prognostic information beyond established risk factors.

In the past few years, pragmatic molecular classifiers have been developed, able to identify four prognostically distinct molecular subgroups that may change the current risk stratification systems (12,13,32). Indeed, the evolution of genomic classification in ECs has the potential to be used routinely in therapeutic protocol selection and in stratifying cases in future clinical trials (13).

Nevertheless, mounting evidence has emphasized the roles and clinical significance of non-coding RNAs and, in particular lncRNAs, among others in endometrial cancer (33,34).

Several studies have shown the potential of lncRNAs as therapeutic targets and investigated their involvement in cancer pathogenesis (35,36). Indeed, limited information is available on EC (37-44). Clarification of their role in the onset, progression and follow-up of this tumor is warranted to help molecular classification and targeted therapy of this cancer histotype.

In the present study, we first evaluated the differential expression of lncRNAs between endometrial tumor samples

and paired normal tissues of six EEC patients and then we compared and validated our results with different EEC datasets present in TCGA database. In this way, we identified a set of lncRNAs discriminating between normal and cancerous endometrium, common between those described here and publicly available data. Moreover, deep investigation of this lncRNAs dataset among a group of 405 EEC RNA-Seq profiles in TGCA revealed that 80 lncRNAs, differentially expressed with a higher fold, were statistically distributed in five clusters, reflecting tumor grade stratification and associated with different survival trends.

A number of studies have demonstrated that a large number of miRNA-binding sites are present on lncRNAs, suggesting how these transcripts can serve also as competing endogenous RNAs controlling 'free' miRNA levels and functions (45,46). Importantly, lncRNAs could compete with mRNAs for miRNAs, and thereby regulate miRNA-mediated transcript repression (45,46). Therefore, we built a ceRNA network, depicting key interactions occurring among differentially expressed mRNAs, lncRNAs and miRNAs in our EEC samples and determining a functional impact within crucial pathways in EC, which may represent potential core regulators to be monitored for early diagnosis and therapeutic purposes. Indeed, understanding molecular interactions has becoming a common tool especially in cancer research, given the limited information provided to date by studies focusing on a single molecule (47-49).

In this regard, our results strengthen and integrate the current knowledge concerning EEC molecular markers, thus providing novel details about their functional correlations. In this way, a functional multi-molecule core has been identified, showing miRNA members mostly deregulated in EEC and the corresponding mRNA and lncRNA targets inversely correlated with miRNA expression. From this snapshot it emerges, for example, the coexistence between downregulation of miR-10b that has been also proved to be downregulated in other types of cancer, such as gastric and cervical ones (50,51) and upregulation of its long-RNA targets. Moreover, we confirmed upregulation of almost the entire miR-200 family (miR-200a/b/c and miR-141), an event already well documented in EEC, where these RNAs target mainly genes involved in tissue transformation responsible for epithelial-to-mesenchymal transition (EMT) (52,53). In this context, we observed an inverse correlation, among others, with the well known miR-200 targets ZEB1 and ZEB2, transcriptional repressors of E-cadherin whose expression is restored in EMT (53) and KLF9, whose downregulation in EEC has been previously demonstrated (54,55). Downregulation was also retrieved for TIMP2 representing, together with the above mentioned mRNAs, a key gene involved in the differentiation of EEC and proposed as a potential therapeutic target (56-58). More interestingly, an inverse correlation was also found with several downregulated lncRNAs, including ADAMTS9-AS2, MEG3 and HAND2-AS1, already demonstrated to act as tumor suppressors. Indeed, ADAMTS9-AS2 has been associated with the inhibition of cancer progression and migration in lung and glioma cells; its expression has been correlated with poor prognosis through a mechanism involving the interaction with DNMT1 (59,60). In addition, a role of MEG3 in EEC tumorigenesis and progression through the modulation of Notch and PI3K pathways has been proved (40,61). In several cancer types, MEG3 has been shown to sequester various microRNAs from protein and/or target mRNAs resulting in altered protein activity, translation and degradation; in particular involvement of this lncRNA in the regulation of the miR-200 family has been demonstrated, confirming what was revealed by the ceRNA model constructed here (Fig. 3) (62). Finally, HAND2-AS1 has been shown to be involved in inhibition of EEC invasion and metastasis, through the downregulation of neuromedin U (63), and it has been proposed to play a role in the tumorigenesis of muscle-invasive bladder cancer through the interaction with many several miRNAs (including, of note, also miR-183) (64).

In conclusion, in the present study we identified a functional core, constituted by a specific combination of miRNAs-mRNAs-lncRNAs expression, which represents a molecular signature of potential usefulness to monitor EEC progression, for follow-up and prognosis of this disease.

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Availability of data and materials

The results shown here are in part based upon data generated by the TCGA Research Network: http://cancergenome.nih.gov/. The datasets generated and analyzed in this study are available in the EBI ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) with Accession Number E-MTAB-7039 (raw data) and E-MTAB-7041.

Authors' contributions

AW, FR, FZ and MG designed and coordinated the study; MAC, AC and MR selected the patients and MAC collected the clinical samples; AC, FR, GN, MR, PS and RT carried out the experimental work; AR and GG performed the data analyses; AC, MR and RT wrote the manuscript; MR, AC, PS, AR, MAC, GN, GG, FZ, AW, RT, FR and MG contributed to data interpretation and discussion and to manuscript revision. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The study protocol received approval by the Ethics Committee of the 'SS. Giovanni di Dio e Ruggi d'Aragona' University of Salerno Hospital (n.er 91/13.12.2013) before the beginning of the study, in accordance with The Code of Ethics of the

Declaration of Helsinki, and informed consent was obtained from all patients included.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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