ROLE OF KEY microRNAs, mRNAs AND ITRACONAZOLE IN ENDOMETRIAL HYPERPLASIA

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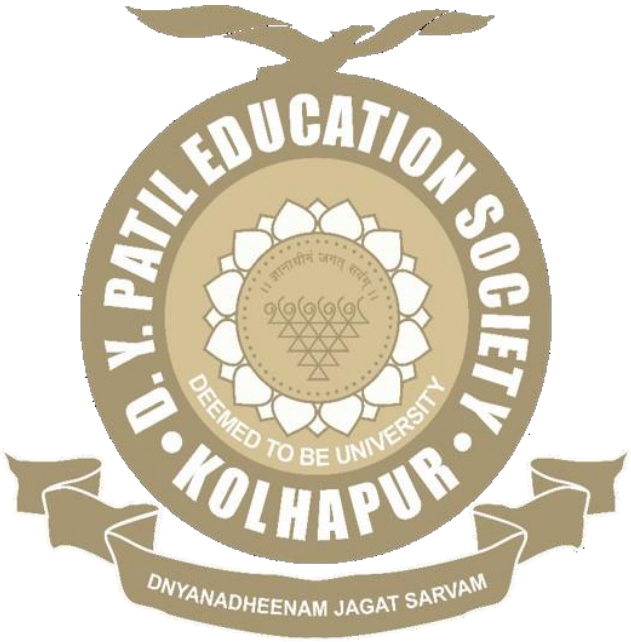

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UNDER THE FACULTY OF

INTERDISCIPLINARY STUDIES

BY

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2024

DECLARATION

I hereby declare that the thesis entitled **"Role of key microRNAs, mRNAs and Itraconazole in Endometrial Hyperplasia"** submitted for the degree of **Doctor of Philosophy (Ph.D.)** in **Biotechnology** under the faculty of **Centre for Interdisciplinary Research** of D. Y. Patil Education Society (Deemed to be University), Kolhapur is completed and written by me, has not before made the basis for the award of any other higher education institute in India or any other country. To the best of my knowledge and belief the thesis contains no material previously published or written by another person except where due reference is made. Further, I declare that I have not violated any of the provisions under the Copyright and Piracy/Cyber/IPR Act amended from time to time.

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CERTIFICATE

This is to certify that the research work presented in this thesis by **Mrs. Apurva R. Birajdar**, titled **'Role of key microRNAs, mRNAs and Itraconazole in Endometrial Hyperplasia'** has been carried out under my supervision at Department of Stem Cell and Regenerative Medicine, Centre for Interdisciplinary Research, D. Y. Patil Education Society (Deemed to be University), Kolhapur, Maharashtra, India. The work is original and has not been submitted either in part or full for award of any other degree or diploma. I hereby recommend the submission of the thesis for the award of **Doctor of Philosophy in Biotechnology**, by D. Y. Patil Education Society (Deemed to be University), Kolhapur.

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LIST OF PUBLICATION

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1. **Best poster award** for 'Inherent angiogenic ability of endometrial stem cells: Their implications in treating vascular disorders.' 7th International conference on Stem cells and Cancer (ICSCC-2016): Proliferation, Differentiation and Apoptosis; held at Margaon, Goa 2016.

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ABBREVIATIONS

BP: Base pair

BEH: Benign Endometrial Hyperplasia

CAM: Chorioallantoic Membrane

cDNA: complementary DNA

CFSE: Carboxyfluorescein diacetate succinimidyl ester

D and C: Dilatation and Curettage

DCM: Dichloro methane

DE: Differentially Expressed

DGCR8: DiGeorge syndrome critical region gene 8

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

DPX: Dibutylphthalate polystyrene xylene

EH: Endometrial hyperplasia

EIN: Endometrial Intraepithelial Neoplasia

EN: Endometrioid Neoplasia

ER: Estrogen

EWG: European Working Group

FBS: Foetal bovine serum

FDA: Food and Drug Administration

FFPE: Formalin fixed paraffin embedded

GnRH: Gonadotropin Releasing Hormone

HBSS: Hank's balanced salt solution

IHC: Immunohistochemistry

ITZ: Itraconazole

KEGG: Kyoto Encyclopedia of Genes and Genomes

MEM: Minimal essential medium

miRNA/ miR: microRNA

NGS: Next generation sequencing

PBS: Phosphate buffered saline

PCOS: Polycystic Ovarian Syndrome

PCR: Polymerase chain reaction

PR: Progesteron

RNAi: RNA interference

siRNA: small interfering RNA

snoRNA: small nucleolar RNA

snRNA: small nuclear RNA

SWI: Sterile Water for Injection

WHO: World Health Organization

PREAMBLE

Obstetrics and Gynaecology is indeed one of the challenging branch of medical science serving all womankind throughout their life. Obstetrics deals with the pre and post care of woman in pregnancy as well as childbirth, whereas Gynaecology deals with diagnosis and treatment of various diseases of the female reproductive system including infertility.

The recent advances in the field of molecular biology and the success of Human Genome Project made new avenues open for research at molecular level whether it may physiologic or disease condition. Being an interdisciplinary research, it tried to find an answer on gynaecological problem with the help of molecular biology, bioinformatics and pharmacology.

This research principally aimed at the Endometrial Hyperplasia, a precursor stage of Endometrial Cancer Type 1 (EC-type I) / Endometrioid Adenocarcinoma.

Endometrioid Adenocarcinoma has been researched on so many aspects including prognostic and therapeutic approaches, however endometrial hyperplasia with cytological atypia (also called as Endometrial Intraepithelial Neoplasia- EIN) and its progression is less explored at molecular level and therapeutic approach other than hysterectomy and hormonal therapy.

This thesis scrutinized EIN at molecular level to reveal key microRNAs (by NGS), m-RNAs (by microarray) and co-relating differentially expressed m-RNAs as targets to the microRNAs by using bioinformatics tool. In addition, it also illuminates the area of repurposed drug- Itraconazole and its effect on EIN.

I hope, this research work will aid to the prognosis of future threat of Endometrial Cancer at precursor stage and make it easy for clinicians to take a call on better treatment modality.

CHAPTER 1

INTRODUCTION

Relevance of endometrial hyperplasia study is primarily due to a high risk for malignant transformation and the problems associated with menstrual irregularities, dysfunctional uterine bleeding in women. Endometrial hyperplasia has a significant place in the structure of gynaecological illness in perimenopausal and postmenopausal women and is one of the most frequent causes of hospitalization. Being a pre-cancerous stage to Endometrioid adenocarcinoma, chances of recurrence and its prevalence to cause carcinoma, a proposal put forward as new approach on miRNA studies to identify key microRNAs and corresponding differentially expressed m-RNA targets involved in disease transition stage, thereby knowing the actual grade/ severity of disease and decreasing the prevalence of endometrial carcinoma by choosing an optimum treatment mode. MicroRNA dysregulation and its association with angiogenesis, apoptosis and cancer stem cells have been reported in several human cancers. However, in Indian population, there are no studies related to the deregulated microRNA profile in endometrial hyperplasia. In addition, there are fewer approaches in targeted therapeutics in endometrial hyperplasia. Hence, it is imperative to study the aforesaid attributes which will aid to discover prognostic biomarkers/ diagnostic profile/ microRNA therapeutics in near future. Also, shedding light on new option in terms of repurposed drug for this condition.

1.1 Background

The word HYPERPLASIA has an ancient Greek origin containing two sub-words, hyper, which means "over" and plasis means "formation". Hyperplasia is a medical condition characterized as increased rate of cell proliferation of respective tissue that may result in gross enlargement of the organ[1]. It may be a physiological response to a stimulus, for example, an increase in milk-secreting glandular cells of the breast as a pregnancy response for being prepared to breastfeed, or a pathological response to the stimulus that is triggered due to excess hormones or growth factors, which if ceased, excess cell proliferation could stop and so hyperplasia could be reversed. Other responses like chronic inflammatory response, hormonal dysfunctions, and compensatory growth after tissue or organ damage are some of the other causes of hyperplasia[2–4].

The most common clinically known forms of hyperplasia are prostatic hyperplasia, adrenal hyperplasia, breast hyperplasia (ductal hyperplasia), Heck’s disease (focal

epithelial hyperplasia), intimal hyperplasia, sebaceous hyperplasia and **Endometrial hyperplasia.**

1.2 Endometrial Hyperplasia (EH)

EH is the most diagnosed gynaecological disease, characterized as an abnormal proliferation of endometrial cells due to excess estrogen stimulation unopposed by the counterbalancing effect of progesterone[5] **(Fig.1.1A**). Histologically, EH **(Fig.1.1B)** is characterized as an increase in gland-to-stroma ratio as compared to normal proliferative endometrium[6]. It has a significant place in the structure of gynaecological morbidity and is one of the most frequent causes of hospitalization in gynaecological hospitals. Developed countries have more EH cases and are found to be 200,000 new cases per annum[7]. Unlike uterine cancer, the statistics of Endometrial hyperplasia cases in India are still unclear. EH is a pre-cancerous stage that originates as benign hyperplasia and progresses to Endometrial Intraepithelial Neoplasia (EIN) to well-differentiated endometrial adenocarcinoma if left untreated. Simple/ benign hyperplasia shows the lowest risk of cancer progression, and most cases (80%) of this natural regress, while EH with cytological atypia/ EIN is characterized as direct precancerous lesions and may carry a higher risk of progression to carcinoma[8].

1.2.1 Classification

Classifying EH using the very first categorization system, experienced to have substantial intra- and inter-observer variability, making it unclear. Based on the level of architectural complexity and nuclear atypia, the previous classification system—which was revised in 2003—classified hyperplasia into four diagnostic groups. It was first created in 1994 (**Fig.1.2**). The 2014 World Health Organisation (WHO) classification system has been demonstrated to be a more accurate and repeatable method for classifying Endometrial Hyperplasia[9–11].

However, the interpretation of the result by following this classification system could also be supported by IHC (immunohistochemistry) markers and molecular alterations in problematic cases[12,13].

1.2.2 Etiology, Symptoms and Diagnosis

The women in perimenopausal as well as postmenopausal stage are more prone to endometrial hyperplasia[14]. Additionally, early menarche, late onset of menopause, nulliparity

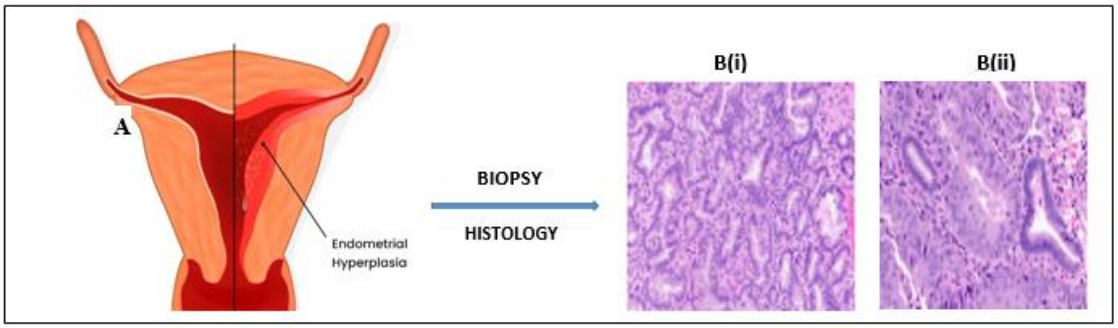

Fig.1.1: Endometrial Hyperplasia. A) LS view of uterus shows normal endometrium (left) and excessive proliferated endometrium (right) showing hyperplasic condition. B) Histology of benign endometrial hyperplasia (i) and EIN (ii).

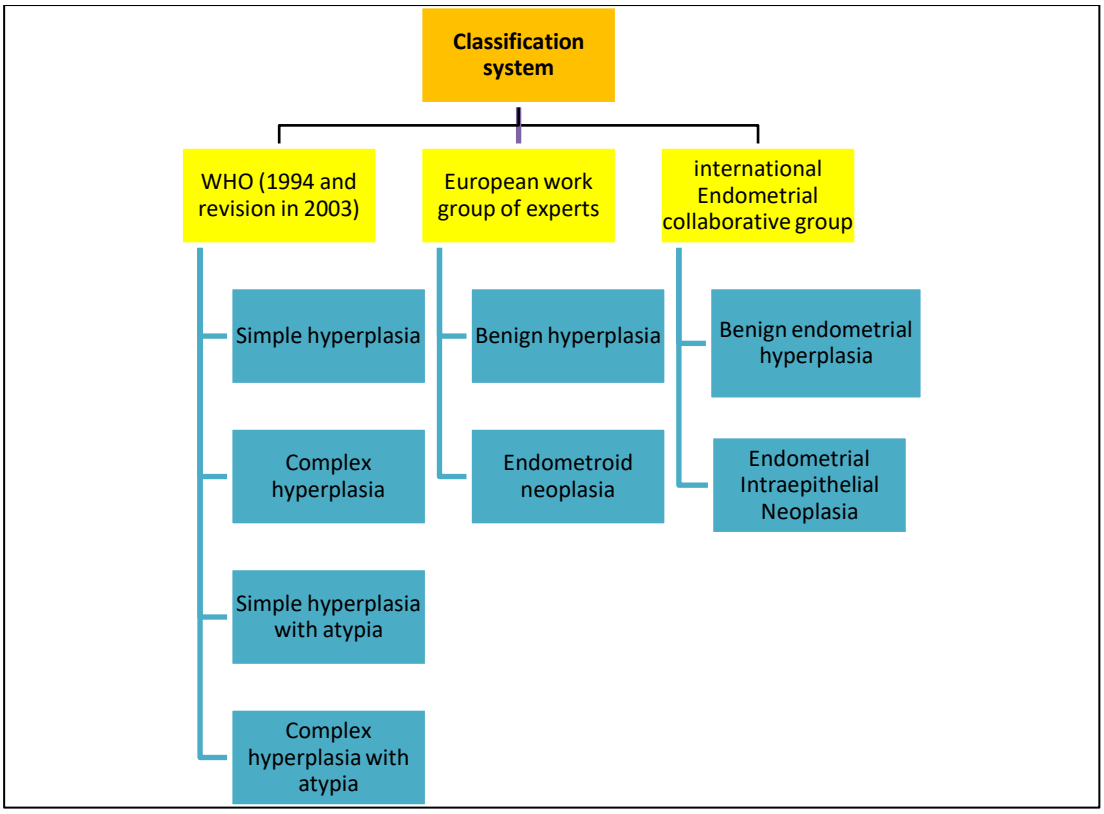

Fig.1.2: Classification system. According to WHO, there are four groups of endometrial hyperplasia whereas according to European work group of experts there are two sub- groups.

In 2003, WHO accepted EIN classification system by the International Endometrial collaborative group, published in 2014 and accepted worldwide till date.

and other conditions associated with increased estrogen levels (either endogenous or exogenous), Diabetes, hypertension, obesity, women with chonic ovulatory dysfunction (eg, polycystic ovary syndrome- PCOS), women under hormone replacement therapy (estrogen only), Tamoxifen exposure are other reasons to be at high risk of developing endometrial hyperplasia[15].

The most common symptoms of EH includes abnormal uterine bleeding including, menorrhagia, intermenstrual bleeding, postmenopausal bleeding, and irregular bleeding, which could be verified diagnostically with techniques such as Endometrial biopsy, Dilation and curettage (D and C,) or Hysteroscopy followed by histopathological analysis of endometrium tissue collected during any of the procedure. The histopathological analysis will confirm the diagnosis and omit other conditions like endometriosis, adenomyosis and endometrial polyp which also cause the endometrium to thicken focally or widely[16].

1.3 Need of better prognosis?

Since EH could be reversed as well as its further transition to Endometrioid Adenocarcinoma could be stopped if treated early, where better prognosis is the key. For benign hyperplasia, the probability of progression to invasive cancer over a 20-year period is less than 5%, whereas for EIN, it is 20–25%. Better prognoses are thus required in order to identify people who are at a high risk of developing cancer and to administer the proper therapy[17].

