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"VALIDATION OF A PANEL OF CIRCULATING MICRO-RNAs AS POTENTIAL BIOMARKERS IN THE DIAGNOSIS AND FOLLOW-UP OF WOMEN WITH ATYPICAL ENDOMETRIAL HYPERPLASIA AND ENDOMETRIAL CANCER"

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ABSTRACT

Objective

The aim of this study was to determine the accuracy of the predictive value of a selected panel of microRNA (microRNA-504, microRNA-429) obtained on endometrial tissue samples, in detecting endometrial cancer (EC) and its precursor, atypical endometrial hyperplasia (AEH).

Materials and Methods

A prospective observational single center study was conducted at the Hysteroscopy Unit, Department of Gynecology and Obstetrics of Tertiary Care University Hospital, University of Naples "Federico II". Patients over the age of 18, with diagnosis of EC or AEH, not receiving any previous treatment were included. Women with other kind of malignancy were excluded. Patients were divided into three groups: women with EC (group 1), women with AEH (Group 2), women with normal proliferative endometrium (Group 3). All patients underwent office hysteroscopic biopsy using a standardized "grasp biopsy" technique. For each sample, after RNA extraction, subsequent reverse transcription in cDNA, the PCR Real Time analysis was performed by using Taqman Advanced microRNA Assay (Thermo Fisher Scientific, MA, USA) to evaluate microRNA expression (microRNA 504-5p, microRNA 429) in order to compare the different level of expression between endometrial cancer tissue, hyperplasia tissue and healthy endometrial tissue. Patient's data and biologic materials were managed according to Helsinki declaration.

RESULTS

A total of 33 women were enrolled. Specifically, 15/33 (45.5%) women were diagnosed with endometrial cancer, 15/33 (45.5%) patients with hyperplasia and 3/33 (9%) with normal proliferative endometrium. In Group 1, a valid amplification plot for the microRNA-504-5p was expressed in 14/15 (93.3%) women, and in 15/15 (100%) of women for miroRNA-429 with a median Ct value of 32.3 and 29.7 for microRNA-504-5p and miroRNA-429 analysis respectively. In Group 2, a valid amplification plot was found in 14/15 (93.3%) patients for microRNA-504-5p and in 13/15 (86.7%) patients for microRNA-429. In Group 3, microRNA-504-5p was detected in 3/3 patients (100%) while microRNA-429 in 2/3 (66.7%) women with a median Ct value of 25.0 and 24.0 respectively.

CONCLUSIONS

This preliminary data demonstrated that this panel of two microRNA could be proposed as a potential biomarker for diagnosis of EC and/or AEH; microRNA-504-5p and microRNA-429 may act as a non-invasive biomarker for early-stage EC. In particular, miroRNA-429 expression could be more related to EC compared to AEH. Further studies are needed in order to validate the role of microRNAs for an earlier and reliable diagnosis.

CHAPTER 1

ENDOMETRIAL CARCINOMA

1.1 Definition and Epidemiology

Endometrial cancer (EC) is a malignancy of the inner epithelial lining of the uterus, with an increasing incidence and disease-associated mortality, worldwide. It occurs when the endometrial cells, lining the myometrium, start to proliferate abnormally, growing too rapidly. Representing the sixth most commonly diagnosed female cancer, its incidence was estimated of approximately 417,336 new cases in 2020, worldwide. Most cases occur in postmenopausal women with a mean age of 61 years, although EC is increasingly affecting younger women, especially for the increased obesity of the population. Fifteen/twenty of diagnosis are made in premenopausal women. Notably, 70% of this young women < 40 years have not yet completed their reproductive desire.

Racial disparity and socioeconomic and geographical differences are important determinants of EC incidence and mortality. Several non-genetic risk factors have been associated with an increased probability to have EC, particularly for the most prevalent histological subtype: endometrioid endometrial adenocarcinoma (EEC), which include obesity, physical

inactivity, excess exogenous oestrogen, insulin resistance, and tamoxifen use after breast cancer.

1.2 Classification

The classification of EC has evolved over time, with the goal of more precisely predicting patient prognosis and guiding management. At the beginning, uterine cancer was subclassified based only on anatomical location, treating tumors from the cervix and uterine corpus as separate entities. With regard to carcinoma of the uterine corpus, in 1983, Bokhman first classified EC on the basis of clinical, epidemiological, metabolic and endocrine features. Two subtypes, Type I and Type II EC, with distinct clinical, pathological, and histological behavior were identified. Type I EC, are mainly low grade, moderately or higly differentiated with favourable outcomes. Being estrogen-dependent, hormone-receptor-positive adenocarcinomas with endometrioid morphology, they are often referred as endometrioid endometrial cancers. It represents the most common type accounting for approximately 85% of all EC usually diagnosed at an early stage and characterized by a good prognostic. Nulliparity and infertility are frequent risk factors for Type I EC. Estrogen therapy that not balanced by the effect of progestins, estrogen- secreting tumors, early menarche and late menopause (with a risk increased twice), represented other risk factors

involved. Conversely, the use of oral contraceptives, and the occurrence of a pregnancy, are protective factors counteracting the onset of disease.

Although these risk factors have been extensively described in the literature, there is currently no evidence on the efficacy of a screening test at an early stage to be extended to the asymptomatic female population at medium risk for Type I EC. The only recommended screening is for women with Lynch Syndrome.

On the other side, Type II EC is characterized by non endometrioid subtypes such as serous, clear-cell, and undifferentiated carcinomas. Not related to hyperestrogenism, they affect non-obese women, often arising in the absence of endocrine and metabolic disturbances. They generally are high-grade, hormone-receptor negative, poorly differentiated, associated with a higher risk of metastatic spread and poor prognosis. Women with type II EC are often multiparous, smokers with a history of breast cancer.

EC is also classified according to histopathological features, with the most common subtypes being endometrioid carcinoma, serous carcinoma, carcinosarcoma and clear-cell carcinoma. Endometrioid adenocarcinomas represent a range of neoplasms, from well to poorly differentiated tumours (ie, low to high grade), whereas serous and clear-cell carcinomas are high grade by definition.

Comparison of classification system of EC are reported in ***Table 1***.

	Bokhman[5]	WHO[6]*	The Cancer Genome Atlas[7]
Basis	Clinical and epidemiological features	Histological features	Genome-wide genomic characterisation
Categories	Type I Type II	Endometrioid Serous Clear cell	*POLE* (ultramutated), MSI (hypermutated), copy-number low (endometrioid), copy-number high (serous-like) Copy-number high (serous-like) NA

MSI=microsatellite instability. *WHO mucinous, squamous-cell, transitional-cell, small-cell, and undifferentiated carcinoma subtypes were not considered in Bokhman's classification.

Table 1. Comparison of classification system.

In support of all previous classifications, molecular data have become an integral component of pathologic evaluation, as endometrioid carcinomas (type I) are preferentially associated with mutations in PTEN (*Phosphatase and Tensin homolog on chromosome* 10), KRAS, CTNNB1 and PIK3CA (*Phosphatidylinositol 3-kinase*) and MLH1 promoter hypermethylation, whereas serous carcinomas (non-endometrioid, type II) show *HER2* amplification, inactivation of the TP16 gene, low expression of E-caderina and recurrent *TP53* mutations.

The need to introduce a new classification covering the genetic and molecular aspects of these tumors has lead the Cancer Genome Atlas (TGCA) Research Network to improve our understanding of the molecular landscape of EC.

Four molecular subtypes were introduced:

1) POLE: ultra - mutated tumours, characterized by unusually high mutations rates of the exonuclease domain of POLE 58, subunit ε of the DNA

polymerase involved in the DNA replication process and a favorable result;

2) MSI hypermutated (microsatellite unstable tumors) a hypermutated group characterized by microsatellite instability secondary to MLH1 promoter methylation and high mutagenicity;

3) copy- number low generally endometrioid G1-G2, it is a group with lower mutation frequency characterized by microsatellite stability with frequent mutations of CTNNB1;

4) copy- number high tumors consists primarily of serous-like cancers characterized by frequent aberrations of the number of gene copies, low mutagenicity, frequent mutations of TP53 and unfavorable outcome.

1.3 Grading and Staging

EC is staged according to the International Federation of Gynecology and Obstetrics system (FIGO) (***Figure 1***). In 1988, they replaced an inaccurate clinical staging system with a surgical staging system, highlighting the importance of histologic

findings. *Stage I* reflect EC that are confined to the uterine corpus. They are further divided into stage IA (no or less than 50% myometrial invasion) and IB (equal to or more than 50% of myometrial invasion). Tumors that invades cervical stromal but does not extend beyond the uterus are

defined as *Stage II*. *Stage III* represents tumor that spread beyond the uterus but not outside the true pelvis. They are further divided in stage IIIA (invade the uterine serosa and/or adnexa), stage IIIB (parametrium and/or vaginal involvement), and stage IIIC1 (positive pelvic nodes) and IIIC2 (positive paraaortic lymph nodes). *Stage IVA* includes tumors with extension to the bladder or bowel and *Stage IVB* tumors with distant metastases. Although 67% of patients present with early-stage disease, which is associated with an 81% 5-year overall survival (OS), the 5-year OS for stage IVA and IVB EC are only 17% and 15%, respectively. Beyond cancer stage, is important to define the grade of it, and so, EC are graded according to the 1988 FIGO classification from grade one to three. Grade 1 tumors exhibit ≤5% solid nonglandular, nonsquamous growth; grade 2 tumors from 6% to 50%; and grade 3 tumors >50%. The main goal of staging classifications is to define groups of patients with similar outlooks to standardise management and allow comparisons of therapeutic strategies. Today, two alternative surgical-pathological classification systems exist, based on surgical staging and including assessment of the extent of myometrial invasion and distant metastatic disease: the 2009 FIGO and the TNM classification.

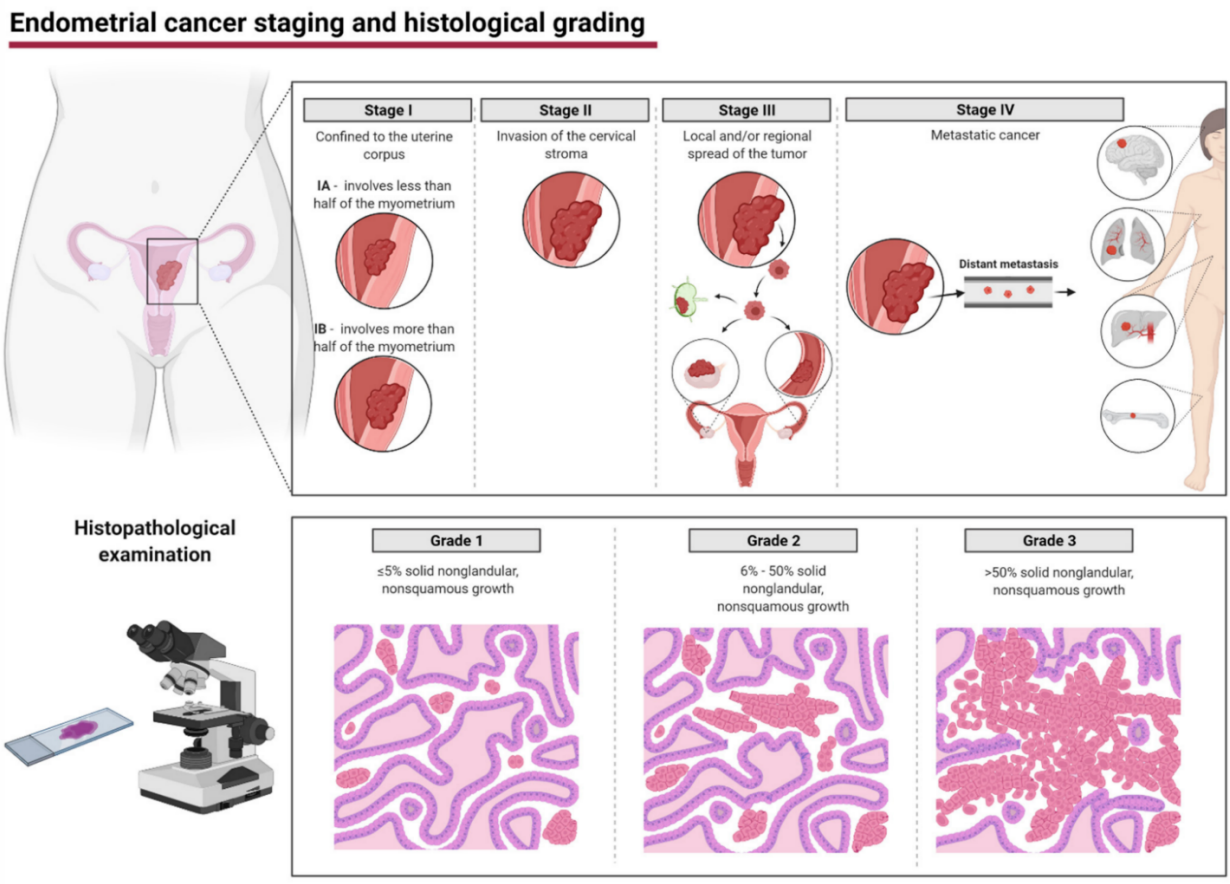

Figure 1. Federation of Gynecology and Obstetrics (FIGO) staging and histological grading of endometrial cancer.

1.4 Diagnosis

1.4.1 Transvaginal ultrasonography

Most guidelines recommend either transvaginal ultrasonography or endometrial biopsy as the initial study for the evaluation of endometrial cancer. Transvaginal ultrasonography is often the initial diagnostic study of choice when evaluating for endometrial cancer because of its availability, cost-effectiveness, and high sensitivity. Transvaginal ultrasonography can be used to measure endometrial thickness. A recent ACOG committee

opinion notes that the cutoff value for a normal transvaginal ultrasonography result should be 4 mm in postmenopausal women. An endometrial thickness greater than 5 mm in this kind of patients, should be evaluated with a tissue sample, especially if bleeding is present. The American College of Radiology uses a cutoff of 5 mm or less. The optimal cutoff for evaluating premenopausal women has not been defined, but recommendations include a cutoff of 16 mm or less. In all patients, if bleeding persists despite a normal transvaginal ultrasonography result, a tissue biopsy is warranted. The ultrasound findings suggestive of malignancy include also heterogeneous endometrial echogenicity, irregularities of the endometrial-myometrial interface and specific features under color or power Doppler (a tortuous and irregular pattern in multiple vessels, with a low resistance velocimetric index) (***Figure 2***).

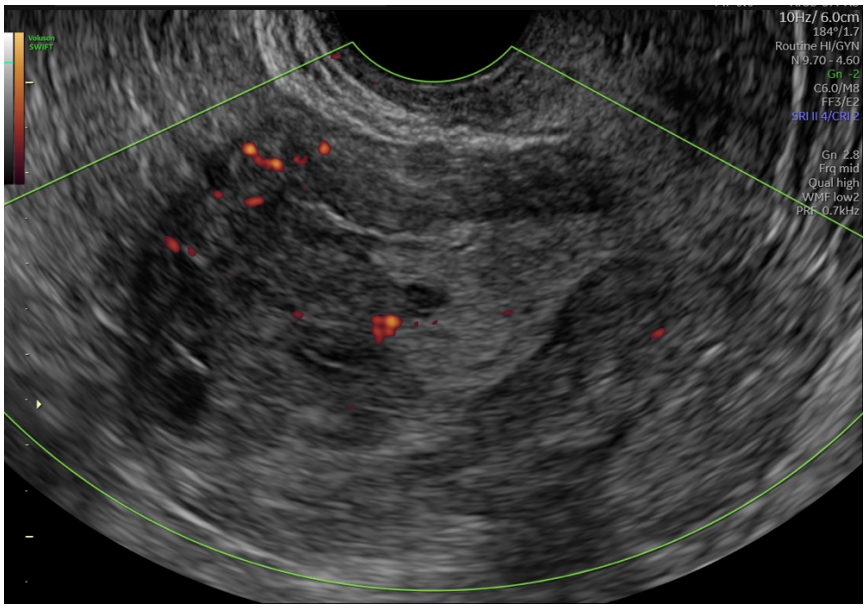

Figure 2. Endometrial cancer at ultrasound.