Prognosis is done based on symptoms and clinical history. Molecular basis of prognosis in endometrial hyperplasia is still an area of research. Research studies suggested the use of MicroRNAs as prognostic markers[18]. According to a research, endometrial intraepithelial neoplasia might be correctly diagnosed using a miRNA signature[19]. Another study discovered that in benign endometrial hyperplasia, miRNAs might predict the likelihood of eventual uterine cancer[20]. As miRNAs have the ability to control the expression of genes involved in the onset and course of these illnesses, their expression-based risk score may be used independently to predict future theat and patient's overall survival[21].

1.3.1 What are microRNAs?

Genetic aberration and altered pathways are the additional reasons to develop cancerous condition. Since the genetic abnormalities like mircosatellite instability and mutations in the tumor suppressor genes may occur in Endometrioid adenocarcinoma, perhaps its roots could be found at precancerous stage as abberent expressions of microRNAs (miRs) or microRNA - m-RNA interaction[22].

MicroRNAs are endogenous, highly conserved, short non-coding RNAs approx. 23-25 bp long and involved in post transcriptional regulation of gene expression. Most miRNAs are transcribed from DNA sequences into primary miRNAs (pri-miRNAs) and processed into precursor miRNAs (pre-miRNAs) and mature miRNAs by either following canonical or non-canonical biosynthetic pathway (**Fig.1.3**)[23].

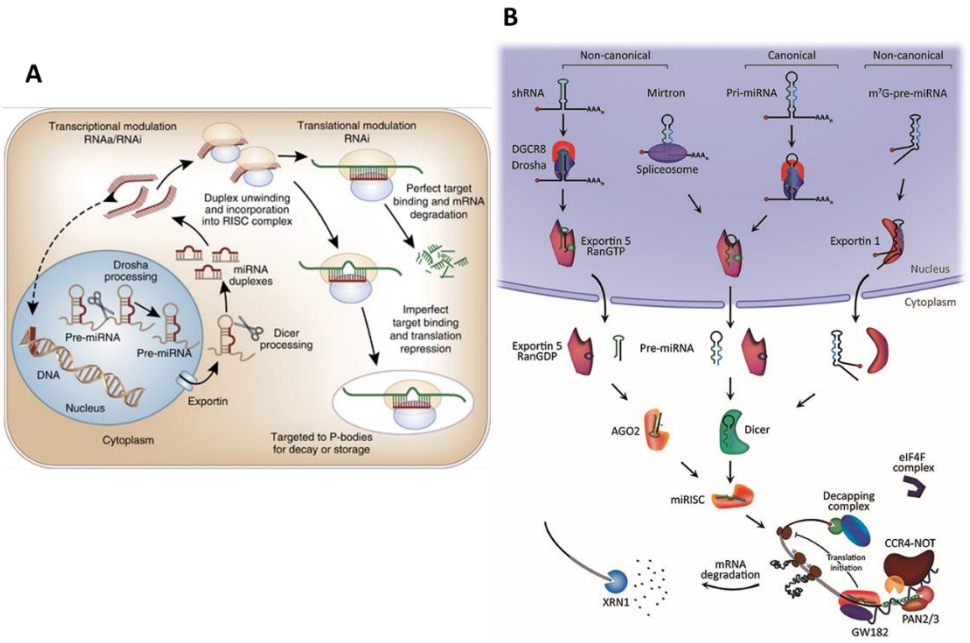

Fig.1.3: Biogenesis of miRNA. A) miRNAs are transcribed from DNA sequences into primary miRNAs (pri-miRNAs) by RNA polymerase II/III. Pri-miRNAs are processed into precursor miRNAs (pre-miRNAs) by the nuclear microprocessor complex, which is composed of Drosha and DGCR8. Pre-miRNAs are exported from the nucleus to the cytoplasm by exportin-5. Pre-miRNAs are processed into mature miRNAs by the cytoplasmic RNase III enzyme Dicer. Mature miRNAs are approximately 22 nucleotides long and are incorporated into the RNA-induced silencing complex (RISC). The RISC complex recognizes and binds to target mRNAs through base pairing between the miRNA and the mRNA. B) A more detailed and classified biogenesis of miRNA which takes place via canonical and non- canonical pathways. These two

pathways involve DICER and Drosha proteins, however, are exported by exportin 5 and exportin 1.

These are important for the normal development and various biological processes of animals while their abberent expression leads to various disorders[24]. They may act as oncomiRs or tumor suppressor miRs and mostly binds to the 3'UTR of its target mRNA and promotes deadenylation, decapping and translational repression[25]. There are reports of binding of miR to the 5'UTR, coding region and promotor region of target mRNA[23]. A single microRNA could regulate 100s of mRNAs. Hence, it is obligatory to do the target enrichment and functional analysis of miRs. Moreover, the cell specificity, stability and abundance make microRNAs potential prognostic/ diagnostic biomarker[26,27].

Since the discovery of microRNA in 1993, it came long way. In addition to its significance as prognostic/ diagnostic biomarker, many leading companies like Regulus therapeutics, Mirna Therapeutics, Miragen therapeutics and Santaris Pharma are engaged in developing microRNA therapeutics as anti-miR or microRNA replacement therapy. Among these companies Santaris Pharma have passed two phase I clinical trial and currently in phase II for mir-122 targeting drug miravirsen[28,29].

1.4 Treatment

Currently, cyclic progestin therapy, GnRH therapy, and hysterectomy are the suggested treatment modalities for endometrial hyperplasia (table 1).

Table 1: List of progestins, GnRH analogues and surgical options in treatment of Endometrial Hyperplasia

Progestins	**GnRH Analogues**	**Surgical modalities**
Progesterone	Native GnRH	Thermal balloon ablation
Medroroxy-Progesterone acetate	Leuprolide acetate	Resectoscopic surgery
Megestrol Acetate	Histrelin (agonist)	Hysterectomy
Levonorgestrel	Nafarelin	----------

Norethisterone acetate	Triptorelin (agonist)	----------
17α-Hydroxy- progesterone caproate	----------	----------

Progestins are the synthetic progesterone that is used effectively in ER+/estrogen-dependent endometrial cancer as well as relapsed endometrial atypical endometrial hyperplasia. Gonadotropin-releasing hormone regulates the secretion of gonadotropins by the pituitary gland and its expression is mediated though its receptor (GnRH-R). Thereby, the use of GnRH analogues (agonist/ antagonist) could be responsible for the withdrawal of estrogen by inhibiting its production which would have an anti-proliferative effect on endometrial cells.

Patient with benign hyperplasia are recommended to go for progestin therapy or GnRH therapy for 6 month with intermittent testing for regression. Whereas the patients with relapse of hyperplasia, having cytological atypia are recommended with surgical options or hysterectomy.

Long-term use of progestin cause depletion in progesterone receptor of target tissue causing response failure in adjuvant setting. Prolonged GnRH therapy cause menopause symptoms, bone demineralization and is costly option. The patients not willing to hysterectomy and wants to retain fertility is still a challenge. Therefore, to overcome these limitations, finding new effective treatment modalities are warranted.

1.4.1 Targeted therapeutics: Repurposing a drug

Considering the limitations of conventional medication and multidrug resistance made Targeted therapeutics a thiving area in cancer research, which could also be effective at precancerous or early stages of cancer inhibiting its further progression.

Discovery of novel drugs is a time-consuming process and requires millions of fundings, hence, drug repurposing is the suitable approach when time is limited and the demand for therapies increase[30]. Drug repurposing refers to the reuse of well-acknowledged non-cancer drugs for their onco-therapy purpose. Although, repurposing could be an approach to treat other diseases as well, for example, infections,

inflammatory disorders, etc. It has its advantages as most of the drugs are FDA approved with all data details generated in preclinical and Phase I clinical trials and could allowed directly to Phase II and Phase III clinical trials which would eventually reduce the cost associated with drug development[31].

Itraconazole (ITZ) is a well-known, broad-spectrum antifungal drug, that belongs to the triazole family, and has gained consensus in recent years as a re-purposed drug exerting anti-cancer activity[32]. It follows a targeted therapeutic approach as an inhibitor of angiogenesis and Hedgehog pathway, inducer of autophagy, and able to reverse multi-drug resistance. In addition, ITZ was found to be effective when used synergistically with other chemotherapy agent[33,34].

In the case of Gynecological oncology, the drug has been proven effective in epithelial ovarian cancer[35], Cervical cancer[36], primary malignant melanoma of vagina[37], and Endometrial cancer[38]. Since Itraconazole was found effective in –vitro in Endometrial cancer (type I), it is hypothesized that Itraconazole could also be effective at precancerous stage Endometrial hyperplasia and could be used synergistically with ongoing medicines[38].

Thus, this study aims at the identification of key microRNAs and mRNAs in endometrial hyperplasia, could be proven prognostic biomarker for early detection and severity as well as the associated risk of its further progression to Endometrial cancer (type I). Also, role of Itraconazole in endometrial hyperplasia would be studied, which will assist clinicians to take a call on better treatment modalities.

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CHAPTER 2

REVIEW OF LITERATURE

Clear understanding of the Endometrial Hyperplasia condition needs the knowledge about physiology of female reproductive cycle.

2.1 Endometrium and the menstrual cycle

The endometrium, which is the uterus' innermost lining layer, is essential for reproduction and eventually survival of our species **(Fig.2.1A).** The multicellular tissue that lines the uterus, sheds during menstruation and regenerates itself in the absence of pregnancy; or undergoes changes accordingly for implantation of foetus to maintain a pregnancy[1]. Menstruation causes the upper two-thirds, or functional layer to shed, while, the basal layer, which is the bottom third of the endometrium and is next to the myometrium, does not shed. Basal layer, the actual niche of endometrial stem and progenitor cells, regenerate the functional layer of the lining cyclically[2]. Histology is shown in **(Fig. 2.1 B).**

2.1.1Menstrual cycle

The reproductive years of a women i.e. the timespan from Menarche (onset of menses) to Menopause (end of menses), is characterized by monthly rhythmic changes in the ovaries and the uterus under the influence of aforementioned female hormones. These monthly changes are generally known as sexual cycle/ reproductive cycle/ menstrual cycle[3].

The menstrual cycle is a period between the first day of menstruation and the first day of menstruation of next cycle, which on an average of 28 days. It can vary person to person from 25-30 days[4]. It is divided into three phases; Follicular, ovulatory and luteal phase **(Fig.2.1 C)**.

Every menstrual cycle begins with the **menstruation (day 0-4)** indicating failure of fertilization due to decreased estrogen and progesterone level, causing functionalis layer to shed. Menstruation comes under **follicular phase (day 1 till ovulation)** where decreased level of estrogen and progesterone act as positive feedback to the GnRH which in turn cause anterior pituitary gland to secrete Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH)[5]. FSH stimulates the growth of 3-30 follicles (primordial follicles), each having an egg, inside the ovary. During the maturation of follicles, **estrogen level gradually increases and endometrium begins to grow due to cell proliferation**[6]**.** The decrease in the FSH level allows dominant follicle to mature

further and continue to secrete estrogen. Thus, the increasing level of estrogen stimulate the regeneration and further growth of the endometrium in the uterus, and signals pituitary gland for the release of Luteinizing hormone (LH)[7].

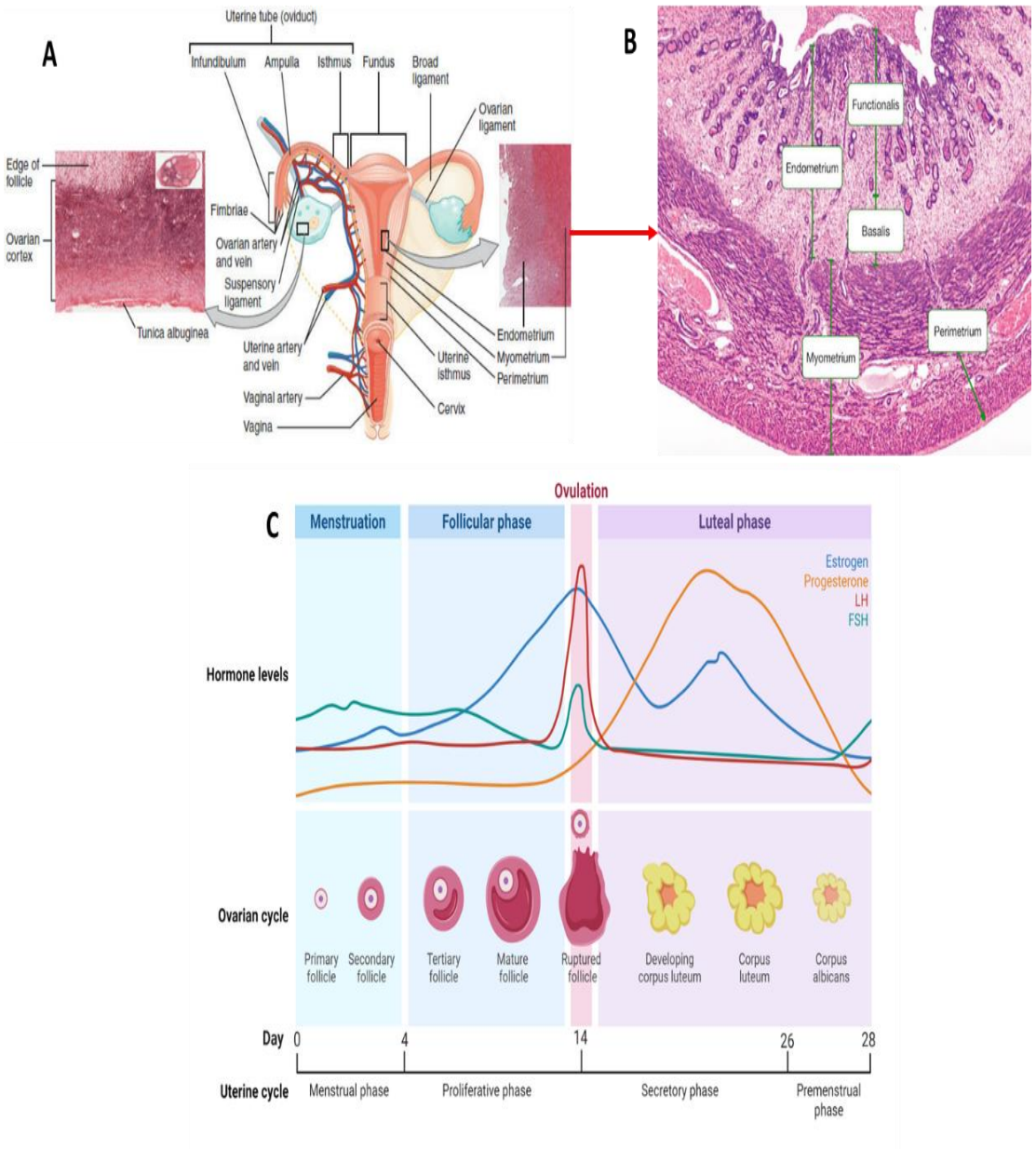

Figure 2.1: Anatomy and physiology of endometrium A) The above panel shows the anatomy of female reproductive part that consists of vagina, uterus, ovaries, fallopian tube and many other sub structures labelled in the figure. B) Histological view; shows three layers viz. perimetrium (outer lining), myometrium (middle lining) and endometrium (innermost lining) which divided into two layers i.e. basalis . C) Diagramatic representation of events of uterine and ovarian cycle and phases as per menstrual day, with controlling hormones.

LH surge is the main characteristic to the onset of **ovulatory phase (16- 32 h)**, causing Graffian follicle to buldge from the surface of ovary and rupture releasing egg[8].

In Luteal phase (day 14- day 1 of menstruation) egg releases and empty follicle (corpus luteum) begin to secrete hormone progesterone and Inhibin. As **progesterone level increases, estrogen level decreases results in secretory changes in the endometrium.** Failure of fertilization leads to degeneration of corpus luteum after 14 days and progesterone and inhibin level decreases, which act as positive feedback to GnRH and a new cycle starts[9].