1.4.2 Hysteroscopy and endometrial biopsy

The gold standard diagnostic procedure for the identification of the intra-uterine pathology is endometrial biopsy. It is carried out thanks hysteroscopy procedure that with direct visualization of the endometrial cavity, it is a better alternative to blind D&C, since it can be performed in an office setting, allowing direct visualization of the lesions, and obtaining targeted biopsies using 5Fr instruments. EC appears hysteroscopically in two types: a circumscribed form and a diffuse form. The circumscribed form most often presents as a polypoid lesion and, more rarely, as an ulceration or a nodular relief limited to a specific endometrial area. Such lesions, unlike benign endometrial polyps, are irregular, friable, and show distinct areas of necrosis and/or hemorrhaging. The diffuse form of endometrial pathology usually occupies a large part of the uterine cavity, and may be due to the spread of a poorly- circumscribed form that begins mainly in the upper third of the cavity, or secondary to a multicentric origin of the tumor. In summary, the specific hysteroscopic features suggestive of an endometrial malignancy are as follows (***Figure 3***):

- whitish, green-gray coloration: the normal endometrial color varies from pale pink to yellowish;
- areas of necrosis, hemorrhage and microcalcification: these findings is strongly suggestive of EC;

- atypical vascularization: diffuse vascular patterns with irregular ramifications or blurred outlines, and inconsistency between the main vascular axis and the lesion's direction of growth;

- irregular or ulcerated surface: whitish thickened areas or surface irregularities or ulcerations should raise a suspicion for malignancy;

- soft consistency: malignant lesions are generally soft in consistency, friable, and susceptible to bleeding on contact with the hysteroscope;

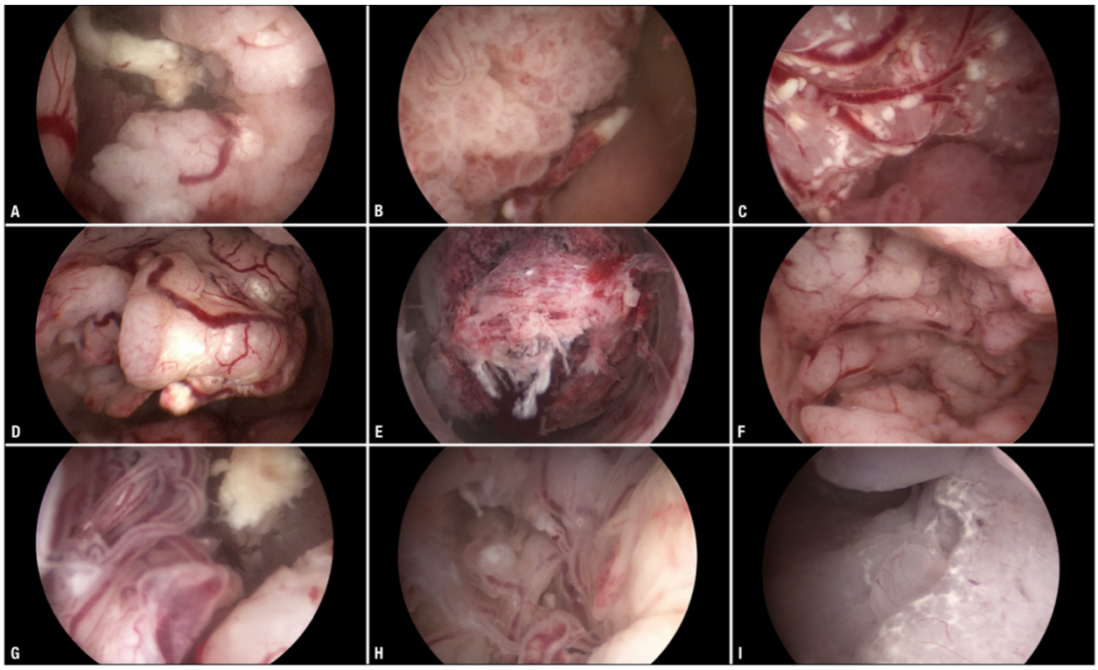

Figure 3. Images of an endometrial carcinoma under hysteroscopic examination.

The proper and complete description of a lesion suggestive of malignancy must also take into account the following aspects: intracavitary tumoral extension, invasion of the cervical canal and patterns of tumoral growth (nodular, polypoid, papillomatous). Recent studies have shown that the

morphology of the tumor does not have any significant correlation with its prognosis.

The diagnostic accuracy of hysteroscopy is improved by targeted endometrial biopsy sampling, yielding a sensitivity of 97.5%, and a specificity of 100%. There are several techniques of endometrial biopsy. In 2002, Bettocchi et al. introduced a new technique defined as "*grasp biopsy*" with the objective of removing a larger amount of endometrial tissue, sufficient for a proper histological analysis (***Figure 4***)

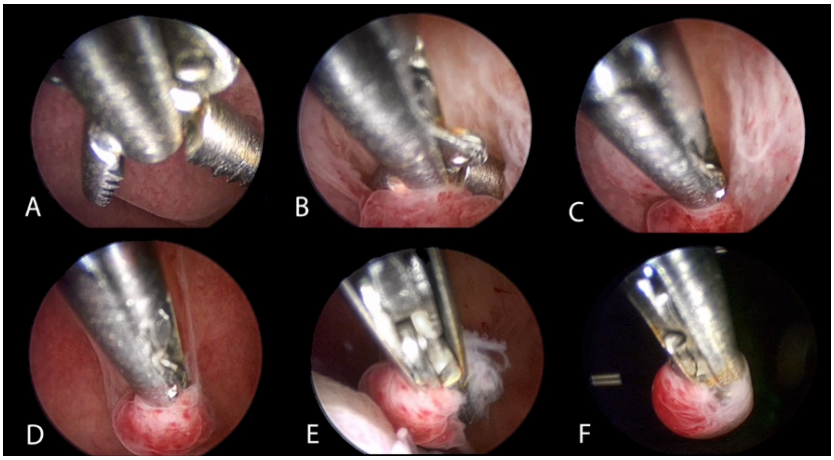

Figure 4. Endometrial grasp biopsy technique.

Recently, Di Spiezio A et al. investigated the accuracy of endometrial biopsy performed with hysteroscopic direct visualization using the "*grasp technique*" for the detection of endometrial cancer histology type and tumor grade. They demonstrated that preoperative hysteroscopic guided "grasp biopsy" provides a more accurate diagnosis of EC histology type

and tumor grade when in presence of endometrioid type tumor compared to blind endometrial biopsy obtained using the Novak curette.

1.5 Treatment

The standard treatment for endometrial carcinoma and its precursors is total hysterectomy with bilateral salpingo-oophorectomy, and surgical staging; while achieving excellent survival outcomes, this remains a radical treatment devastating for women, especially if still eager for offspring. Therefore, fertility-sparing treatment approaches for women who desire offspring are now well established worldwide.

CHAPTER 2

MICRO-RNA

2.1 MicroRNA Biogenesis

MicroRNAs are a class of small RNAs that are important regulatory molecules in plants, animals and viruses. They are very short, ~ 20–25 nucleotides, non-coding RNA.

They were discovered in 1993 during the studies carried out on Caenorhabditis elegans and then it was expeditiously recognized that existence of distinctly conserved miRNA sequences caper a paramount role in regulatory pathways among unicellular and multicellular eu-karyotic organisms. The canonical role of these small non-coding RNAs is to influence messenger RNA (mRNA) via recognition sites in the 3′untranslated region (UTR), which regulates their stability. microRNAs primarily affect gene expression levels via targeting mRNA. Any changes in microRNA expression may affect the extent of target regulation, and thus influence cell homeostasis. Therefore, the relative levels of microRNA, and consequently mRNA, have a major role in carcinogenesis and other diseases. It has become clear that microRNAs regulate several key cellular processes including developmental timing, stem cell division and apoptosis. MicroRNAs likely influence these processes by post-transcriptional negative

regulation of gene expression through binding to messenger RNA (mRNA) targets, causing mRNA cleavage, translational repression, or mRNA decay. The biogenesis of microRNAs follows a series of cleavage stages in the nucleus and in the cytoplasm (***Figure 5***). The primary (pri)-miRNA transcript is cleaved in the nucleus by Microprocessor, a catalytic complex composed of Drosha and Di George critical region 8 (DGCR8). Recent reports have shown that the stem-looped pri-miRNA is correctly oriented for cleavage through the interaction of Drosha with the basal UG motif and alignment of the DGCR8 dimer with the apical UGU motif. Microprocessor cleavage forms precursor (pre)-microRNA, which is transported into the cytoplasm by exportin-5. It is here that Dicer (also known as DICER1) cleaves pre-microRNA, and the resulting double-stranded mature microRNA is subsequently bound by Argonaute (AGO). The guide strand remains bound to AGO to form the miRNA-induced silencing complex (miRISC), whereas the passenger strand, denoted as microRNA*, is removed and degraded.

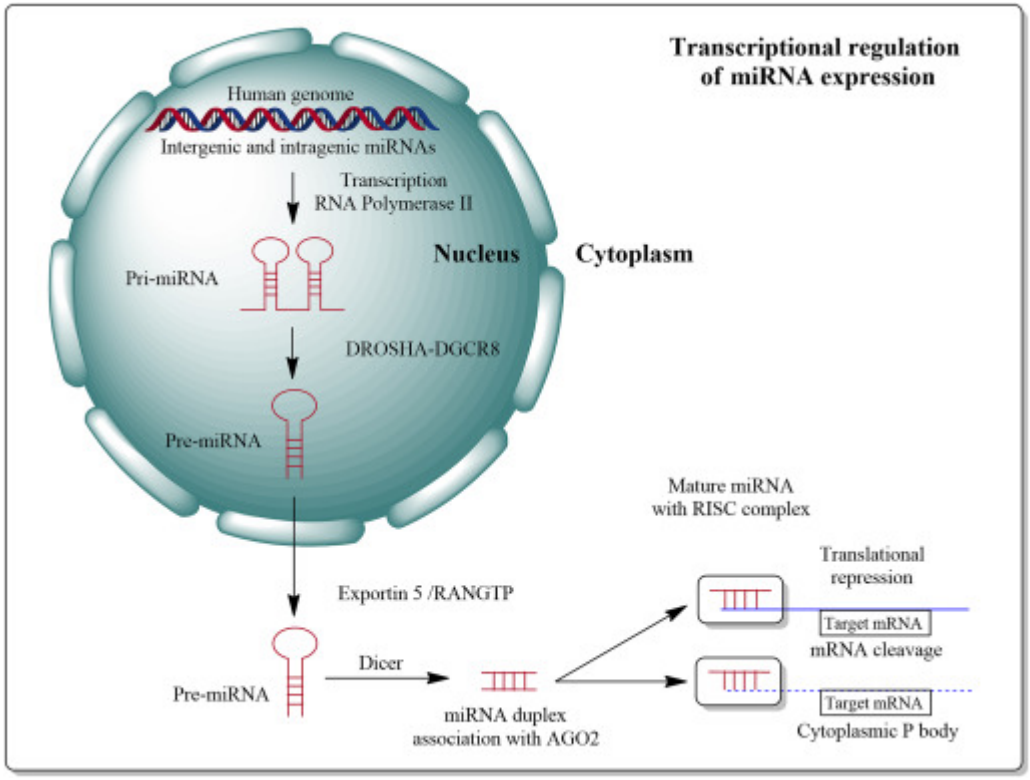

Figure 5. MicroRNA biogenesis and role in gene regulation.

The main role of miRISC is to enable the RNA interference (RNAi) pathway, whereby the seed region of the microRNA, spanning nucleotides 2-8 from the 5′ end, recognises Watson–Crick complementary binding sites in the 3′UTR of mRNA. Although the main role of microRNAs is to perform post-transcriptional gene regulation, their control of other non-coding RNAs has reshaped our understanding of RNA biology. microRNAs have been found to interact with long non-coding RNAs (lncRNAs), circular RNA (circRNA) and pseudogenes to either induce microRNA suppression or increase cellular competition for microRNA binding sites.

2.2 MicroRNA and Carcinogenesis

Mutations that generate oncogenes or that alter the functions of tumor suppressor genes are crucial for the development of a tumor, but are not sufficient to explain all the changes that accompany the conversion from normal to tumor cell. Many of the properties of cancer cells are not caused by mutations, but by epigenetic modifications that involving microRNAs. In addition to being produced in altered quantities in cancer cells, some microRNAs can be themselves changed. The cause of the different expression of microRNA genes among the malignant and normal cells can be explained by different mechanisms, including:

a) chromosomal alterations involving regions containing microRNA genes;
b) DNA point mutations;
c) epigenetic mechanisms;
d) alterations of the machinery responsible for microRNA production.

The development of different technologies has allowed the characterization of expression profiles of microRNAs for several cancers, including chronic lymphocytic leukemia, breast cancer, cancer of lung, papillary thyroid carcinoma, pancreatic tumors, glioblastoma, the gastric cancer, prostate cancer, hepatocellular carcinoma. Several studies suggest the presence of alterations in some microRNAs which could therefore

identified as new diagnostic and/or prognostic markers of EEC. It has already been demonstrated that MicroRNA-200 Family Is Upregulated in EC playing a role as biomarkers for cancer and as markers for prognostication. Cascione et al. revealed that the cluster miR-17/92 and miR-200 family were upregulated, while two members of the let-7 family (let-7b and let-7c) were downregulated from the 116 deregulated miRNAs in the first set of primary Triple Negative Breast Cancer and normal tissues. A recent study in lung cancer cells found that the tumour suppressor miR-660-5p controls the expression of miR-486-5p via mouse double minute 2 (MDM2) and p53 (also known as TP53). In this model, miR-660 silences its direct target *MDM2*, which consequently results in an increase in p53. Therefore, this network demonstrates the wider impact of microRNA:microRNA modulation via their control of transcriptional regulation. A study in tongue squamous cell carcinoma tissues demonstrated that miR-29b downregulates the DNA methyltransferase gene *DMNT3B*, which in turn alters the methylation pattern of the miR-195 promoter. This induces an increase in miR-195 production, generating a positive regulatory system in which upregulation of miR-29b increases the levels of miR-195. As both microRNAs are tumour suppressors that are downregulated in cancer, this mechanism may offer a therapeutic window for tongue squamous cell carcinomas. These examples show how indirect control of

microRNAs via transcription factors, promoters and epigenetics has wider implications on microRNA expression, and the capacity to influence several cellular pathways, including those in cancer development.

2.3 MicroRNA and Endometrial carcinoma

Several studies suggest the presence of alterations in some microRNAs which could therefore identified as new diagnostic and/or prognostic markers of EEC. Since their discovery, it has become clear that microRNAs regulate several key cellular processes including developmental timing, stem cell division and apoptosis. Therefore, microRNAs have the potential to act as diagnostic and prognostic biomarkers of EC, as demonstrated in a multitude of studies. Extracellular Vescicals (EV) facilitate cellular crosstalk and show selective microRNA packaging, and, as a result, EV, tissue and extracellular microRNA biomarkers can have differing performance and predictive ability. Fan et al. demonstrated this in the EC context, identifying a six miRNA whole serum signature with diagnostic potential, with only one of these microRNAs showing differential abundance in enriched serum EVs. Multiple studies have identified EV microRNAs that are differentially abundant in EVs between EC patients and healthy controls. Jaime Snowdon et al. identified 43 microRNAs that are dysregulated in EEC and AEH compared to normal controls.

Clustering analysis shows that these 43 microRNAs can differentiate EEC from both AEH and normal controls. Furthermore, the dysregulated microRNAs show intermediate expression level changes in the precursor lesion, AEH. In their study the most striking similarity is the up-regulation of the miR-200 family (miR-141, 200a, 200b, 200c, 429) in EEC relative to normal controls. The microRNA-200 family consists of five members localized on two genomic clusters (microRNA-200a/b, microRNA-429 on chromosome 1, and microRNA-200c, microRNA-141 on chromosome 12). The microRNA-200 family has been implicated in the epithelial-to-mesenchymal transition (EMT) that occurs as a part of tumour invasion and metastasis.