2.2 Pathophysiology of Endometrial hyperplasia

Since the menstrual cycle is critically regulated at hormonal levels, the disturbances in this hormonal co-ordination could lead to the menstrual disorders, like either related to duration (Polymenorrhea, Oligomenorrhea, Amenorrhea) or related to discharge (AUB, Hypomenorrhea, Menorrhagia, Metrorrhagia). Thus, disturbed hormonal co-ordination becomes the root cause of some gynaecological diseases including Endometrial hyperplasia[10].

Endometrial hyperplasia is influenced by the menstrual cycle because endometrium regenerates cyclically from stem cells in the basal layer when progesterone and oestrogen are present. Estrogen stimulates synthesis of receptors (ER α and β) though which it acts, located in nuclei of epithelial and stromal cells of the endometrium. Progesterone inhibits the synthesis of these receptors[11]. Since estrogen remains unopposed by the progesterone, the cells go on proliferating resulting in crowding of cells causing hyperplasia. Cytologic atypia may represent early neoplastic process[17]. This abnormal proliferation further leads to neoplasm, in which genetically abnormal cells manage to proliferate in a non-physiological manner which is unresponsive to normal stimuli[8].

2.3 miRNA and hallmarks of cancer

The journey from pre-cancer stage to well differentiated cancer involves basic hallmarks of cancer such as angiogenesis, and cell death escape (apoptosis). Additionally, cancer stem cells (CSCs) have also been found to be involved in not only the progression and maintenance of cancer but recurrences of cancer as well[14].

The process of creating new blood vessels from pre-existing ones is known as angiogenesis, and it is crucial to the development of endometrial malignancies as it provides cancer cells with nutrients and oxygen and removal of metabolic waste products[15]. Angiogenesis is another aspect in the complex illness endometriosis. The key protein that regulates and enables the process of angiogenesis is vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), tumor necrosis factor-alpha (TNF-α), transforming growth factor-beta (TGF-β), and the angiopoietins (Ang)[16]. Various therapeutic strategies that target these proteins and inhibit the process of angiogenesis has been approved by FDA against cancer. For example, bevacizumab, cabozantinib, lenvatinib, regorafenib, axitinib, sunitinib, sorafen etc. The miRNAs miR-10 and the miR-200 family for VEGFR1, miR-15b-5p, miR-16-5p, miR-17-3p, miR-195, the miR-200 family and miR-370 for VEGFR2, and miR-1236-3p for VEGFR3 are the ones that directly control the VEGFRs[17]. More than 40 miRNAs that specifically target VEGF have been verified so far. Angiogenesis is observed in endometrial hyperplasia and endometrial cancer, and it is correlated with increasing tumour grade and deeper penetration[18].

Continuous proliferation of endometrial cells in endometrial hyperplasia point towards the failed apoptosis, which regulates cell homeostasis in the endometrium during regular menstrual cycles. Similar to angiogenesis, apoptotic factors have also been targeted in cancer[19]. Poly (ADP-ribose) polymerases (PARP), a key protein regulating cell death and cell cycle has been found to be targeted by miR-124, miR-216b, miR-223-3p, miR-335, and miR-708-5p[20]. Both normal and hyperplastic endometrium express Bcl-2 and Bax. Bcl-2 is not connected to additional cancer, although it may contribute to the development of endometrial hyperplasia and early carcinogenesis[21]. It has been discovered that miR-135a stimulates the growth, migration, invasion, and chemoresistance of endometrial cancer cells. It controls the expression of BAX and Bcl-2, preventing endometrial cancer cells from undergoing apoptosis brought on by cisplatin[22]. It has been discovered that miR-21 targets PTEN to encourage the growth and invasion of endometrial cancer[23]. miR-145-5p and miR-449a downregulation function as predictive indicators for endometrial cancer as they play a role in controlling cancer cells' programmed cell death[24].

It is believed that CSCs are essential for the development, progression, and recurrence of tumours[25]. Although the exact involvement of CSCs in endometrial hyperplasia and

the development of endometrial cancer remains unclear, new research has provided some insight into this area. In one study, 60% of endometrial hyperplasia cases were found to have upregulation of NANOG, a key CSCs marker[26]. It has been demonstrated that Wnt/β-Catenin signalling activation in endometrial cancer cells promotes tumour development and quickens cell proliferation. Wnt/β-Catenin signalling is involved in maintenance of CSCs population[27]. It was shown that macrophage M0 was highly correlated with CDH18 and PAGE2B[28]. This finding may have an impact on the transition from atypical endometrial hyperplasia to endometrial cancer[29]. However, no studies have highlighted the role of miRNA in CSCs in endometrial hyperplasia.

More research has been done on the function of apoptosis in endometrial hyperplasia and endometrial cancer than on CSCs[30]. It is recognised, therefore, that CSCs may contribute to treatment resistance and tumour recurrence due to their resistance to apoptosis[31]. To completely comprehend the involvement of CSCs in endometrial hyperplasia and the advancement of endometrial cancer, more study is required[32].

To conclude, the molecular signature of miRNA is important to study as it directly regulates the normal functioning of cells which when disrupted leads to abnormal cellular proliferation[33]. In this section, we noticed a shift in trend. Several miRNA's downregulation led to the development and progression of carcinoma as compared to hyperplasia, where, the miRNAs were found to be parallelly increasing and decreasing in their expression[34].

Since miRNA attaches to and interacts with mRNA, it is necessary to understand the expression pattern of several mRNAs and correspond it to the upregulation or downregulation of miRNAs. Therefore, in the next section, we are going to understand the key players among mRNAs that lead to the development of hyperplasia and carcinoma.

2.4 miRNA and Endometrial Hyperplasia

As mentioned, miRNA has been well studied and established to be associated with malignancy, disease progression, and severity. Each miRNA can regulate the expression of several other genes, some of which could be either oncogenes or tumor suppressor genes[35].

Expression of miR-182, miR-183, miR-200a, miR-200c, and miR-205 was found to be upregulated in endometrial carcinoma when compared to hyperplasia, as studied by Lee et. al[36]. However, no direct comparison between normal endometrial tissue and hyperplasia tissue was made to comment upon. A mere look at the data shows that the miR- 200 family is the key player in hyperplasia and carcinoma[37]. miR-30a-3p, miR-141, miR-200a, and miR-200b were found to be significantly correlated with endometrial hyperplasia in a study conducted by Yun Lin et. al[38]. The expression of these miRNAs was also correlated with the loss of phosphatase and tensin homolog (PTEN) protein, a tumor suppressor protein[39]. Hence, the authors suggest that a combination of the mentioned miRNAs and PTEN loss could predict the risk of endometrial cancer as well[38].

In another study, atypical endometrial hyperplasia samples were assessed for their expression of miR-577, miR-182-5p, and miR-183-5p. These were found to be significantly upregulated and associated with the AMPK signal pathway and Wnt signaling pathways[40]. miR-204-5p was found to be downregulated in hyperplasia, however, the comparison was made between endometrial cancer and hyperplasia patients; healthy controls were not assessed[41]. miR-503 level was also downregulated in hyperplasia tissue examined in patients. This study also provided evidence that a cell cycle-associated oncogene encoding cyclin D1 (CCND1) is inversely correlated with miR-503 levels, hence contributing to the development of carcinoma[42]. In another in silico study, miR-149 expression was postulated and shown to be downregulated which decreased the expression of gene repair proteins ADP-ribosylation factor 6) and tumor protein p53 (TP53) and upregulated the expression of cyclin E2 to abnormalize the cell cycle[43].

A common trend that could be noticed in the studies mentioned above is that of miRNA selection. Studies have analyzed hundreds of miRNAs, however, selected a few that showed correlation with signaling pathways that could affect the progression of carcinoma in endometrial hyperplasia patients. Therefore, researchers are trying to understand the role of miRNA, as a biomarker, to track the prognosis of the disease[44]. Additionally, the miRNAs are known to be binding to mRNAs to exert their effect, therefore, it is also important to analyze the corresponding mRNA to understand the activity of miRNAs. Several researchers denoted endometrial hyperplasia as “control” groups and compared the miRNA expression with carcinoma cases.

It could be concluded that not many studies have been conducted to understand the expression of miRNA in endometrial hyperplasia and endometrial cancer, especially in the Indian Asian population. The studies mentioned above have elucidated the pathways that could potentially lead to cancer; therefore, their findings are important to consider.

2.5 mRNA expression in Endometrial Hyperplasia

mRNAs or messenger RNA are single-stranded RNA molecules that carry genetic information from DNA to ribosomes, where they are translated into proteins. The study of mRNAs is crucial for understanding gene expression and regulation, particularly in the context of disease development and progression[45].

In the case of endometrial hyperplasia, the study of mRNAs is especially important because dysregulation of mRNA expression has been linked to various processes implicated in the disease, including angiogenesis, apoptosis, and cancer stem cell proliferation[46]. Recent advancements in microarray technology have enabled researchers to investigate the expression of thousands of genes simultaneously, providing valuable insight into the underlying mechanisms of endometrial hyperplasia[47].

Understanding the role of mRNAs in endometrial hyperplasia will not only improve our understanding of the disease at the molecular level but also have the potential to lead to the development of more effective diagnostic and therapeutic strategies[8]. Additionally, mRNA studies can complement miRNA studies, as miRNAs can regulate the expression of target genes at the post-transcriptional level. Therefore, identifying dysregulated miRNAs and their target genes can provide a more comprehensive understanding of the gene expression changes in endometrial hyperplasia[48].

Overall, the study of mRNA expression patterns in endometrial hyperplasia can provide valuable information on the underlying molecular mechanisms of the disease, potentially leading to the development of better diagnostic and therapeutic tools.

Since miRNAs often reduce the amount of mRNA expression, miRNAs that target oncogenes inhibit tumours by regulating the cell cycle. Endometrial cancer development and aberrant sex steroid hormone signaling have been connected to the dysregulation of miRNAs[49]. In this study, we have focused on the miRNA-targeted

mRNA expression that leads to the development of endometrioid adenocarcinoma from endometrial hyperplasia[50]. Not many studies have analyzed the mRNA targets and commented on the molecular signature associated with miRNA and mRNA in endometrial hyperplasia and carcinoma.

2.6 Itraconazole and its repurposing

Identifying abberent miRs, their target enrichment and functional analysis aids in finding newer biomarkers for early detection of the disease. Additionally, MiR based therapy has the ability to target several genes in a given pathway. However, when it comes to translational use, the off-target effect of miR drug hampers the success rate in clinical trials[51]. Hence, less than 20 anti-miRs are in clinical trials[52]. Therefore, alongside searching for newer therapeutic approach is imperative.

Here, use of repurposed drug in targeted therapy approach seems the promising area. As mentioned earlier (Introduction 1.4.1) Itraconazole is such a repurposed drug found to be effective in Endometrial cancer.

As an anti-angiogenic agent, this drug inhibits the proliferation of endothelial cells with little or no effect on non-endothelial cells[53] by lowering cholesterol levels and inhibiting mTOR[54]. In addition, it interferes with VEGF binding to VEGFR2, thereby failing downstream signal transduction[55]. As one would recall, endothelial cells are responsible for angiogenesis, the process of formation of blood vessels which is a key hallmark of cancer. As the tumor cells grow, they require more nutrition which is met by the formation of new blood vessels at the site of the tumour[56].

There are several other ways by which Itraconazole works. It inhibits the hedgehog pathway and acts as an inducer of autophagy[57]. It has been proven effective in multi-drug resistance, a common condition acquired by the cancer cells towards various chemotherapy agent, which is responsible for the 90% failure of chemotherapy in invasive and metastatic cancer[58]. Itraconazole have proved as potent inhibitor of p-glycoprotein (p-gp) and able to reduce p-gp function by 50% at dose approx[59]. 2µM, in cell line overexpressing p-gp. Itraconazole with ketokonazole on Topotecan resistant HEK cell line over-expressing BCRP have significantly reverse the resistance of Topotecan[60].

Itraconazole has also been used against gynaecological-associated carcinoma. For example, Itraconazole with Paclitaxel was found effective in epithelial ovarian cancer in mouse model[61]. Itraconazole could also suppress the proliferation of AN3-CA, HEC-1A, and Ishikawa cells, as reported by Tsubamoto and colleagues[62]. Since the efficacy of Itraconazole was demonstrated in Endometrial carcinoma, it is hypothesized that Itraconazole might work similarly in Endometrial hyperplasia and could stop its progression to Endometrioid Adenocarcinoma.

Considering the research problem and the relevant review of literature, this study has following aim and objectives:

AIM:

1. To identify key micoRNAs and corresponding mRNA targets responsible for Endometrial Hyperplasia and its further progression.
2. To check the efficacy of Itraconazole in Endometrial Hyperplasia.

OBJECTIVES:

1. To identify key microRNAs responsible for Endometrial hyperplasia and its further progression.
2. To identify the differentially expressed m-RNA s in endometrial hyperplasia.
3. To identify differentially expressed m-RNA targets to shortlisted miRs using bioinformatics tools.
4. To identify the role of Itraconazole in Endometrial Hyperplasia.

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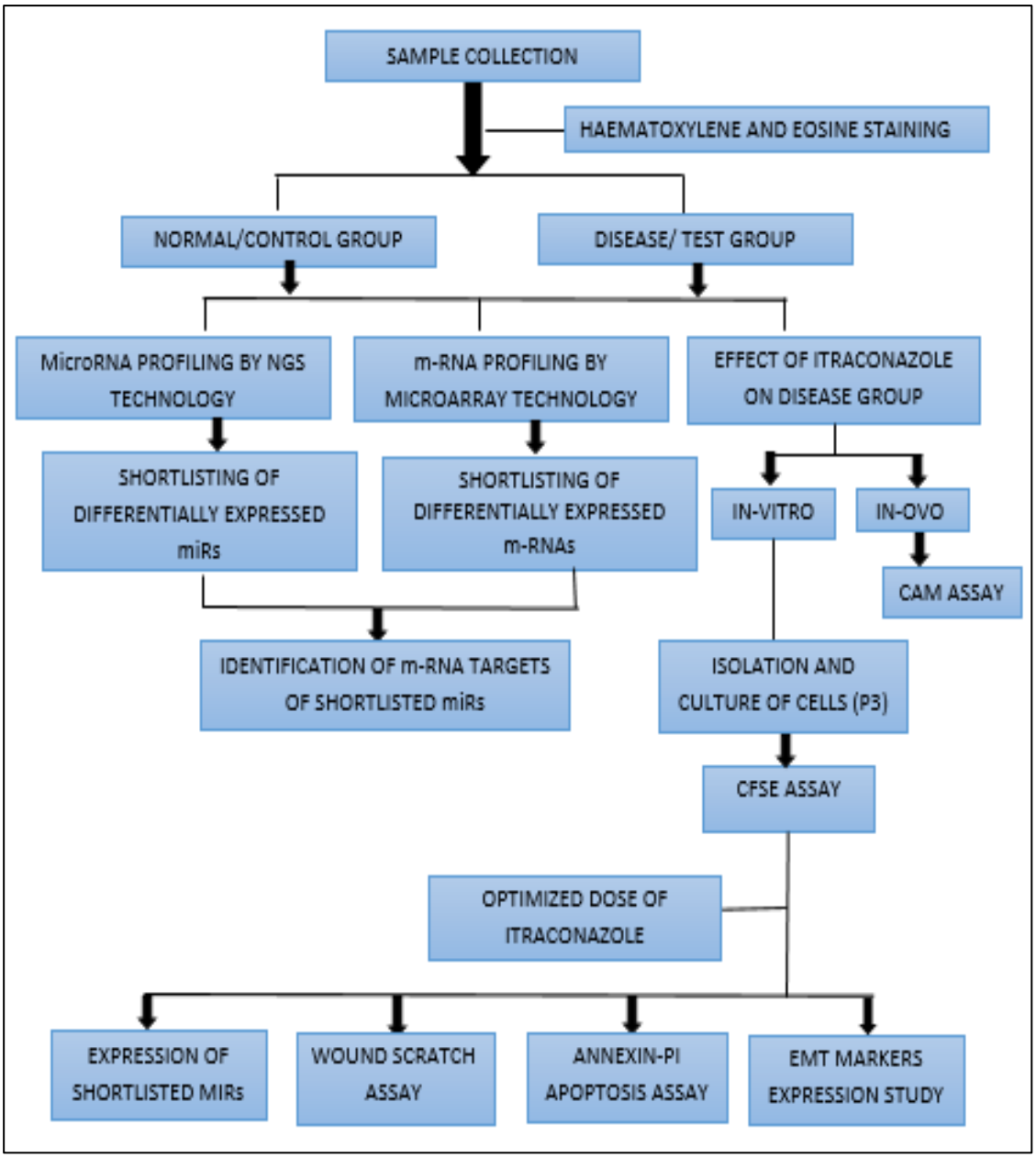
CHAPTER 3

MATERIALS AND METHODS

To execute the objectives listed in previous chapter, following research plan was used. Materials required for the same and methods followed are explained in detail.