Increased expression of oncogenic microRNAs in cancerous cells inhibits tumor suppressor genes. Decreased expression of tumor suppressor microRNAs potentially enhances the expression of oncogenes. Consequently, both oncogenic and tumor suppressor microRNAs lead to tumor-development by stimulating cell proliferation, antiapoptotic response, invasion, metastatis and angiogenesis (***Figure 6***).

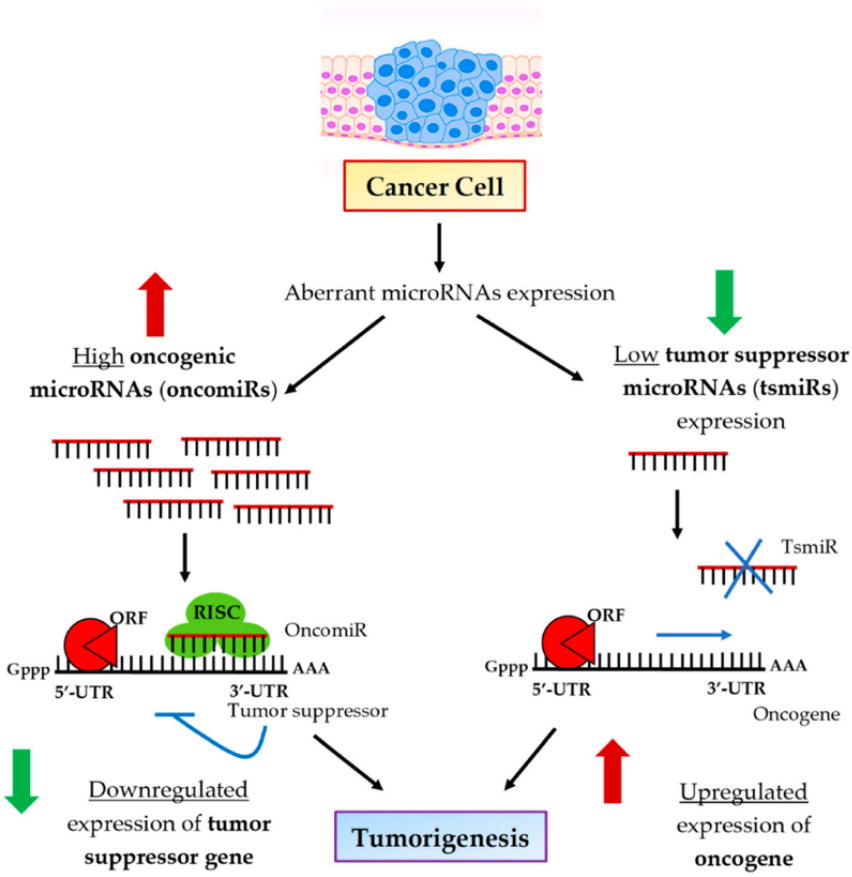

Figure 6. Regulatory mechanism of oncogenic and tumor suppressor microRNAs in tumorigenic events.

CHAPTER 3

OBJECTIVE

This study aimed to establish the diagnostic accuracy of microRNA panel in a series of diagnostic routine endometrial cancer patients (EC). Moreover, negative and positive predictive value for clinical stratification of EC in diagnostic stage was assessed.

CHAPTER 4

MATERIALS AND METHODS

4.1 Study design

This study was designed as a perspective observational study in accordance with STROBE guidelines. For each patient, microRNAs expression profile was evaluated in order to distinguish common molecular signatures in EC patients from control group. Clinical and molecular records were anonymously collected in internal database. All the managing and clinical procedures were performed in accordance with Helsinki Declaration and the Good Clinical Practice Guidelines; each patient had received detailed information about the study and signed informed consent.

Institution Review Board (IRB) approval was obtained before initiating the study.

4.2 Patients

Patients diagnosed with either AEH or EC of each histotype and grading at endometrial biopsy obtained under hysterosopic direct visualization, meeting the following inclusion criteria were invited to participate: age > 18 years, absence of malignancy and other medical intercurrent conditions such as autoimmune disorders and metabolic disease. Exclusion criteria

included: age <18 years, presence of other gynecologic pathology (i.e. polyps, myomas), previous hormonal therapy with Tamoxifen, Progesterone or Estro-Progestin.

All data were collected in a dedicate database, including: age, BMI, family history of EC, tumor characteristics of the lesion at the hysteroscopic direct visualization including pattern of tumor growth, intracavitary tumor extensions, vascularization.

To further validate our findings, we included a group of patients with the same characteristics of the study populations who presented a negative endometrial pathology histology obtained at endometrial biopsy.

4.3 Surgical procedure

All patients performed office hysteroscopies with a vaginoscopic approach and continuous flow hysteroscopes with a foreblique view and an operating 5 Fr channel (Office Continuous Flow Operative Hysteroscopy "size 5" or "size 4", Karl Storz, Tuttlingen, Germany). Normal saline was used as a distension media, and constant intrauterine pressure of 30–40 mmHg was maintained by a fluid pump-machine (Endomat, Karl Storz, Tuttlingen, Germany). No analgesics or anesthetics were administered before, during or after the hysteroscopic procedure which was performed in

ambulatory outpatient setting without cervical dilatation. Through direct identification of endometrial lesions suggestive of EC, targeted endometrial biopsies were collected with the hysteroscopic "grasp biopsy" technique as follows: using a hysteroscopic 5Fr toothed grasping forceps with the jaws opened, the instrument was brought in near contact to the target area desired to biopsy; then, the opened jaws of the forceps were advanced, "plowing" along with the tissue for about 0.5–1 cm. At this point, the jaws were closed, catching the piece of endometrial tissue, which was then retrieved from the uterine cavity together along with the hysteroscope, without, retracting the tip of the forceps into the operating channel. In cases where the endometrium was diffusely thickened and irregular, a new technique called "Visual D&C" was performed. This consisted of the use of Tissue Removal Device as a type of atraumatic curettage which obviates the need for using electric current while offering the added benefit of direct vision of the uterine cavity.

4.4 Technical Analysis

4.4.1 *FFPE microdissection and RNA extraction*

For each patient, a formalin fixed paraffin embedded specimen (FFPE) was available. Overall, a set of n=5 sections of 5 μm were prepared to

perform total RNA extraction following manufacturer instructions. Briefly, a representative slide was stained with Hematoxlin and Eosin (H&E) to evaluate neoplastic, hyperplastic area and cell content in healthy control group. Each E/E stained slide was digitalized and collected in virtual archive of Predictive Molecular Pathology Unit of University of Naples Federico II. Remaining slides were adopted to perform RNA extraction. RNA was manually extracted by using QIAsymphony RNA Kit (Qiagen, Hilden, Germany) on QIA symphony (Qiagen, Hilden, Germany) automatic platform following standardized procedures. Finally, RNA was resuspended in 60 µl of RNAse free water and immediately stored at -20ºC until molecular analysis.

4.4.2 PCR Real Time Analysis

Each patient was analyzed by using a RT-PCR based system to evaluate miRNAs expression profile. In details, RNA samples were prepared for retro-transcriptin by approaching poly (A) tailing and the adaptor ligation reactions. Then, samples were retro-Transcripted following the manufacturer instructions of Taqman Advanced miRNA Assay (Thermo Fisher Scientific, MA, USA). In addition, complementary-DNA (cDNA) was diluted 1:5 and a final volume of 20 µl was amplified on QuantStudio 5 platform (Applied Biosystems; Thermo Fisher) following manufacturer

thermal conditions: Enzyme activation 95°C for 20 seconds repeated for 1 cycle; denaturation at 95°C for 1 second and annealing at 60°C for 20 seconds (X40). As regards, amplification plot, Ct values for miR 504-5p and miR 429 were inspected. Data interpretation was carried out by using proprietary software (Quant Studio Design Analysis Software v.1.5.2, Thermofisher Scientifics).

CHAPTER 5

RESULTS

A total of 33 patients were enrolled. Specifically, 15/33 (45.5%) were diagnosed with EC, 15/33 (45.5%) with AEH and 3/33 (9%) with normal endometrium. The main baseline characteristics of the patients are reported in ***Table 2.*** All the endometrial biopsy provided an adequate specimen for the evaluation of microRNA expression.