SCHEMATIC REPRESENTATION OF OVERALL RESEARCH PURSUIT



3.1 MATERIALS:

3.1.1 ENDOMETRIUM TISSUE SAMPLES:

- **Physiologic endometrium tissue:**

Endometrium tissue devoid of any pathology.

Such type of endometrial tissue was collected from the patient undergoing Endometrial Biopsy (EB) for infertility diagnosis / hysterectomy for non-malignant reason such as prolapse and fibroid.

- **Diseased endometrium tissue:**

Endometrium tissue derived from Endometrial Hyperplasia/ Adenocarcinoma condition.

Such type of endometrial tissue was collected from the patient undergoing Endometrial Biopsy/ hysteroscopy/ hysterectomy for Endometrial Hyperplasia condition; provided that patient must not have underwent any hormonal treatment 3 month prior to the procedure.

3.1.2 FERTILIZED EGGS:

Zero hour fertilized eggs of *Black australorp* procured from Central Egg Hatching Centre, Kolhapur (MH), India.

3.1.3. BUFFERS:

- **10x Phosphate buffered saline (PBS):**

The stock solution of 10x PBS [Himedia TL1032] was diluted in 1:9 proportion by using sterile distilled water/ sterile water for injection to get working 1x PBS.

- **1x Hank's Balanced Salt Solution (HBSS) [HiMedia -TL1003]**

3.1.4 CULTURE MEDIA:

- **For sample collection:**

  1x Minimum Essential Medium (MEM) [HiMedia- AL080] supplemented with 1% Antibiotic Antimycotic Solution (1x).

- **For primary culture of physiologic endometrium:**

  Dulbecco's Modified Eagle Medium (DMEM)/F12 [Gibco - 11320033] or [HiMedia -AL215A] supplemented with 10% Foetal Bovine Serum (FBS) and 1% antibiotic solution (1x).

- **For primary culture of endometrium derived from Endometrial Hyperplasia:**

  1x Minimum Essential Medium (MEM) [HiMedia- AL047S] supplemented with 5% Fetal Bovine Serum (FBS) [HiMedia -RM9954] and 1% Antibiotic Antimycotic solution (1x)

3.1.5 CHEMICALS:

- **RNA later**
- **Buffered formalin (10%): (Composition for 1000 ml)**

  4 g of sodium phosphate monobasic (Na_2HPO_4) and 6.5 g sodium phosphate dibasic (NaH_2PO_4) were dissolved in minimum quantity of distilled water. To this, 100 ml stock formaldehyde (commercial) was added and made up the volume to 1000 ml with distilled water.

- Xylene, ethanol, HCL, paraffin
- **Itraconazole**
- DMSO, Dichloro methane

3.1.6 TOOLS AND SOFTWARES FOR DATA ANALYSIS

- **For MicroRNA data analysis:**

Following are the pre-requisites for performing analysis:

Table 3.1: Tools and softwares for NGS data analysis

Tools	Description
Fastx Toolkit	Tools utilized for pre-processing of Fastq files.
Bowtie2	Software utilized for mapping of reads onto genome.
MirDeep2	Software to perform prediction and quantification of miRNA.
DESeq2	Differential expression analysis is performed using DESeq2 in R Package
TargertScan	Tool utilized to perform target analysis of human miRNA

- **For Microarray data analysis:**

Table 3.2: Tools and softwares for microarray data analysis

Tools	Description
Feature Extraction software Version 11.5 of Agilent.	Software for data extraction from image
GeneSpring GX software	Analysis of extracted raw data
Genotypic Biointerpreter-Biological Analysis Software	For pathway analysis

- **For identification of m-RNA target:**

MiRSystem (ver. 20160511): A user friendly tool predicting m-RNA target associated microRNAs, from a database of seven well known miRNA target gene prediction programs: DIANA, miRanda, miRBridge, PicTar, PITA, rna22, and TargetScan.

3.1.7 Plastic and glasswares:

All plasticwares used in the experiments were from HiMedia and SIGMA and glasswares from Borosil.

3.1.8 Kits:

Kits from the Thermofisher Scientific and Quiagen were used for the in vitro experiments.

3.2 METHODS

3.2.1 SAMPLE COLLECTION:

The protocol was approved by the Institutional Ethical Committee (IEC). Patients were informed prior to sample collection.

Sample collection was done in strict aseptic condition, inside Operation Theatre.

1. **In case of hysterectomy**, the uterus was cut open to the cavity and the endometrium was scraped with the help of sterile scalpel and collected in tube.

In case of EB/ hysteroscopy, clinician only gives the endometrium tissue during procedure.

2. Endometrium tissue was collected in three test tubes as follows:

   i. **Tube 1 (sterile): RNA later**

   ii. **Tube 2 (Sterile): Transport medium**

   iii. **Tube 3 (non-sterile): 10% buffered formalin**

3. All the tubes were transported to the laboratory with appropriate precautions and labelled.

4. Tube1 was shifted to refrigerator at 2-8^{0}C, kept for 24h followed by -40^{0}C for 24h and finally at -80^{0}C till RNA extraction.

5. Tube 2 was subjected to isolation and culture of cells derived from the collected tissue.

6. Tube 3 was kept at RT for 24 h. for tissue fixation and subjected to block preparation and Hematoxylin and Eosin (H and E) staining.

3.2.2 HISTOLOGY:

- **FFPE Block preparation**

1. **Tissue fixation**: The endometrium tissue was fixed in 10% buffered formalin for 48 h. (Sample collection step 6).

2. **Dehydration**: Post 48 h, the tissue was dehydrated by placing the tissue in a series of graded ethanol starting with 70%, 80%, 95% and 100%.

3. **Clearing:** The tissue was further cleared in xylene to remove alcohol.

4. **Paraffin infiltration:** The cleared tissue was immersed in molten paraffin (58^{0}C) with gentle agitation throughout the process. Two changes, each 1 h.

5. **Paraffin embedding:** The tissue was shifted on the cassette/ mold (sample ID on sidewall of mold) according to the desired position. Then immersed in molten paraffin (58^{0}C) and allowed to cool at RT.

- **H and E staining**

1. **Sectioning:** A series of 5 µm thick sections (called as ribbon) of FFPE block were made by using Microtome () and placed on a clean grease free glass slide.

2. **De-waxing:** Xylene treatment was given twice for 5 min each.

3. **Hydration:** The slide was pass through a series of graded alcohol from 100%, 95%, 80% and 70% for removal of xylene and rinsed thoroughly with water.

4. **Hematoxylin nuclear staining:** The sections were stained with Harris hematoxylin for 5 min and rinsed with water.

5. **Bluing:** Bluing was done with weak alkaline solution for 5 min, where hematoxylin converts to deep blue colour.

6. **Background de-staining:** The non-specific background staining was removed and contrast was improved with weak acid alcohol treatment (1 ml HCL in 70% alcohol) and the slide was thoroughly rinsed with water.

7. **Eosin staining:** The sections were stained with eosin for 1 min and rinsed with water.

8. **Dehydration and clearing:** The slide was kept in absolute ethanol (thee changes, 2 min. each. Cleared in xylene (three changes, 2 min. each), to remove alcohol.

9. **Mounting:** A thin layer of DPX was applied on the slide and a coverslip was placed over it carefully, without any air bubble and allowed to dry.

3.2.3 ENDOMETRIAL CELL ISOLATION AND CULTURE:

Cell isolation:

1. Before starting the cell isolation, all reagents and culture medium were maintained at room temperature.

2. The transport medium was discarded [Sample collection; step 2(ii)] and the endometrium tissue was thoroughly washed with 1x PBS twice to remove the blood clots and the mucus.

3. The tissue was minced mechanically with the help of surgical blade and transferred in the tube containing enzyme cocktail for tissue digestion. Temperature was maintained at 37^0C and the reaction was stopped by adding cold culture medium.

4. The digested tissue was removed carefully and centrifuged at 3000rpm for 3min in cooling centrifuge.

5. Supernatant was decanted and the cell pellet obtained was suspended in 1-2ml complete medium; which is then re centrifuged at 3000rpm for 3min in cooling centrifuge.

6. The supernatant was decanted and the cell pellet was re suspended in 1ml complete medium.

Culture:

7. Cell viability was tested by using Trypan blue dye exclusion test and the viable cells were counted by using Neubauer's chamber.

8. $1x10^4$ cells/ml were seeded in vented T25 culture flask with additional 2ml complete medium and incubated at 37^0C in CO_2 incubator maintained at 5% CO_2, for 48h.

9. Post 48h, the cells were observed for their adherence and the old culture medium was replaced with fresh culture medium. The flask was re incubated until the cells attain confluency with intermittent addition of fresh culture medium.

Subculture/ passage:

10. Once the cells attain confluency (confirmed under microscope), the culture medium was discarded carefully and the cells were washed gently with 1x PBS.

11. The cells were detached by using adequate amount of trypsin (0.25%) and the enzymatic reaction was stopped with cold culture medium.

12. Trypsinized cells were then centrifuged at 1200rpm for 3min in cooling centrifuge.

13. Supernatant was decanted and cell pellet was wash with 2ml culture medium, then subjected to re-centrifugation at 1200rpm for 3min.

14. Supernatant was decanted and cells were re-suspended in fresh 2ml culture medium; which is then distributed equally in two different T25 culture flask, each with additional 2ml culture medium. The flasks were then incubated in CO_2 incubator with aforementioned condition till cells were ready for next passage**.**

3.2.4 Purification of Total RNA from Endometrium Tissues:

The protocol is according to **QiagenRNeasy Lipid tissue Kit (Cat.No. 74106)** with DNase treatment:

1. The endometrium tissue stored in RNA later was taken out and subjected for disruption in presence of 1 ml QIAzol Lysis Reagent. Homogenization of lysate was done by using TissueRuptor II.

   The tube containing homogenate was kept at RT (15-25^{0}C) for 5 minutes.

2. 200 µl chloroform was added and shake vigorously for 15 s, then kept at RT for 2-3 min.

3. Centrifugation was done at 12,000 x g for 15 min at 4°C which resulted in three-layer separation.

4. The upper, colourless aqueous phase, containing RNA, was collected in new tube and equal amount of 70% ethanol was added and vortexed.

5. 700 µl of the sample was transferred to an RNeasy Mini spin column placed in a 2 ml collection tube and centrifuged at 8000 x g for 15 s at RT. Flow-though was discarded. The step was repeated if some left over sample is there.

6. 350 µl Buffer RW1 was added to the same spin column and centrifuged at 8000 x g for 15 s. Flow-though was discarded.

7. 80 µl of DNase I incubation mix (prepared by adding 10 µl of stock DNase I solution to 70 µl Buffer RDD) was directly added to the spin column membrane and kept at RT for 15 min.

8. 350 µl Buffer RW1 was added to the spin column and centrifuged at 8000 x g for 15 s. Flow-though was discarded.

9. 500 µl Buffer RPE was added to the spin column and centrifuged at 8000 x g for 2 min. Flow-though was discarded. The spin column with new collection tube was centrifuged at high speed for 1 min to eliminate any leftover buffer RPE, if needed.

10. The RNeasy Mini spin column was carefully paced in a 1.5ml collection tube and 30–50 µl of RNase-free water was added directly to the spin column membrane. Centrifuged at 8000 x g for 1 min.

11. The final elute containing purified RNA was then used for further downstream experiments or stored at -80^{0}C.

3.2.5 Purification of Total RNA from cultured endometrial cells:

The protocol is according to ambion **PureLink RNA Mini Kit (Cat. No. 12183018A) using TRIzol reagent.**

1. The harvested cells of both the groups were transferred to the RNase-free tube and centrifuge at 2,000 × g for 5 minutes at 4°C to pellet. The supernatant (culture medium) was discarded and the pellet was suspended in 500 µl TRIzol® Reagent for 5 minutes at RT.

2. 200 µl of chloroform was added and the tubes were shake vigorously for 15 seconds and incubated at RT for 2-3 minutes.

3. The tubes were centrifuged at 12,000 × g for 15 minutes at 4ºC. Post centrifugation, the upper colourless aqueous phase (400 µl) was transferred to fresh RNase–free tubes.

4. To this aqueous phase, 400 µl of 70% ethanol was added and vortexed to mix well.

5. Out of 800 µl of sample in above tube, 700 µl sample was transferred to respective spin columns (control and test) with collection tube.

6. The spin columns were centrifuged at 12,000 × g for 15 seconds at room temperature and flow-though was discarded.

7. To the same spin columns, 700 µl of wash buffer I was added and centrifuged at 12,000 × g for 15 seconds at room temperature. The collection tubes with flow-though were discarded and a new collection tubes were inserted.

8. To the same spin columns, 500 µl of wash buffer II with ethanol was added and centrifuged at 12,000 × g for 15 seconds at room temperature. The flow-though was discarded.

9. Step-8 was repeated and the collection tubes were replaced with recovery tubes.

10. To the same spin columns, 80 µl of elution buffer was added, incubated at RT for 1 minute and centrifuged at 12,000 × g for 2 minutes at room temperature.

11. The final elute containing purified RNA was then used for further downstream experiments or stored at -80^0C.

3.2.6 RNA quantification and quality check by Nanodrop:

1. The purified RNA samples as well as nuclease free water were kept ready on icebath.

2. Post cleaning the sample reader with molecular grade water, following the software instruction, 2 µl of nuclease free water was loaded as blank.

3. The computer setting was changed to RNA and 2 µl of RNA sample was loaded.

4. Post read complete, 260/280 and 260/230 ratios were recorded along with RNA quantity in ng/ µl.

   The sample reader was cleaned and dried using wipes each time before sample change.

3.2.7 Small RNA (miR) sequencing by Next Generation Sequencing (NGS) on Ion Torrent S5 platform: (Performed at Thermofisher scientific, Gurgaon, India**)**

1. The endometrium tissue samples of physiologic condition (Proliferative phase and secretory phase) as well as disease condition (non-atypical Hyperplasia, atypical Hyperplasia and Endometrioid Adenocarcinoma) were collected in 10% buffered formalin and RNA later separately.

2. Post histology confirmation by H and E staining, corresponding samples stored in RNA later were progressed for RNA isolation, Quality testing of RNA by using the Nanodrop Spectrophotometer (Thermo Scientific; 1000) and RNA integrity.

   The samples with RIN value ≥ 6.5 were selected for next generation sequencing on Ion Torrent S5 platform as follows:

3. **Library construction:** The cDNA prepared from isolated RNA sample of each experimental group processed into relatively short double-stranded fragments (100–800 bp) which were ligated to the technology specific adaptor sequence, forming a fragment library.

4. **Templating:** Here, each DNA fragment/molecule in the library was bound to the surface of a bead or flow cell and amplified by PCR to create identical clones. The amplification will further aid to easy detection of each target during sequencing.