CHARACTERISTICS	**N = 33**
Age, yr (mean ± SD)	54.16 ± 13.78
BMI, kg/m2 (mean ± SD)	29.67 ± 7.11
Caucasian Ethnicity, n (%)	33/33 (100 %)
Premenopausal patients, n (%)	13/33 (39.4 %)
Postmenopausal patients, n (%)	20/33 (60.6 %)
Nulliparity, n (%)	10/33 (30.3 %)
Pluriparity, n (%)	23/33 (69.7 %)
Vaginal bleeding, n (%)	26/33 (78,8 %)

Table 2. Baseline patients characteristics

Molecular results

Overall, miR 429 n=15/15 (100%) was successfully analyzed in all instances while 504-5p failed in a single case (n=14/15; 93,3%). Data showed a median Ct value of 32.2 (ranged from 29.0-35.0) and 29.7 (ranged from 24.0 to 35.0) for miR 504-5p and miR 429 analysis, respectively (***Table 3***).

Regarding Hyperplastic tissue samples, a successful analysis was observed in 14 out 15 93.3%) and 13 out of 15 (86.7%) for miR 504-5p and miR 429 cases, respectively (***Table 4***).

Regarding the normal tissue samples miR 504-5p was successfully analyzed in all cases, while miR 429 was successfully amplified in 2 out3 cases (66.7%) with a median Ct value of 25.0 (ranging from 23.0 to 28.0) and 24.0 (ranging from 23.0 to 26.0) (***Table 5***).

ID	Age	FFPE ID	Diagnosis	Ct miRNA 504-5p	Ct miRNA 429
1	66	I/2021/15268	Endometrial Ca (G2)	32	29
2	58	I/2022/1313	Endometrial Ca (G3)	29	31
3	64	I/2022/684	Endometrial Ca (G3)	31	30
4	75	I/2022/685	Endometrial Ca (G3)	32	31
5	79	I/2022/7822	Endometrial Ca (G3)	Not Amplified	35
6	62	I/2022/3913	Endometrial Ca (G3)	34	26
7	52	I/2022/2896	Endometrial Ca (G3)	35	28
8	55	I/2021/14963	Endometrial Ca (G2)	34	27
9	59	I/2021/15278	Endometrial Ca (G3)	32	30
10	80	I/2021/12111 A1	Endometrial Ca (G3)	31	24
11	79	I/2022/8991	Endometrial Ca (G3)	32	26
12	70	I/2022/4673	Endometrial Ca (G2)	31	33
13	54	I/2022/4678	ADC	31	35
14	59	I/2022/33	Endometrial Ca (G2)	32	29
15	55	I/2022/10833	ADC	35	32

Table 3. List of Ct Values from miRNA 504-5p and miRNA 429 amplification in EC patients.

ID	Age	FFPE ID	Diagnosis	Ct miRNA 504-5p	Ct miRNA 429
16	60	I/2021/14857	Atypical hyperplasia	30	32
17	48	I/2022/879	Atypical hyperplasia	31	Not Amplified
18	60	I/2022/2633	Atypical hyperplasia	30	Not Amplified
19	46	I/2021/15912	Atypical hyperplasia	30	31
20	49	I/2022/1845	Atypical hyperplasia	29	28
21	50	I/2021/11965	Atypical hyperplasia	Not Amplified	32
22	54	I/2021/15508	Atypical hyperplasia	30	31
23	43	I/2022/1311	Atypical hyperplasia	29	32
24	50	I/2022/1936	Atypical hyperplasia	28	35
25	30	I/2022/7071	Atypical hyperplasia	27	24
26	36	I/2022/6954	Atypical hyperplasia	25	27
27	35	I/2022/2983	Atypical hyperplasia	30	29
28	73	I/2022/10153	hyperplasia	26	27
29	51	I/22/16770	hyperplasia	26	30
30	61	I/2022/2942	Atypical hyperplasia	26	29

Table 4. List of Ct Values from miRNA 504-5p and miRNA 429 amplification in Hyperplastic endometrial patients

ID	Age	FFPE ID	Diagnosis	Ct miRNA 504-5p	Ct miRNA 429
31	37	I/2021/16033	Normal	28	Not Amplified
32	33	I/2021/16239	Normal	24	26
33	38	I/2022/14460	Normal	23	23

Table 5. List of Ct Values from miRNA 504-5p and miRNA 429 amplification in control group

CHAPTER 6

DISCUSSION

To date, microRNAs represents an heterogeneous group of non-codifying RNA that play a crucial role in physiological cell homeostasis. Despite their small size, they can regulate the expression of hundreds of target genes. Indeed, when this regulatory complex is damaged, cell stability is not preserved and several death mechanisms (e.g. apoptotis) are activate.

Several literature efforts underline how microRNAs may drive cell homeostasis. In particular, microRNAs deregulation may represent a leading effect in cancer development. The first example of microRNAs found to be aberrantly expressed in cancer patients was miRNA-15a and 16-1, which are clustered at chromosome 13q14, a frequently deleted region in B cell chronic lymphocytic leukemia (CLL). It was observed that a decreasing level of these two microRNAs was significantly found in tumor samples than matched normal controls.

Recently, several studies have elucidated the role of microRNAs expression profile in carcinogenesis processes of several types of solid tumors, in which they may act as tumor suppressors or as oncogenes, such as breast cancer, colorectal cancer and endometrium carcinoma.

As regards, great attention was paid to identify the action of miRNA-related pathways in Endometrial Cancer patients. These efforts aimed to evaluate diagnostic attitude of microRNAs profile in clinical setting of solid tumor patients. Then, emerging considerations encouraged the application of microRNAs as clinically valuable diagnostic biomarkers in tumor patients.

Due largely to the rise in obesity and prolonged life expectancy, Endometrial Cancer diagnosis showed an increasing rate to 56% since the early 90s. Unfortunately, lack of diagnostic sensitivity screening approaches is routinely available. This criticism reduces the number of patients that could benefit from tempestive clinical strategy.

In this regard, our study tries to identify the dysregulated patterns of expression of two kind of microRNAs leading useful information on the biological processes involved in the development or progression of Endometrial Atypical Hyperplasia and Endometrial Cancer. These microRNAs derive from an accurate selection of microRNA that have demonstrated a pivotal role in the guidance of tumor processes. We set out to find microRNAs that might differentiate between EC and AEH and differentiate both from normal controls.

Interestingly, We identified two microRNAs that resulted dysregulated in EC and AEH. A more accurate analysis revealed that these microRNAs

can differentiate EC from both AEH and normal tissue. Indeed, microRNA-504-5p and microRNA-429, show intermediate expression level changes in AEH, that represent the precursor lesion.

Of note, a previous review conducted by Donkers H et al. in 2020, evaluated the overall diagnostic accuracy of microRNAs in detecting endometrial cancer. They included 26 studies and reported the most frequently upregulated microRNAs: microRNA-205, microRNA-200 family, microRNA-135b, -182, -183 and 223. However, they pointed out that some of the same microRNAs are also upregulated in colorectal cancer, therefore suggesting the need to associate the presence of specific symptoms such as abnormal or postmenopausal bleeding for the diagnosis of Endometrial Cancer.

Donkers et al. also reported in their review that there was less consensus in the literature about down-regulated microRNAs, which are counted to be 44.

Indeed, just as several microRNAs are found to be upregulated, as many microRNAs are found to be downregulated in cancer samples compared to normal tissue. MicroRNAs have different behavior towards cancer development, they can be tumor promoters and tumor suppressor. Reports from several studies have demonstrated that different group of microRNAs appear to have a positive or negative influence on development

and progression of Endometrial Cancer, implying that a reduction or elimination of tumor suppressor microRNA or an amplification or up-regulation of a microRNAs oncogenicity may play a role in the onset and/or progression of this type.

In our series, microRNA-504-5p and microRNA-429 have been investigated in tissue sample of patients with AEH, EC or without any pathologies (normal proliferative endometrium). Our data share some important similarities with previously published reports, demonstrating that these two microRNAs are related to the diagnosis of AEH and EC. Moreover, their expression level was significantly lower in patients with AEH than those in healthy controls. Furthermore, they were found to be expressed at even lower levels in patients with Endometrial Cancer compared to those with AEH.

These data represent encouraging findings because the new WHO classification is more likely to successfully identify premalignant uterine lesions, the low interobserver reproducibility among gynecological pathologist in diagnosing AEH, distinguishing it from EC, should be improved. In fact, molecular analysis in EC patients may be considered a valid strategy to consider the evaluate the best clinical administration of diagnostic routine patients.

To our knowledge, there are few studies in literature that have identified microRNAs able to discriminate between AEH and EC.

Our results support the concept that these are microRNAs that control an oncogenic protein, therefore the downregulation of microRNA-504-5p and microRNA-429 in patients with AEH and even more in those with EC compared to healthy controls, results a lack of control over a protein which, when overexpressed, determines the onset or progression of the tumor.

Conversely, Donkey et al. obtained different results, reporting that micro RNA-429 resulted upregulated in EC.

Member of microRNA-200 family, microRNA-429 is involved in the epithelial-mesenchymal transition, progression, development, invasion, metastasis of a variety of cancers, reporting a different behavior depending on the type of tumor in which it is involved. Therefore, may specifically work as a tumor suppressor for breast cancer or gastric cancer, while it has a tumor-promoting role in endometrial cancer and lung cancer. Castilla et al. have demonstrated differential microRNA expression levels between the epithelial and mesenchymal elements in uterine carcinosarcoma, with up-regulation of the miR-200 family in the epithelial part.

Recent studies have also reported that low microRNA-200 family levels are associated with a more aggressive tumor phenotype. In our study, there

was no significant difference in microRNA levels between lower grade 1 and 2 tumors compared to grade 3 tumors, maybe because of the small sample size not sufficient to see such a difference.

Regarding microRNA-504, multiple evidence has suggested that it is dysregulated in several type of tumors, functioning as an oncogenic microRNA or a tumor suppressive microRNA. According to Quan H. et al, this microRNA plays an important role in hepatocellular carcinoma development and progression. To our knowledge, no other studies have indicated its role in endometrial cancer.

Although not completely in line with previous studies, our findings demonstrated to be valid in diagnosis of AEH and EC, and useful to differentiate between tumor and precursor lesions.

6.1 Future Perspectives

The major challenge in the field of endometrial cancer is the lack of screening tests and the presence of minimally invasive methods for early detection of EC. Another challenging point concerns with the scant diagnostic material available in diagnostic routine practice. As regards, low quality nucleic acids extracted from biopsy sample may drastically impact on the successful rate of molecular techniques. This controversial point

may be solved applying optimized diagnostic workflow based on high-sensitive technologies.

The first step for the diagnosis of EC and AEH is transvaginal ultrasound, but it is established in literature that the final diagnosis is based only on histological features. This is why endometrial sampling continues to play a key role in the diagnostic work-up of this pathology. Nowadays, endometrial biopsy performed using hysteroscopic grasp technique represents the best choice.

However, hysteroscopic approach represents, even if minimally, an invasive technique that is still not easily acceptable as a routine examination. Therefore, we are in continuous search for novel approaches to overcome this problem and the emergence of the role of microRNA in cancer field has illuminated us on this topic. The identification of validated and non-invasive diagnostic biomarkers to reduce the number of women who undergo invasive diagnostic testing, such as microRNA could be useful, as well as prognostic marker that can aid timing of treatment for endometrial hyperplasia. The results we obtained in our study, recognized their role as potential biomarkers in the early detection of EC.

As reported in literature, they can be extracted for analysis from blood, plasma, serum and other different body fluids, such as urine. This point represents an important advantage of this small but powerful molecules.

Their capability to be detectable all these different tissues, opens future perspective on the use of the microRNAs as biomarkers for early detection of endometrial cancer.

As well as adding clues about the biological processes that lead to the development or progression of cancer and providing rapid methods for diagnostic or prognostic testing, the identification of these markers can lead to the identification of novel and specific druggable targets.

On the basis of our data, we have probably identified a future therapeutic target, therefore if we induce the expression of this microRNA from the outside, we can bring down the protein levels keeping the development of the lesion under control. In fact, numerous microRNAs influence the growth of cancer cells when overexpressed or inhibited. This means that the growth of cancer cells can be controlled by manipulating microRNAs. Synthetic microRNA mimics can be used to achieve overexpression or inhibition of microRNA.

CHAPTER 7

CONCLUSION

The identification of new biomarkers which could be used for risk stratification and early detection strategies in the future is of particular significance in view of the rise in EC incidence and mortality. Worldwide, endometrial cancer represents 4% of all cancers in women.

Our preliminary data demonstrated that this panel of two miRNA could be proposed as a potential biomarker for diagnosis of EC and/or AEH; microRNA-504-5p and miccroRNA-429 may act as a non-invasive biomarker for early-stage EC. In particular, miRNA 429 expression could be more related to EC compared to AEH.

Our study has harnessed the power of microRNAs as master regulators of gene expression to develop a biomarker assay for early detection of EEC and AEH.

However, it is too soon to recommend the routine use of microRNAs in EC diagnosis.

Moreover, a greater number of well-designed preclinical studies are required to explore the promising potential to the full extent, so that this novel approach would become realistic.

REFERENCES

1. ACOG practice Bullettin, Clinical management Guidelines for Obstetrician-Gynecologists; management of endometrial cancer. Obstetrics & Gynecology, number 65, August 2005.
2. Aghanoori, MR, Mirzaei B, Tavallaei M. MiRNA molecular profiles in human medical conditions: connecting lung cancer and lung development phenomena. Asian Pac. J. Cancer Prev. 2014;15(22):9557-65.
3. Ali Syeda Z, Langden, SSS, Munkhzul C et al. Regulatory mechanism of MicroRNA expression in cancer. Int. J. Mol. Sci. 2020 Mar 3;21(5):1723.
4. Amant F, Mirza MR, Koskas M et al. Cancer of the corpus uteri. Int J Gynaecol Obstet. 2018 Oct; 143 Suppl 2:37-50.
5. Associazione Italiana di Oncologia Medica (AIOM). Linee guida: neoplasie dell'utero: endometrio e cervice. Agg Settembre 2022.
6. Bettocchi S, Di Venere R, Pansini N et al. Endometrial biopsies using small diameter hysteroscopes and 5 Fr instruments: how can we obtaine enough material for a correct histologic diagnosis? J Am Assoc Gynecol Laparosc. 2002 Aug;9(3):290-2.
7. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004 Jan 23;116(2): 281–97.
8. Borzi C, Calzolari L, Centonze G et al. mir-660-p53-mir-486 Network: a new key regulatory pathway in lung tumorigenesis. Int. J. Mol. Sci. 2017 Jan 23;18(1): 222.

9. Braun MM, Overbeek-Wager EA, Grumbo RJ. Diagnosis and Management of Endometrial Cancer. Am Fam Physician. 2016 Mar 15;93(6):468-74.
10. Cascione L, Gasparini P, Lovat F et al. Integrated microRNA and mRNA signatures associated with survival in triple negative breast cancer. PLoS ONE. 2013;8:e55910.
11. Cheng LC, Tavazoie M, Doetsch F. Stem cells: from epigenetics to microRNAs. Neuron 2005 May 5;46(3): 363–367.
12. Creasman, W. Revised FIGO staging for carcinoma of the endometrium. Int J Gynecol Obstet. 2009 May;105(2):109.
13. Di Leva G, Croce CM. miRNA profiling of cancer. Curr Opin Genet Dev. 2013 Feb; 23(1): 3–11.
14. Di Spiezio Sardo A & Campo R. State-of-the-art Hysteroscopic Approaches to Pathologies of the genital trac. Endo Press 2021.
15. Di Spiezio Sardo, M.C. De Angelis, L. Della Corte, et al., Should endometrial biopsy under direct hysteroscopic visualization using the grasp technique become the new gold standard for the preoperative evaluation of the patient with endometrial cancer ? Gynecol Oncol. 2020 Aug;158(2):347-53.
16. Donkers H, Bekkers R and Galaal K. Diagnostic value of microRNA panel in endometrial cancer: A systematic review. Oncotarget. 2020 May 26;11(21):2010-23.
17. Duska LR, Garrett A, Rueda BR, et al. Endometrial cancer in women 40 years old or younger. Gynecol Oncol 2001; 83: 388–93.
18. Ellenson LH, Ronnett BM, Kurman RJ. Precursor lesions of endometrial carcinoma In: Kurman RJ, Ellenson LH, Ronnett BM (eds).. Blaustein's Pathology of the Female Genital Tract. Boston, MA: Springer, 2011;359–392.