5. **Sequencing:** The sequencing of library was done by using sequencing instrument, Ion torrent S5 platform, which is based on the principle of sequencing by synthesis, where incorporated bases were detected optically followed by subsequent removal of reactants to restart the cycle.

6. **Data Analysis stages:** A huge complex data generated during sequencing was analyzed as follows-

   Preprocessing of reads: Raw reads are preprocessed before alignment stage. Only reads passing the theshold were utilized for alignment. Cutadapt (https://cutadapt.readthedocs.io/en/stable/guide.html) was utilized to perform these analyses where reads were trimmed up to minimum length of 15 bp thus

keeping only those reads which are above 15 bp. Reads without adaptor sequences were stored separately.

Genome mapping: After preprocessing, High quality reads were mapped onto the reference genome using Bowtie2 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml) using the default parameters. Genome was indexed using the command provided from bowtie2 package. Reads aligned other than small RNA regions and unaligned are segregated using Picard and utilized along with earlier filtered out reads and aligned to genome using Bowtie2 local alignment parameters.

Known small RNA Quantification: Reads mapped on genome were utilized further using FeatureCount tool for performing quantification of knows small RNA. These counts are utilized further for performing differential expression studies.

Differential expression analysis: Counts generated for known miRNA are utilized to study differential expressed miRNA using the R package DESeq2 (http://bioconductor.org/packages/release/bioc/html/DESeq2.html) using default parameters for individual sample comparison.

Novel Small RNA Prediction: Novel miRNA regions were predicted using Mirdeep2 algorithm which considers miRNA candidates based on the reads mapped onto the reference genome. Mapped reads are filtered to have reads with minimum length of 17 and above using Samtools. These candidate regions were screened for different features such as number of reads aligning, prediction of secondary structures, fold energy, etc. Scores are assigned to each predicted small RNA though different analysis. A score theshold was utilized to filter novel small RNA and performed further downstream analysis.

Target discovery: miRNA found to be significantly expressed after differential expression was utilized further to study gene targets by utilizing different softwares. TargetScan 7.1 (http://www.targetscan.org/vert_71/) where, miRNA IDs as input and providing genes lists with targets and their probability values for each gene target..

3.2.8 m-RNA profiling by Microarray: (Performed at Genotypic, Bangalore, India)

1. The endometrium tissue samples of physiologic as well as disease condition (non-atypical Hyperplasia and atypical Hyperplasia) were collected in 10% buffered formalin and RNA later separately.

2. Post histology confirmation by H and E staining (detailed protocol), corresponding samples stored in RNA later were progressed for RNA isolation, Quality testing of RNA by using the Nanodrop Spectrophotometer (Thermo Scientific; 1000) and RNA integrity by Bioanalyzer (Agilent; 2100 expert). A good quality RNA, based on the 260/280 values, rRNA 28S/18S ratios and RNA integrity number (RIN) were selected for following steps.

3. **Labeling and microarray hybridization:**

   The samples for Gene expression were labeled using Agilent Quick-Amp labeling Kit (p/n5190-0442). 500ng each of total RNA were reverse transcribed at 40°C using oligodT primer tagged to a T7 polymerase promoter and converted to double stranded cDNA. Synthesized double stranded cDNA were used as template for cRNA generation. cRNA was generated by in vitro transcription and the dye Cy3 CTP(Agilent) was incorporated during this step. The cDNA synthesis and in vitro transcription steps were carried out at 40°C. Labeled cRNA was cleaned up using QiagenRNeasyMini kit columns (Qiagen, Cat No: 74106) and quality assessed for yields and specific activity using the Nanodrop ND-1000.

4. **Hybridization and scanning:**

   600ng of labeled cRNA sample were fragmented at 60ºC and hybridized on to a Agilent designed Human Gene expression Microarray 8x60K (AMADID No: **G4858A_39494**) arrays. Fragmentation of labeled cRNA and hybridization were done using the Gene Expression Hybridization kit of (Agilent Technologies, In situ Hybridization kit, Part Number 5190-0404). Hybridization was carried out in Agilent's Surehyb Chambers at 65º C for 16 hours. The hybridized slides were washed using Agilent Gene Expression wash buffers (Agilent Technologies, Part Number 5188-5327) and scanned using the Agilent Microarray Scanner (AgilentTechnologies, Part Number G2600D).

5. **Feature Extraction:**

   Data extraction from Images was done using Agilent Feature Extraction software Version 11.5.

   Microarray Data Analysis:

   Images were quantified using Agilent Feature Extraction Software (Version-11.5).

   Feature extracted raw data was analyzed using Agilent GeneSpring GX software.

   Differential expression patterns were identified among the samples.

   Significant genes up regulated fold change> 0.6 (logbase2) and down regulated <-0.6 (logbase2) in the test samples with respect to control sample were identified.

   Statistical student T-test, p-value among the replicates was calculated based on volcano plot algorithm.

   Differentially regulated genes were clustered using hierarchical clustering based on Pearson coefficient correlation algorithm to identify significant gene expression patterns. Pathway analysis for the differentially regulated genes was performed using Genotypic Biointerpreter-Biological Analysis Software.

   The Significant Functional classification of differentially regulated genes was performed using GeneSpring GX software gene ontology.

IN-VITRO METHODS

3.2.9 Wound scratch assay:

The cells used for wound scratch assay were of P3 (passage-3). Endometrial cells derived from EIN tissue were considered as control, while the similar cells treated with Itraconazole (0.8 µM) considered as test.

1. $1x10^4$ cells/ml were seeded in 24 well plate with additional 1ml culture medium and allowed to grow at 37^0C, 5% CO_2, in humidified incubator, till cells reach 80% confluency.

2. The culture medium was discarded and a wound was created by making a longitudinal scratch with sterile 100 µl tip.

3. The cells were washed gently with 1x PBS to remove the detached cells and fresh serum- free culture medium was added to control wells, while serum free culture medium supplemented with Itraconazole (0.8 µM) was added to the test wells.

4. Each well was monitored for cell migration though phase contrast microscope and photographed, at the time interval of 6 h. until closure of wound.

5. Post wound closure, the cells of control and test group were harvested, washed with 1x PBS and progressed for expression of migration marker.

3.2.10 Annexin V- PI assay:

1. The P3 cells derived from EIN tissue were counted and 1x 10^6 cell/ml were seeded in 24 well plate and grown for 24h in humidified CO_2 incubator at 37^0C.

2. The culture medium of each well was replaced with fresh medium supplemented with different concentration of Itraconazole i.e. 0.2, 0.5, 0.8 and 1 µM. and re-incubated at same aforementioned condition for next 24 h.

3. Post 24 h of incubation, the cells of each well were harvested separately in Eppendorf tubes.

4. 5µl of alexa 488 annexinV as well as 1 µl of PI were added to each cell suspension and incubated at RT for 15 mins, followed by addition of 400 µl ABB (1x).

5. Finally, flowcytometric analysis was done at 530nm and 575nm wavelength.

3.2.11 CFSE assay:

1. The P3 cells derived from EIN tissue were counted and 1x 10^6 cell/ml were seeded in 24 well plate and grown for 24h in humidified CO_2 incubator at 37^0C.

2. The culture medium of each well was replaced with fresh medium supplemented with different concentration of Itraconazole i.e. 0.2, 0.5, 0.8 and 1 µM. and re-incubated at same aforementioned condition for next 24 h.

3. The culture medium was removed and replaced with loading solution for labeling the cells with CFSE and incubated for 20 min.at 37^0C.

4. The loading solution was removed. Cells were washed twice with culture medium and then fresh pre-warmed complete medium was added and incubated further.

5. The dye dilution was analysed flowcytometrically on day 3 and 5 of incubation. The % MFI was recorded.

3.2.11 IN-OVO METHOD:

Preparations:

Itraconazole stock solution (1 mM):

Itraconazole was obtained from Smilax Laboratories Ltd (B. No. IT H 1605023).

7.06 mg of Itraconazole dissolved in minimum quantity of DMSO till complete dissolution and make up to 10ml volumetrically by sterile water for injection (SWI).

Whatman filter paper (No.2) discs:

1. Whatman filter paper discs were made with the help of punching machine. All the discs were sterilized prior use.

Following steps were done in LAF cabinet.

2. The sterilized discs were dipped in aforementioned working concentrations of Itraconazole separately and kept in petri dishes accordingly.

3. The discs were allowed to dry for some time, wrapped in aluminium foil and kept at RT according to the experimental groups.

Chick Chorioallantoic Membrane (CAM) assay:

1. Zero h fertilized eggs of *Black australorp* (N=40) were procured from Central Egg Hatching Centre, Kolhapur. The good quality eggs were selected based on their shape, uniformity and transported to the lab carefully.

2. The eggs were cleaned, disinfected with 70% ethanol and incubated at 37^0C with 70-75% humidity (monitored by hygrometer) for 72 h (D-3), in an egg incubator.

Following steps were done inside LAF cabinet

3. Post 72 h (D-4), 2-3ml of egg albumin was removed from narrow end and a small window was created at blunt end of each egg. The windows made were sealed with cellophane tape and re-incubated the eggs at same aforementioned conditioned for further 72 h.

   The eggs were divided into four groups, viz. Control, positive control, vehicle control and test.

4. Post 72 h (D-8), the sterile filter paper discs were placed on the CAM vasculature as follows:

   Control: Plain filter paper disc

   Vehicle control: Filter paper disc treated with DMSO and 0.9% NS (medical grade)

   Positive control: Filter paper disc saturated with Cyclophosphamide

   Test: Filter paper disc saturated with Itraconazole

   The egg windows were re-sealed and incubated at same condition (step-2) for 48h, in an egg incubator.

Macroscopic and microscopic observation:

1. Post 48 h (D10), the window at blunt end of egg was widened. Photograph of CAM vasculature was taken with digital camera and the images were exported to Image J software for image analysis.

2. The CAM vasculature around the disc was examined microscopically and scored based on following scoring system (Table 1).

3. Average anti-angiogenic score was calculated by using following formula.

Table 3.3: Angiogenesis scoring system

SCORE	**ANTI-ANGIOGENIC EFFECT**
0	Absent
0.5	Weak
1	Moderate
2	Strong

$$\text{Average score} = \frac{[\text{Number of eggs (Score 2)} \times 2] + [\text{Egg number (Score 1)} \times 1]}{\text{Total number of eggs (Score 0, 0.5, 1, 2)}}$$

CHAPTER 4

Identification of DE microRNAs responsible for Endometrial Hyperplasia and its progression

4.1 Background

MicroRNAs have emerged as important regulators of gene expression and have been found to be dysregulated in various diseases, including endometrial hyperplasia and endometrioid adenocarcinoma. Over the past five years, there has been a growing body of literature highlighting specific miRNAs that are associated with these conditions. The identification of these miRNAs and their downstream targets provides a valuable tool for developing better diagnosis/ prognosis. Moreover, they have also been found to regulate tumor growth and metastasis, making them potential targets for cancer therapy. The identification of key miRNAs responsible for endometrial hyperplasia and its further progression is a promising area of research that could lead to the development of novel therapeutic strategies.

4.2 NGS Technology

Next-generation sequencing (NGS) technology has transformed the field of genomics by enabling the rapid and cost-effective sequencing of large amounts of DNA and RNA. It has played a vital role in identifying novel miRNAs and studying their expression patterns in various diseases[1]. This technology has the potential to uncover new insights into the complex regulatory networks involving miRNAs and their target genes, providing a better understanding of the molecular mechanisms underlying cancer development and progression[2].

NGS has several advantages over traditional sequencing methods, like, it can generate massive amounts of data in a short period and at a much lower cost. It allows researchers to sequence not only the coding regions of the genome but also the non-coding regions, such as miRNAs. Several studies have used NGS to identify miRNAs that are associated with different stages of endometrial hyperplasia and endometrioid adenocarcinoma[3]. For example, a study by Jiang et al. used NGS to profile miRNA expression in endometrial hyperplasia and endometrioid adenocarcinoma tissues[4]. They identified several differentially expressed miRNAs that were associated with the progression from endometrial hyperplasia to endometrioid adenocarcinoma. Another study by Zhang et al. used NGS to identify miRNAs that were dysregulated in endometrial cancer and to study their potential target genes. They found that several miRNAs, including miR-21, miR-34a, and miR-182, were upregulated in endometrial cancer and were associated with poor prognosis[5].

Despite having enriched data globally, the data of Indian ethnicity is lacking. Considering the geographical condition, lifestyle, food habits etc. there are possibility of getting different miR profile. This forms the basis and thus, this chapter deals with the identification of key DE miRs (Indian ethnicity), involved in the transition of Endometrial hyperplasia to Endometrioid Adenocarcinoma using NGS technology**.**

4.3 METHODS: (Detailed method in chapter 3)

Briefly, the endometrial sample collection was done from the peri/postmenopausal women undergoing diagnostic hysteroscopy/ hysterectomy having likely symptoms of endometrial hyperplasia, like AUB/ menorrhagia etc. Post histology, the collected samples were segregated as control, benign hyperplasia, Endometrial Intraepithelial Neoplasia and Endometrioid Adenocarcinoma and carefully transported to the Thermofisher, Gurgaon for microRNA profiling by NGS. Analysis was done by using bioinformatics tools to get differentially expressed miRs in all respective groups. The overlapped miRs between EIN and Endometrioid Adenocarcinoma were considered further and 4 miRs were shortlisted for qPCR validation in retrospect study.

4.4 RESULTS:

4.4.1 Sample collection and histology:

The women in peri/ postmenopausal stage, having Abnormal Uterine Bleeding (AUB) and undergoing diagnostic hysteroscopy/ hysterectomy were selected for sample collection **(Fig.4.1)**. During/Post procedure, the sample was collected in RNA later and 10% buffered formalin. The sample in RNA later was stored at -80^0 C until receiving histopathology report.

The samples collected from diagnostic hysteroscopy/ hysterectomy for non-malignant reasons were considered as control.

The sample in 10% buffered formalin was subjected to FFPE block preparation and then H & E staining. Microscopy of 5 um thick H & E stained sections reveal either of the stage shown in **(Fig.4.2)**

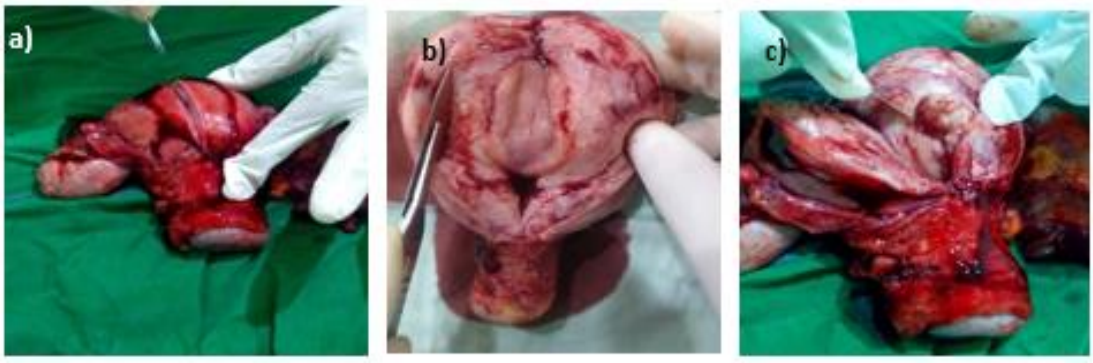


Fig.4.1: process of sample collection. a) Gross uterus with longitudinal cut; b) opened uterus with hyperplasic endometrium; c) uterus with Endometrioid adenocarcinoma

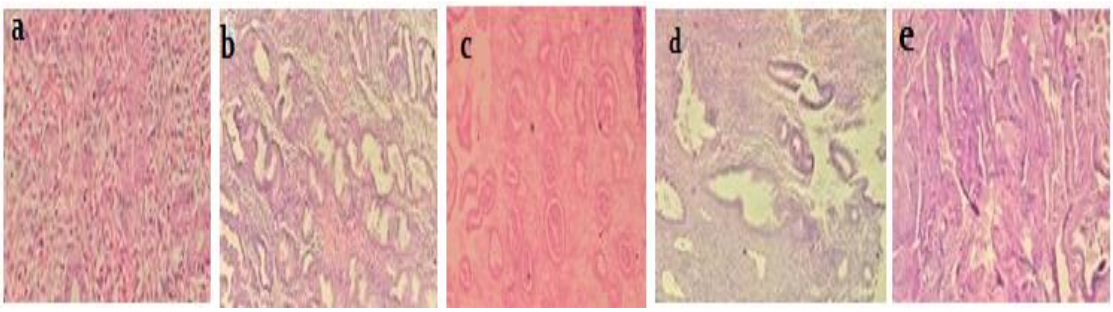


Fig. 4.2: Histology. a) Proliferative phase ; b) Secretory phase; c) Benign (non-atypical) hyperplasia; d) EIN (atypical hyperplasia); e) Endometrioid adenocarcinoma

Proliferative phase is characterized by round tubular glands evenly spaced in dense stroma. The columnar cells were also seen in the glands **(Fig.4.2a).** The secretory phase was confirmed on the basis of cork-screw shape of glands, which is late secretory phase **(Fig.4.2b)**. The increased density of glands relative to stroma was seen in **(Fig.4.2c)**. The glands were dialated and irregular in shape, showing benign hyperplasia. EIN or Atypical hyperplasia is a celluar atypia characterized by large round or elongated nuclei. The glands were irregular shape with little intervening stroma **(Fig.4.2d)**. As the disease progress, the gland to stroma ratio increases, which is clearly visible in **(Fig.4.2e)**, asserting Endometrioid Adenocarcinoma. The glands were dense and tightly packed back to back with very little to no intervening stroma.