19. Epstein E, Ramirez A, Skoog L et al.Transvaginal sonography, saline contrast sonohysterography and hysteroscopy for the investigation of women with postmenopausal bleeding and endometrium > 5 mm. Ultrasound Obstet Gynecol 2001;18:157-62.
20. Evans-Metcalf ER, Brooks SE, Reale FR et al. Profile of women 45 years of age and younger with endometrial cancer. Obstet Gynecol. 1998 mar; 91(3):349-54.
21. Faria SF, Devine CE, Rao B et al. Imaging and Staging of Endometrial Cancer, Semin Ultrasound CT MRI.2019 Aug;40(4):287-94.
22. Gebert, LF and Macrae IJ. Regulation of microRNA function in animals. Nat. Rev. Mol. Cell Biol. 2019 Jan;20(1):21-37.
23. Giglio S., Annibali V, Cirombella R., et al. miRNA as candidate biomarker for the accurate Detection of atypical endometrial Hyperplasia / Endometrial Intraepithelial Neoplasia. Front. Oncol. 2019 Jun 21;9:526.
24. Guo CM, Liu SQ, Sun MZ. miR-429 as biomarker for diagnosis, treatment and prognosis of cancer and its potential action mechanisms: a systematic review. Neoplasma. 2020 Mar; 67(2):215-28.
25. Hatfield SD, Shcherbata HR, Fischer KA et al. Stem cell division is regulated by the microRNA pathway. Nature 2005 Jun 16;435(7044):974-8.
26. Henley SJ, ward Em, Scott S et al. Annual report to the nation on the status of cancer, part I: National cancer statistics. Cancer 2020 May 15;126(10):2225–49.
27. Hill M and Tran N. miRNA interplay: mechanisms and consequences in cancer. Dis Model Mech. 2021 Apr 1;14(4):dmm047662.

28. Hutt S, Tailor A, Ellis P, et al. The role of biomarkers in endometrial cancer and hyperplasia: a literature review. Acta Oncol. 2019 Mar; 58(3):342-52.
29. Iqbal MA, Arora S, Prakasam G et al. MicroRNA in lung cancer: role, mechanisms, pathways and therapeutic relevance. Mol Aspects Med. 2019 Dec;70:3-20.
30. Jia, LF, Zheng, YF, Lyu MY et al.miR-29b upregulates miR-195 by targeting DNMT3B in tongue squamous cell carcinoma. Sci. Bull. 61, 212-219. 10.1007/s11434-016-1001-6.
31. Klicka K, Grzywa TM, Klinke A et al. The role of miRNAs in the regulation of endometrial cancer invasiveness and metastasis – a systematic review. Cancers (Basel). 2021 Jul 6;13(14):3393.
32. Kurman R, Carcangiu M, Herrington C. World Health Organisation Classification of Tumors of Female Reproductive Organs, 4th edn Lyon France: International Agency for Research on Cancer (IARC) Press, 2014.
33. Lee YS and Dutta A. MicroRNAs in cancer. Annu rev Pathol. 2009;4:199-227.
34. Lee, R.C., Feinbaum, R.L., et al., 1993. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75 (5), 843–854.
35. Li Min, Shengtao Zhu, Lei Chen et al. Evaluation of circulating small extracellular vesicles derived miRNAs as biomarkers of early colon cancer: a comparison with plasma total miRNAs. J Extracell Vesicles. V.8(1);2019.
36. Lidor A, Ismajovich B, Confino E, David MP. Histopathological findings in 226 women with post-menopausal uterine bleeding. Acta Obstet Gynecol Scand 1986;65:41–43.

37. Loh HY, Normsn BP, Lai KS et al. The regulatory role of microRNAs in breast cancer. Int J Mol Sci. 2019 Oct 6;20(19):4940.
38. Morice P, Leary A, Creutzberg C, Abu-Rustum N & Darai E Endometrial cancer. Lancet. 387, 1094–1108 (2016).
39. Nguyen, TA, Jo MH, Choi YG et al. Functional anatomy of the human microprocessor. Cell. 2015 Jun 4;161(6):1374-87.
40. Ozdegirmenci O, Kayikcioglu F, Bozkurt U. Comparison of the efficacy of three progestins in the treatment of simple endometrial hyperplasia without atypia. Gynecol Obstet Invest. 2011;72(1):10-4.
41. Prip CM, Stentebjerg M, Bennetsen MH et al. Risk of atypical hyperplasia and endometrial carcinoma after initial diagnosis of non-atypical endometrial hyperplasia: A long-term follow-up study. PLoS One. 2022 Apr 12;17(4):e0266339.
42. Raglan O, Kalliala I, Markozannes G et al. Risk factors for endometrial cancer: An umbrella review of the literature. Int. J. Cancer. 2019 Oct 1;145(7): 1719-30.
43. Ransohoff, JD, Wei Y and Khavari PA. The functions and unique features of long intergenic non-coding RNA. Nat. Rev. Mol. Cell Biol. 2018 Mar; 19(3):143-157.
44. Ravegnini G, De Leo A, Coada C et al. Identification of miR-499°-5p as a potential novel biomarker for risk stratification in endometrial cancer. Front Oncol. 2021 Oct 29;11:757678.
45. Royal College of Obstetricians & Gynaecologists. Management of Endometrial Hyperplasia, Green Top Guildeline 67. 2016.
46. Shi Y, Liu Z, Lin Q et al. MiRNAs and cancer: key link in diagnosis and therapy. Genes (Basel). 2021 Aug 23;12(8):1289.

47. Siegel RL, Miller KD & Jemal A. Cancer statistics, 2018. CA. Cancer. J. Clin 2018 Jan;68(1):7–30.
48. Schwarz DS, Hutvágner G, Du T et al. Asymmetry in the assembly of the RNAi enzyme complex. Cell 2003 Oct 17;115(2):199-208.
49. Snowdon J, Zhang X, Childs T. et al. The MicroRNA-200 Family Is Upregulated in Endometrial Carcinoma. PLoS ONE. 2011;6(8): e22828.
50. Soslow RA, Tornos C, Park KJ et al. Endometrial Carcinoma Diagnosis: Use of FIGO Grading and Genomic Subcategories in Clinical Practice: Recommendations of the International Society of Gynecological Pathologists. Int J Gynecol Pathol. 2019 Jan;38 Suppl 1(Iss 1 Suppl 1):S64-S74.
51. Sung H, Ferlay J, Siegel RL et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA. Cancer. J. Clin 2021, 71, 209–49.
52. Trimble CL, Method M, Leitao M et al.Management of endometrial precancers. Obstet Gynecol 2012 Nov;120(5):1160–75.
53. Ulitsky, I. (2018). Interactions between short and long noncoding RNAs. FEBS Lett. 592, 2874-2883. 10.1002/1873-3468.13085
54. World Health Organization. WHO Classification of tumors of female reproductive organs, IV Edition. World Health Organization. WHO Classification of tumors, 2014.
55. Fan X, Zou X, Liu C et al. MicroRNA expression profile in serum reveals novel diagnostic biomarkers for endometrial cancer, Biosci. Rep. 2021 jun 25;41(6)BSR20210111.
56. Yiu AJ and Yiu CY. Biomarkers in Colorectal Cancer. Anticancer Res.2016 Mar;36(3):1093-102.

57. Zhang B, Wang Q, Pan X (2007) MicroRNAs and their regulatory roles in animals and plants. J Cell Physiol 210: 279–89.
58. Zhang B, Wang Q, Pan X (2007) MicroRNAs and their regulatory roles in animals and plants. J Cell Physiol 210: 279–89.