4.4.2 Quality control and genome mapping :

Histologically confirmed samples were transported to Thermofisher Scientific, Gurgaon for Next generation of sequencing of small RNAs, by using Ion Torrent platform. The samples with RIN ≥ 6 were selected for sequencing **(Table 4.1).** Sample size post quality control check was N= 2/ group.

Table 4.1: RNA quality control

Experimental group	RIN value
Control	7.8
Endometrial non-atypical hyperplasia	6.5
Endometrial atypical hyperplasia (EIN)	8.1
Endometrioid Adenocarcinoma	8.2

Post sequencing, raw reads were obtained and those reads passing the threshold were utilized for alignment. Cutadapt was used to perform these analyses where reads were trimmed upto minimum length of 15 bp. High quality reads were mapped onto the reference genome using Bowtie2 with default parameters. The respective data is summarized in **(Table 4.2)**.

4.4.3 Known small RNA quantification and differential expression:

Reads mapped on genome were used in FeatureCount tool for quantification where counts were generated and these were further used to know differential expression of miRs.

4.4.4 Identification of DE miRs

In six samples, corresponding to thee experimental disease group i.e Benign hyperplasia (50), atypical hyperplasia (122) and Endometrioid adenocarcinoma (279), total 451 mirs were differentially expressed with p value (≤ 0.05) as compared to control. Since atypical hyperplasia is considered as precursor stage to endometrial adenocarcinoma, these groups were considered further. The venn diagram showed 13 up-regulated and 19 down-regulated miRs in atypical hyperplasia whereas 51 up-regulated and 100 down-regulated miRs in Endometrioid Adenocarcinoma. Out of these, 14 miRs were found overlapped which includes 6 up-regulated and 8 down-regulated miRs **(Fig. 4.3 a and b).**

Table 4.2: Genome mapping

Sample	Total Reads	Reads after processing	Reads mapped on genome	Mean length
Control	11,331,462	6,717,008	6,199,879	17 bp
	13,983,057	8,715,303	7,966,837	18 bp
Benign hyperplasia	11,096,752	6,239,344	5,810,439	16 bp
	10,216,229	4,584,153	4,255,709	15bp
EIN	10,398,905	8,413,022	8,001,112	20 bp
	14,759,511	12,993,234	12,332,952	21 bp
Endometrioid Adenocarcinoma	10,670,350	7,674,244	7,221,214	18 bp
	13,901,357	9,527,983	8,883,007	18 bp

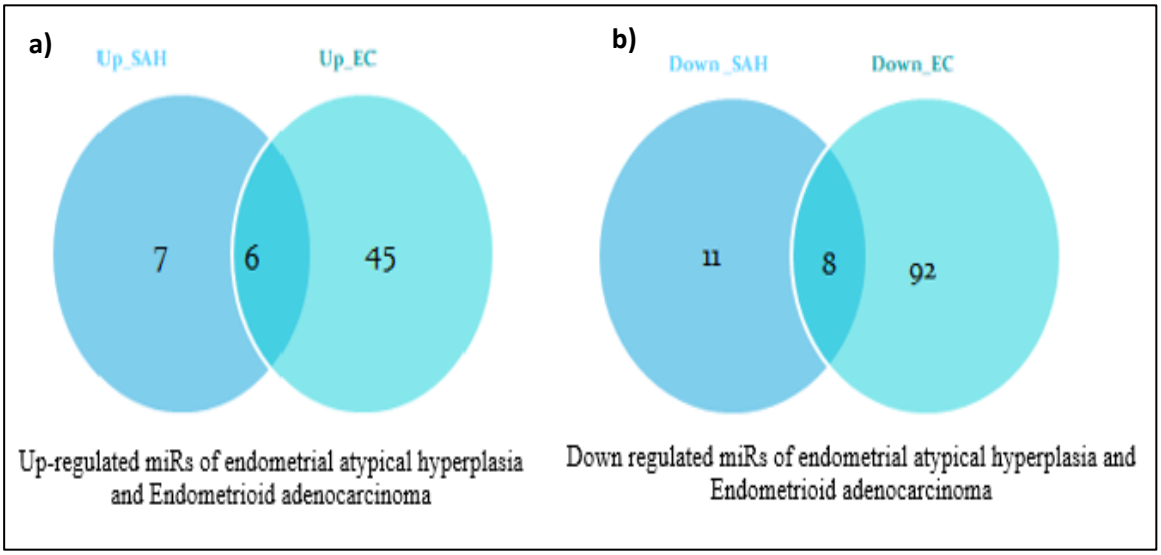


Fig. 4.3: Venn diagram representation of differentially expressed miRs in endometrial atypical hyperplasia (SAH) and Endometrioid adenocarcinoma (EC). A) Up-regulated miRs, b) down-regulated miRs.

The overlapped miRs with |log2FC| and p value ≤ 0.05are given in table 4.3 and table 4.4 below. The highly significant, unique differential miRs of endometrial atypical hyperplasia are listed in table 4.5.

Table 4.3: Common up-regulated miRs between endometrial atypical hyperplasia (EIN) and Endometrioid Adenocarcinoma

	Control	Sec vs. BEH	Sec vs. EIN	Sec vs. EC
hsa-miR-205-5p	-2.591120247	1.43121545	3.973443586	8.659220954
hsa-miR-181c-5p	-1.097827676	0.070750332	2.223713318	2.39164059
hsa-miR-509-3-5p	-3.551547173	3.110650243	4.599832982	3.907975406
hsa-miR-181c-3p	-1.993445508	1.025015532	2.471053835	2.735463907
hsa-miR-181d-5p	-1.191965876	0.682678732	2.542230536	2.23329398
hsa-miR-509-5p	-3.553553414	3.800250931	5.685201424	5.124029327

Table 4.4: Common down-regulated miRs between endometrial atypical hyperplasia and Endometrioid Adenocarcinoma

	Control	Sec vs. BEH	Sec vs. EIN	Sec vs. EC
hsa-miR-876-5p	2.166120548	-0.091317457	-6.484027084	-6.290071087
hsa-miR-873-5p	2.139522491	-1.769124141	-4.875449219	-7.039017371
hsa-miR-876-3p	1.72347663	-0.348542977	-5.492546413	-7.157955883
hsa-miR-483-3p	0.093735064	-1.124365653	-2.825533088	-7.372115981
hsa-miR-6718-5p	6.603865156	-6.820150111	-9.406731977	-6.238635399
hsa-miR-585-3p	2.039140852	1.10092889	-4.333290039	-4.248529758
hsa-miR-875-5p	0.519428439	1.560396842	-9.3428077	-6.361049307
hsa-miR-18b-3p	2.554023788	-0.573770753	-8.902346051	-5.713054737

Table 4.5: Highly significant unique differential miRs of EIN

Endometrial Atypical Hyperplasia (EIN)	UP regulated	Down regulated
	hsa-miR-135a-5p	hsa-miR-3910
	hsa-miR-135a-3p	hsa-miR-653-3p
	hsa-miR-153-5p	hsa-miR-489-5p
		hsa-miR-599

Aforementioned miRs in table 4.5 are with |log2FC|≥ 3 for up and |log2FC|≤ -3 for down having p value≤ 0.01.

4.4.5 Shortlist miRs and q-PCR

According to the |log2FC| of microRNAs and the relative literature available, two up and two down regulated miRs were shortlisted for qPCR validation. Ha-miR-205-5p and Ha-miR-509-5p in up regulated while Ha-miR-875-5p and Ha-miR-585-3p in down regulated were chosen. These were retrospect for their expression in EIN and Endometrioid adenocarcinoma in biological replicates (N=3). Formalin fixed paraffin embedded (FFPE) tissues were selected for the experiment. microRNA was carefully purified from the blocks by using miRNeasy FFPE Kit (Cat.No. 217504) using manufacturer's protocol.

The RT-PCR analysis showed the statistically significant expression of all shortlisted miRs in endometrial hyperplasia and adenocarcinoma. In hyperplasia, miR 205-5p was 0.5 times up while 0.7 times up in cancer than normal **(Fig. 4.4 a)**. In case of miR 509-5p, it was 0.3 times up than normal while 0.8 times up than normal in hyperplasia and cancer respectively **(Fig. 4.4 b).** In down-regulated miRs, miR 585-3p was 0.2 times down than normal in both condition **(Fig. 4.4. c)**, while miR 875-5p was 0.3 times and 0.2 times down in hyperplasia and cancer respectively **(Fig. 4.4. d).**

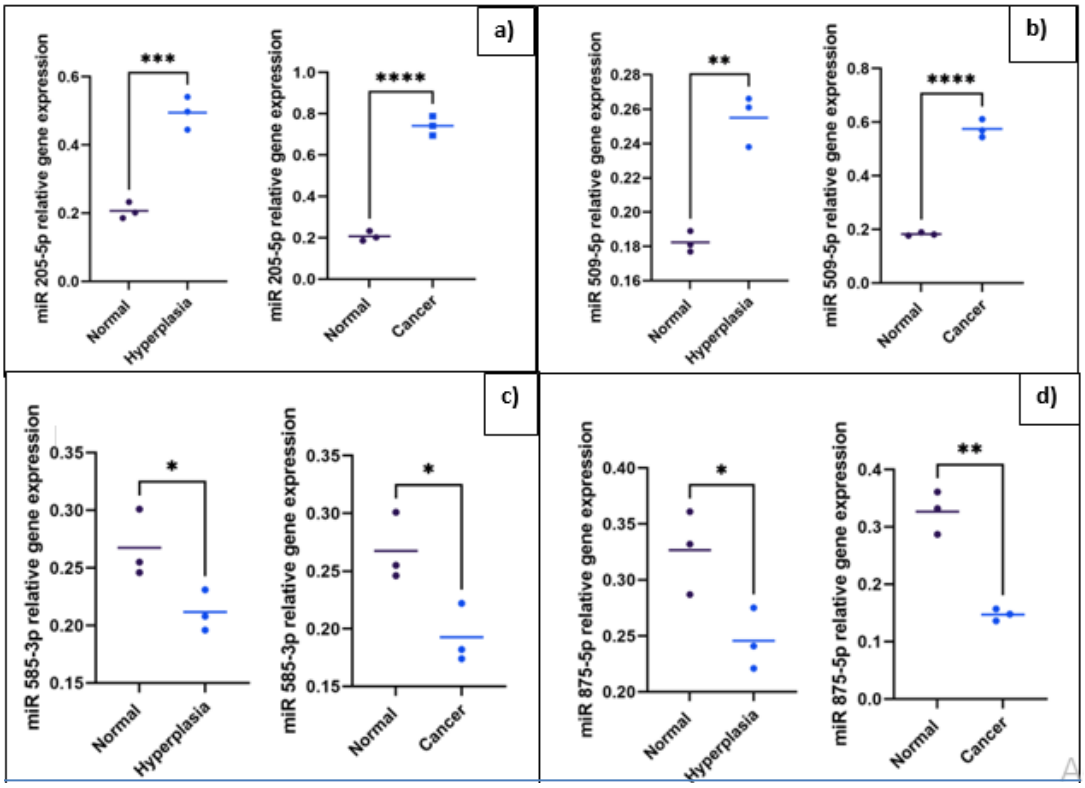


Fig. 4.4: qPCR of shortlisted miRs in EIN and Endometrioid adenocarcinoma. a) ha-miR-205-5p, b) ha-miR-509-5p, c) ha-miR-585-3p and d) ha-miR-875-5p.

4.5 Discussion:

The present study demonstrated the key significant microRNAs which are differentially expressed in series of transitional stages of normal to non-atypia to atypia to adenocarcinoma conditions of human endometrium. Plenty of research on microRNA signature in endometrial malignancy is available as compared to endometrial hyperplasia. However, the microRNA profiling by NGS, in endometrial hyperplasia in aforementioned stages, that too in Indian ethnicity is first of its kind.

In this regard, histologically confirmed samples (Fig.4.2), post microRNA profiling showed 6 onco-miRs and 8 tumor suppressor miRs overlapped in pre-cancerous to cancer transition (Fig. 4.3). Selection of control is of utmost importance for comparison and to get a concrete conclusion. The physiologic endometrium shows two phases i.e. proliferative and secretory as a response to sex steroids. However, in most of the AUB cases, a key symptom in gynae disorder, secretory phase was revealed upon histology. This forms the basis for taking secretory endometrium as control. This is due to the fact

that, all the significant microRNAs which are upregulated in normal control of secretory phase have been down regulated in diseased and down regulated miR in secretory phase have been upregulated in diseased, causing dysregulation leading to atypical hyperplasia and adenocarcinoma (Table 4.3 and 4.4).

Upon further analysis, it has been found that miR-205-5p is consistently down regulated in secretory phase, whereas consistently and gradually showing upregulated in non-atypia, atypia and drastic up regulation in adenocarcinoma conditions. Similarly, mir-181c-3p is also down regulated in secretory phase, and gradually upregulated in various phases of diseased condition (Table 4.3). Similar results were obtained in down-regulated miRs too. The miRs which are upregulated in control were down regulated in disease. For instance, miR-875-5p is slightly upregulated in control, whereas drastically down regulated in atypia and adenocarcinoma conditions. Similarly, miR-876-5p and miR-873-5p also showed an up regulation in control with gradual up regulation from non-atypia to atypia to cancer, with drastic up regulation in atypia and cancer. The miR-585-3p has the similar down regulation in atypia and cancer as compared to control. According to this analysis, the continuous increase/ decrease in log2FC of miRs, 2 up-regulated and 2-down-regulated miRs were shortlisted for further studies.

Out of the onco-miRs, has-miR-205-5p has been studied extensively. It was reported earlier as prognostic marker[6] and its involvement in the initiation and progression of cancer via tumor proliferation and invasion[7], which was observed in this study too. Reports already have established the upregulation of miR-205-5p and miR-181c in serous carcinoma of endometrium[8] and endometrial adenocarcinoma cell line[9]. Zhou et al, studied that miR-205 inhibition leads to death of Ishikawa cell line[10] (Endometrial cancer cell line). Additionally, this miR is involved in numerous signaling pathways, in particular angiogenesis and EMT[11,12]. miR-205 plays a central role in tumour vascularisation as well as tumour invasion though targeting VEGF-A and ZEB1 in cancers including melanoma, glioblastoma, ovarian carcinoma and breast carcinoma[13-17].

Hsa-miR-181 is a highly conserved family includes four 5p mature forms 181a/b/c/d, whose role in physiology and pathology is known. Out of 4 mature forms, 181c-5p and 181d-5p were found up regulated in endometrial hyperplasia and adenocarcinoma (table 4.3). Mir 181 family act as either oncomiR or tumor suppressor miR in different

cancers. Similar results obtained by Zuang et al reported that miR181c is one of the culprit in Endometrioid adenocarcinoma (estrogen dependant endometrial cancer) by targeting PTEN[18]. Most of the research literature related to miR-509-5p indicates its tumor suppressor role in different cancer such as non-small cell lung cancer, pancreatic cancer[19]. In contrary to this, miR509-5p and 509-3-5p were up regulated in endometrial hyperplasia and adenocarcinoma in this study (table 4.3). This could be attest by the recent research on miR profiling in Endometrioid adenocarcinoma in Indian population[20].

On the other hand, out of the 8 tumor suppressor miRs, miR876 was highly down regulated in Endometrioid adenocarcinoma while miR 875-5p and miR 6718-5p were highly down regulated in hyperplasia with atypia (table 4.4). Tumor suppressor role of miR 875-5p was reported in colorectal cancer and in uterine cancer by its direct target NOTCH3[21]. Hu et al proved that miR-875-5p inhibited cell migration, invasion, EMT progression and angiogenesis in his study[22]. In accordance with this, miR 875-5p is down regulated in diseased population, thereby promoting cell migration and invasion. There are also reports establishing the role of miR-585-3p and their down regulation, leading to cancer progression, as they act as tumor suppressor gene, and hence, acting a down regulation gene in disease[23].

While talking about the exclusive, highly significant differential miRs of precursor EIN stage, hsa-miR-135a and 153-5p found to be overexpressed and both exert the dual role. They may act as oncomir or tumor suppressor miR in various cancers (saghar yousefnia). In Endometrial cancer cells it promotes proliferation, migration, invasion and induces chemoresistance (JIping wang)

Although a wide comparative analysis using bioinformatics tools and comparative research publication confirmed the key significant up and down miRs coupled with the perfectly matching control, still to validate the results obtained, a qPCR validation of two shortlisted miRs for both up-and down-regulated miRs was done in retrospect manner, which found in accordance with miR analysis and proved their involvement in progression of pre-cancerous to cancer stage.

4.6 Conclusions:

Upon conclusion, we demonstrated the key significant microRNAs which are differentially expressed in series of transitional stages of normal to non-atypia to atypia to adenocarcinoma conditions. For the first time, we explored the complete profiling of this transition in Indian ethnicity. Secretory phase could act as an ideal control and 4 shortlisted miR panel could prove promising prognostic/ diagnostic markers in endometrial hyperplasia. For instance, in healthy endometrium, miR 205-5p is down regulated, if this miR in IHC or PCR of endometrial sample seems up regulated, then prediction of such result would identify the likelihood of the women prone to hyperplasia or cancer. Similarly, if the miR that is generally down regulated in healthy women seems to be up regulated, she may likely to possess the disease in near future. Thereby, this significant miRs having potential vice versa effects on healthy and diseased endometrium could be a worthwhile panel for prediction of benign and malignant gynaecological abnormalities.

Additionally, miR-205-5p could be considered as global prognostic/diagnostic marker in transition of atypical endometrial hyperplasia to Endometrioid adenocarcinoma.

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CHAPTER 5

Identification of DE m-RNAs and microRNA target enrichment in Endometrial Hyperplasia

5.1 BACKGROUND

Messenger RNA/ mRNA is a single-stranded RNA molecule that carries genetic information from the DNA in the nucleus to the ribosomes in the cytoplasm, where it serves as a template for protein synthesis. The study of mRNA is crucial in understanding the gene expression patterns in various diseases[1]. In the context of endometrial hyperplasia, studying mRNA expression patterns is important for understanding the dysregulation of genes involved in angiogenesis, apoptosis, and cancer stem cell regulation. Dysregulation of these genes can contribute to the development and progression of endometrial hyperplasia to its ultimate lead endometrial cancer (typr-1)[2].

Additionally, the study of mRNA expression in endometrial hyperplasia is critical to identify genes regulated by deregulated miRs, which play a vital role in post-transcriptional gene regulation. MiRs target mRNAs and inhibit gene expression though translational repression or mRNA degradation, thereby controlling various physiological and pathological processes in the body[3]. Deregulated miRs can target oncogenes or tumor suppressor genes, leading to the development and progression of various diseases, including endometrial cancer[4].

This chapter deals with the identification of differentially expressed m-RNAs, emphasised on angiogenesis, apoptosis and cancer stem cells, in Endometrial Hyperplasia by Microarray and further correlating it with shortlisted miRs as their targets using bioinformatics tool.

5.2 MICROARRAY TECHNOLOGY AND TARGET ENRICHMENT

Microarray technology is a powerful tool for high-thoughput gene expression analysis, allowing for the simultaneous detection of thousands of mRNAs in a single experiment[5]. It involves the printing of cDNA or oligonucleotide probes onto a solid surface, such as a glass slide or a microchip, followed by hybridization with labeled cDNA or RNA samples. The resulting hybridization patterns are then analyzed to determine the relative expression levels of each mRNA transcript.

The microarray data of differentially expressed mRNAs could be used to identify miR targets with the help of bioinformatics tools. It can predict the mRNA targets of miRNAs by analyzing the complementary base pairing between the miRNA and the

target mRNA[6]. This method is based on the principle that miRNAs bind to the 3' untranslated region (UTR) of target mRNAs, resulting in their degradation or translational repression. Several computational algorithms have been developed for miRNA target prediction, including TargetScan, miRanda, and PicTar. These algorithms use different parameters to predict miRNA target sites, such as the free energy of base pairing, the conservation of the target site across species, and the accessibility of the target site[7]. The combination of multiple algorithms can increase the accuracy of miRNA target prediction. Here, for target identification, MIRSYSTEM ver.20160513 (http://mirsystem.cgm.ntu.edu.tw/) bioinformatics tool was used. It is a microRNA integration system for target gene prediction. It integrates seven well known miRNA target gene prediction programs: DIANA, miRanda, miRBridge, PicTar, PITA, rna22, and TargetScan. This also contains validated data from TarBase and miRecords on interaction between miRNA and its target genes[8].

5.3 METHODS:

Briefly, histologically confirmed samples of benign endometrial hyperplasia, endometrial intraepithelial Neoplasia (atypical endometrial hyperplasia) along with control were carefully transported to Genotypic, Bengaluru for microarray study to get differentially expressed m-RNA data, in angiogenesis apoptosis and cancer stem cell pathway, in each condition as compared to control.

The genes included in the pathway analysis of microarray data, especially Angiogenesis, Apoptosis and Cancer Stem Cell were analyzed by using MIRSYSTEM ver.20160513 (http://mirsystem.cgm.ntu.edu.tw/) bioinformatics tool, where the genes of each pathway were analyzed separately as query to find corresponding miR.

5.4 RESULTS:

5.4.1 Sample collection and Histology

Since the study samples are of same disease group, the results are similar to the chapter 4, except Endometrioid Adenocarcinoma.

5.4.2 RNA extraction and quality control

To perform microarray, total RNA was extracted from respective samples and its quality was examined by spectrophotometry and RNA was considered of good quality

based on the 260/280 values of ~2.0 (Nanodrop) and rRNA 28S/18S ratios of 2:1. The integrity of RNA was analysed by RNA integrity number (RIN) using Bioanalyzer. The results showed RIN value of 6.5 for non-atypical hyperplasia while atypical hyperplasia samples demonstrated RIN value of 6.6.

5.4.3 Microarray and data analysis

The extracted RNA was then processed and microarray analysis was performed and data extraction from Images was done using Feature Extraction software Version 11.5 of Agilent. The extracted raw data was analyzed using GeneSpring GX software from Agilent and normalized by GeneSpring GX using the 75th percentile shift and differential expression patterns were identified among the samples. Significant genes up regulated fold> 0.6 (logbase2) and down regulated <-0.6 (logbase2) in the test samples with respect to control sample were identified.

The data revealed the differentially expressed genes in Endometrial hyperplasia without atypia and with atypia as compared to physiologic endometrium**.** Total 247 genes were upregulated out of which 155 genes were upregulated in BEH while 146 genes were upregulated in EIN. Among these 101 genes were uniquely upregulated in BEH and expression of 92 genes was up only in EIN. Further, 54 genes were found commonly upregulated in both groups as compared to normal control **[Fig. 5.2A]**. Similarly, total 143 genes were downregulated out of which 77 genes were downregulated in Endometrial hyperplasia without atypia and 96 genes were downregulated in Endometrial hyperplasia with atypia. Out of 77 genes, 47 were distinctively downregulated in without atypia group. In case of with atypia group, the expression of 66 out of 96 genes was exclusively decreased. Moreover, 30 genes were commonly downregulated in both with and without atypia groups as compared to control. **[Fig. 5.2B].**

With the help of bioinformatic tools, differentially expressed mRNAs were assessed and analysed according to their classical pathways and involvement in various cellular processes.

The data demonstrated that the differentially expressed mRNAs in both groups were associated with various key cellular processes and molecular signalling pathways associated with cancer such as angiogenesis, apoptosis, stem cell regulation and cancer

progression. In case of BEH, majority of upregulated mRNAs (53.23%) were associated with apoptosis followed by angiogenesis (26.11%) and stem cell regulation (20.9%). Further, the expression of mRNAs associated with breast cancer (19.8%), endometrial cancer (10.4 %) and cancer stem cells (CSCs) (17.7 %) were also found to be enhanced. Moreover, mRNAs associated with MAP kinase (7.3%), P53 (13.6%), PI3K-AKT (19.8%), cytokine and chemokine (17.7%), VEGF (15.6%) and WNT (18.8%) signalling were also observed to be upregulated in BEH samples **(Fig. 5.3A)**. Similar trends were observed in EIN samples, where top thee

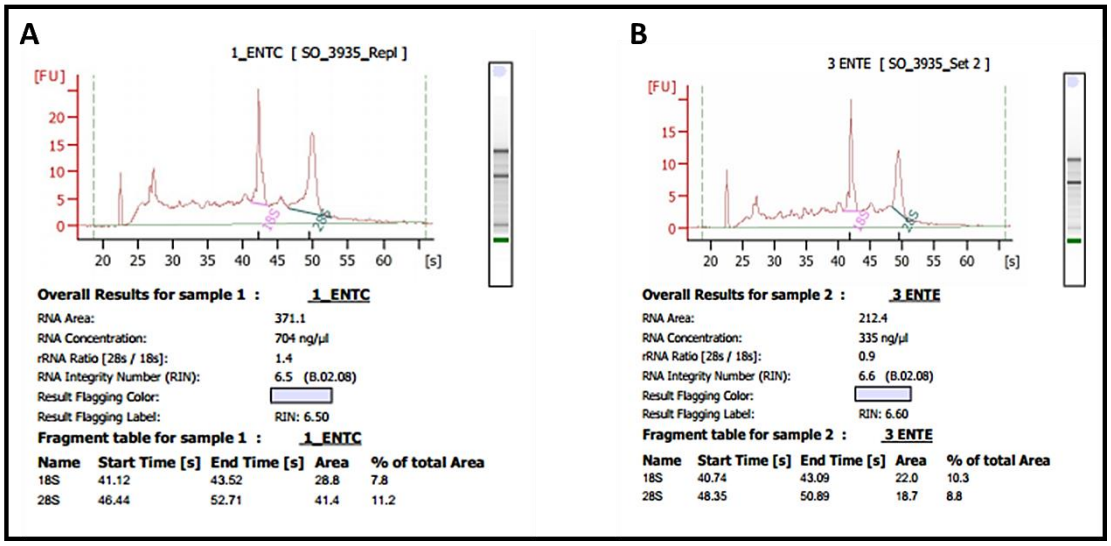

Figure 5.1: RNA quality control by bioanalyser for **(A)** non-atypical hyperplasia and (**B)** atypical hyperplasia.

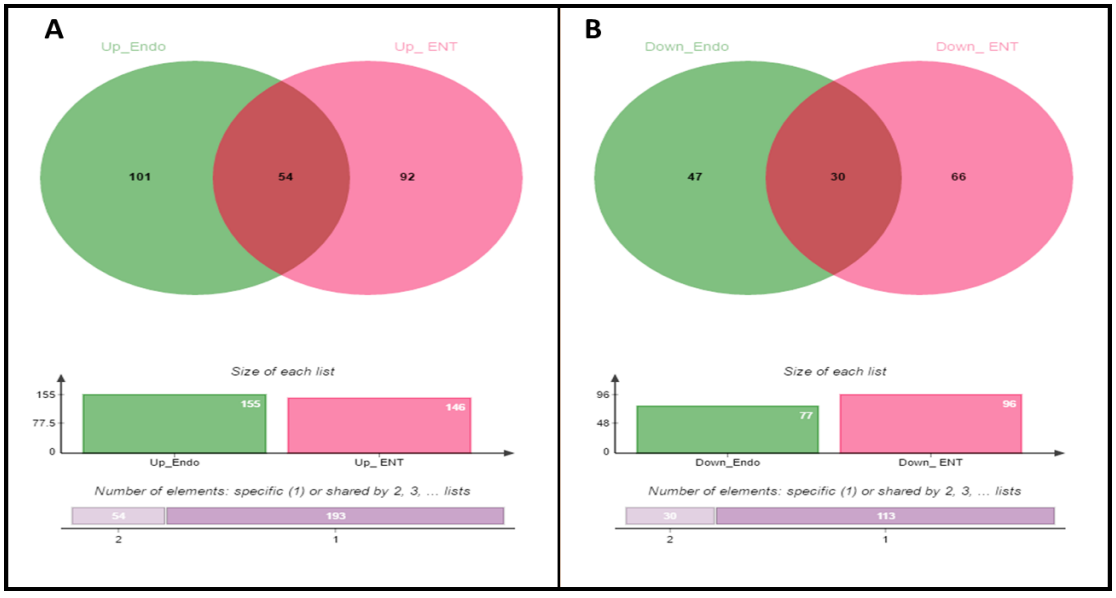

Figure 5.2. Venn diagram representation of DEGs in BEH and EIN (A) upregulated and **(B)** downregulated mRNAs in BEH (Endo) and EIN (ENT).

upregulated mRNA groups were associated with apoptosis (50.22%), angiogenesis (26.12%) and stem cell regulation (24.11%). Further, in case of cancer associated mRNAs, higher number of CSC related RNAs were upregulated (19.8%) followed by breast cancer (18.8%) and endometrial cancer (6.3%). MAP kinase (7.3%), P53 (13.6%), PI3K-AKT (19.8%), cytokine and chemokine (17.7%), VEGF (15.6%) and WNT (18.8%) signalling associated mRNA expression was also upregulated in atypia group as compared to normal control **(Fig. 5.3B)**.

Further, in silico analysis also revealed that there was differential downregulation of mRNAs associated with angiogenesis, apoptosis, cancer, stem cell regulation and various signalling pathways in BEH and EIN compared to control. As demonstrated in **figure 5.4A**, 28.26%, 7.65% and 15.14% of downregulated mRNAs were related to apoptosis, angiognesis and stem cell regulation respectively in BEH group. Further, breast cancer, CSCs and endometrial cancer associated mRNAs consist 6.5%, 10.9% and 4.4% of total downregulated mRNAs. In case of molecular pathways, MAP kinase (3.3%), P53 (7.6%), PI3K-AKT (5.5%), cytokine and chemokine (5.4%), VEGF (5.5%) and WNT (14.13%) signalling associated mRNAs were found downregulated of total population as compared to control group **(Fig. 5.4B)**.

In EIN group, significantly higher number of downregulated mRNAs were associated with apoptosis (41.29%) as compared to BEH group. Downregulated mRNAs involved in angiogenesis and stem cell regulation made 6.4% and 17.12% of total lot. Further, breast cancer CSCs and endometrial cancer contributed 9.6%, 12.9% and 4.3% to total mRNAs population with decreased expression. Moreover, downregulated mRNAs were also consisting of MAP kinase (5.4%), P53 (11.8%), PI3K-AKT (5.4%), cytokine and chemokine (10.7%), VEGF (2.1%) and WNT (18.13%) signalling associated mRNAs **(Fig. 5.4B)**.

5.4.4 Common up regulated and down regulated genes in BEH (w/o atypia) and EIN (with atypia)

The common up and down regulated genes in angiogenesis, apoptosis and cancer stem cell with $|\log2FC| > 1$ for up and $|\log2FC| < -1$ and p value < 0.05, are shown in **table 5.1 and 5.2** respectively.

Highly up regulated and down regulated unique differentials of EIN with |log2FC| ≥ 5,

|log2FC| ≤ -2 and p value < 0.05, are shown in **table 5.3.**

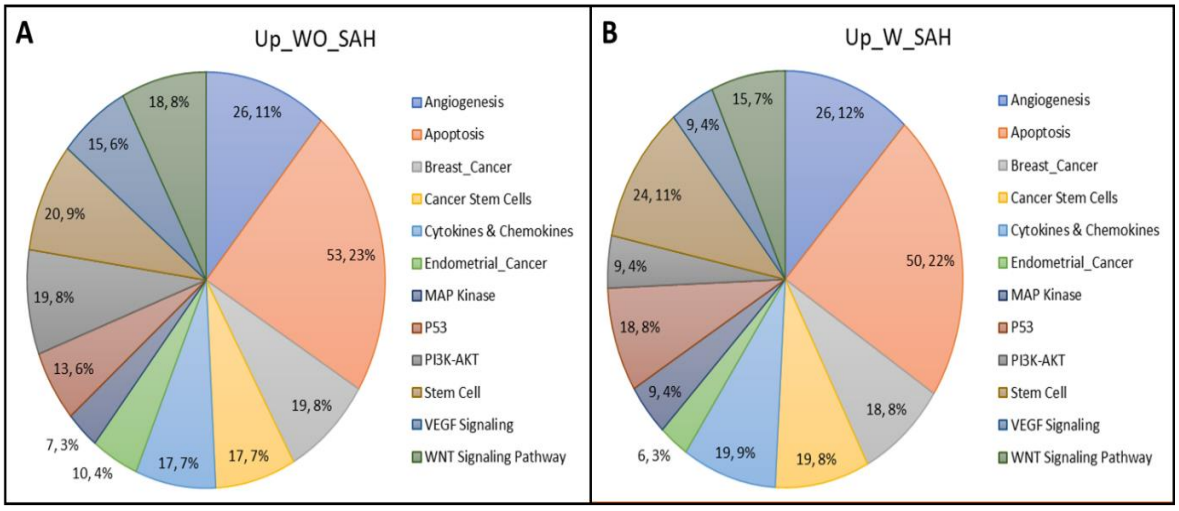

Figure 5.3: Pie chart of pathway's distribution for up regulated genes in BEH and EIN. Shows upregulated mRNAs distribution in various pathways in **(A)** BEH **(B)** EIN, compared to control determined by microarray analysis.

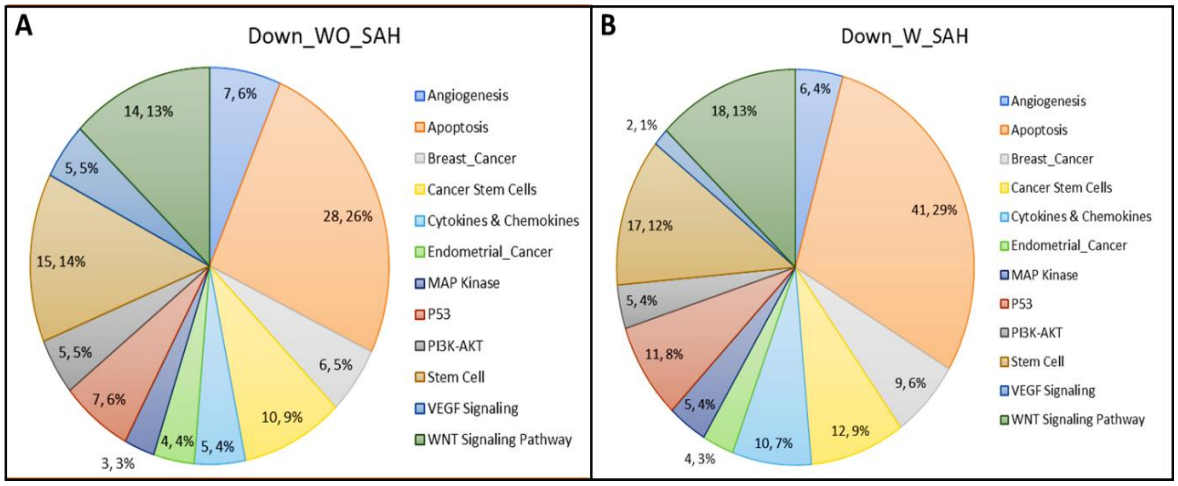

Figure 5.4: Pie chart of pathway's distribution for down regulated genes in BEH and EIN. Shows down regulated mRNAs distribution in various pathways in **(A)** BEH **(B)** EIN, compared to control determined by microarray analysis.

Table 5.1: Common up regulated genes between BEH and EIN

Angiogenesis	Apoptosis	Cancer stem cell
ANK3	BCL2A1	DKK2
CAMK2D	BCL2L11	FZD4
FAS	ERC1	JUN
FGFR1	FAS	NKD2
FN1	HIP1	RAC2
FZD4	IER3	ATM
HBEGF	INHBA	PLAUR
HPSE2	MCL1	
HSPB2	NLRC4	
LUM	PML	
PLAUR	SOCS3	
PXN	SSTR3	
THBS1	STK17B	
ANPEP	TNFSF14	
CXCL1	TPT1	
EDN1		
FN1		
HGF		
IL6		
MMP14		
PF4		
SERPINE1		
THBS1		
THBS2		
TIMP2		

Table 5.2: Common down regulated genes between BEH and EIN

angiogenesis	apoptosis	Cancer stem cell
CAMK2B	BBC3	CAMK2B
EGFR	FADD	DKK4
EIF4B	API5	FZD8
ERBB3	BBC3	PRKACG
ERBB4	CUL3	PLCB2
FGFR1	EDA	WNT1
PRKACG	EEF1A2	WNT10A
PLG	FADD	WNT2
	GADD45A	WNT2B
	IFI6	ITGA2
	NLRP2	MYCN
	NME5	
	PEA15	

Table 5.3: Highly significant unique differential genes of EIN

Up-regulated	Down regulated
IL8	FGFR1
IL8RB	DKK4
IL8RA	PCLB2
PPBP	-
PROK2	-

5.4.5 TARGET ENRICHMENT OF SHORTLISTED MIRS

Identification of the m-RNA targets of shortlisted miRs (has-miR-205-5p, has-miR-509-5p, has-miR875-5p and has-miR-585-3p) were done by using gene list involved in angiogenesis, apoptosis and cancer stem cell pathway from SABioscience and KEGG database. Altogether 798 genes; out of which 270 angiogenesis, 444 apoptosis and 84 cancer stem cell genes were analyzed for their regulator miR. The distribution is shown in table below.

Table 5.4: Distribution of genes among pathways

	Total genes analyzed	Targets found	DEGs
Angiogenesis	270	205	63
Apoptosis	444	269	58
Cancer Stem Cells	84	56	36

The gene list of each pathway was analyzed for its corresponding miR as query for two up regulated miRs (ha-miR-205-5p and ha-miR-509-5p) and two down-regulated miRs (ha-miR875-5p and ha-miR-585-3p) were correlated with unique differentially expressed genes of Benign Endometrial Hyperplasia (BEH) and Endometrial Intraepithelial Neoplasia (EIN).

The data is represented as microRNAs and their computationally predicted/validated mRNA targets. The differential expression of respective gene is given in colour code below, as well as the percentage of DEGs of each miR in each pathway is represented graphically [Fig. 5.5].

Common up	Common down	Unique up BEH	Unique up EIN	Unique down BEH	Unique down EIN

5.4.5.1 ANGIOGENIC GENE TARGETS

Out of 270 genes involved in angiogenesis, 205 genes are found to be targets of aforementioned shortlisted miRs. Among which 63 genes were differentially expressed **(table 5.5**). The percent distribution of DEGs of respective miR in each pathway is represented graphically [Fig. 5.5]

5.4.5.2 APOPTOSIS GENE TARGETS

Out of 444 genes involved in apoptosis, 269 genes were found to be targets of aforementioned shortlisted miRs. Among which 58 genes were differentially expressed **(Table 5.6**). The number of target genes and the DE genes of each miR is shown graphically **(Fig. 5.7)**

5.4.5.3 CANCER STEM CELL GENE TARGETS

Out of 84 genes analyzed in cancer stem cell pathway, 56 genes were found to be targets of aforementioned shortlisted miRs. Among which 36 genes were differentially expressed **(table 5.7).** The number of target genes and the DE genes of each miR is shown graphically **(Fig. 5.7)**

The number in bracket displayed with some of the genes indicates the number of target validating bioinformatics tool, out of seven. Greater the number, more are the chances of target confirmation to the respective miR. The genes with number ≥ 3 could be consider to construct PPI network. Genes with no number indicates only one tool validated it as target.

Table 5.5: DEG target enrichment (involved in angiogenesis pathway)

Table 5.6: DEG target enrichment (involved in apoptosis pathway)

Hsa-miR-205-5p	Hsa-miR-509-5p	Hsa-miR-875-5p	Hsa-miR-585-3p
CTSC	PRF1	EIF2S1 (2)	CTSS
CTSB (2)	HIP1	BCL2	STAT1
TUBA4A	FADD	TNF	PCBP4
EIF2S1	EDAR	CARD6	PIK3R2
COL4A3	PEA15 (3)	MALT1	BCL2L11
PMAIP1	BCL2L11 (3)	PCBP4	MAPK1
TNFSF15	INHBA (3)	PRLR	PTEN
TNFRSF21	PLG	TBX3	CD40
EDAR		TNFAIP3	LTB
DYRK2		INHBA	
CD28 (3)		ERC1	
ALOX12 (2)			
GDNF			
SOCS3			
SON			
TBX3 (4)			
TSC22D3 (2)			
VEGFA (7)			
BCL2L11			
DAPK2 (2)			
FASLG			
INHBA (6)			
PRUNE2			
PTEN (4)			
BCL6 (3)			
STAT5B			
ZAK			
TP53I3			
ERC1 (3)			
EDA (3)			

Table 5.7: DEG target enrichment (involved in Cancer Stem Cell pathway)

Hsa-miR-205-5p	Hsa-miR-509-5p	Hsa-miR-875-5p	Hsa-miR-585-3p
ALCAM (2)	ATXN1 (3)	ALCAM	FGFR2
ATXN1	BMI1 (3)	ATXN1	SOX2
CD24 (2)	CD24(2)	ITGA4	
CD44	CHEK1	LATS1	
FOXP1 (4)	DKK1	WEE1	
GATA3 (5)	FGFR2		
ITGB1	FOXP1 (3)		
KIT	FZD7(2)		
PECAM1 (3)	GATA3		
PTPRC	ITGA4		
SOX2	ITGB1		
THY1	PTPRC		
WWC1 (4)	YAP1		
YAP1 (2)			
ZEB1			
ZEB2 (5)			

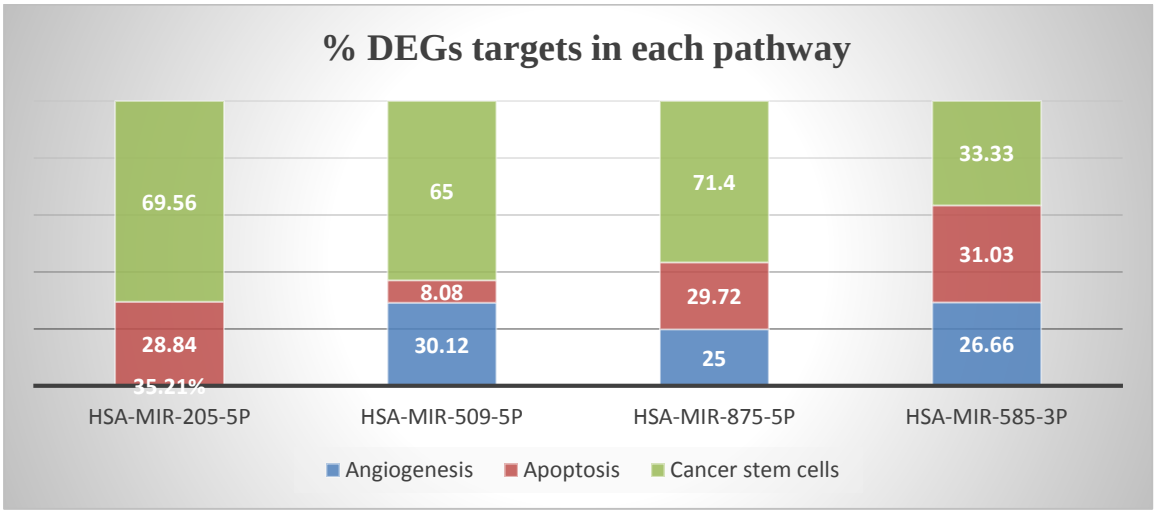

Fig. 5.5: Graphical representation of percent distribution of DEGs targets in each pathway

Discussion:

Based on the dynamic tissue remodelling in uterus, during the menstrual cycle and pregnancy, it has been suggested that adult stem cells from the endometrial tissue plays a vital role in regeneration. However, this property of endometrium is the culprit in causing hyperplasic condition. A thorough characterization of human endometrium at its transcriptome level is of utmost importance. Once this classical pathway/function of microarray has been constructed, it then becomes easier to understand the complex mechanisms underlying the malignancy. Although studies on mRNA profiling of endometrioid adenocarcinoma condition exist, there are uncertainties in the expression profiling of non-atypical and atypical mRNA profiling and their correlation towards adenocarcinoma. This forms the basis of this study with special emphasize on basic hallmarks of cancer, i.e angiogenesis, failed apoptosis and cancer stem cells; for their involvement in transition and establishment of the disease. While analysing the results two databases were taken into consideration viz. KEGG and SABioscience. Accordingly, the number of differentially expressed m-RNAs are represented in venn diagram (**Fig.5.2 A and B**) and common up as well as down regulated genes in angiogenesis, apoptosis and cancer stem cells are listed in (**Table5.1**). Since atypical hyperplasia (EIN) is considered as precursor to cancer, highly expressed unique differentials are tabulated in **Table 5.2**. It shows expression of IL8 and its G protein coupled receptors α (CXCR1) and β (CXCR2) to be highly up regulated, which is in connection to immunohistochemistry study on 101 tumor by L. Ewigton 2012, which demonstrated over expression of IL8, IL8RA, IL8RB[9]. Expression of IL8RA is stronger than other two in endometrial cancer. Another study supports estrogen and prokineticin1 induce IL8 expression[10] which eventually results in increased angiogenesis supporting hyperplasia and further progression. Another chemokine PPBP /CXCL7 though its receptors CXCR1 and CXCR2, is involved in progression of various cancer in autocrine or paracrine manner though multiple signalling pathways[11]. Recent studies on PROK2 reported its role in angiogenesis in various cancers like Glioblastomas, colorectal[12], breast and cervical cancer, and found to be involved in all steps of tumorigenesis, hence could be used as biomarker. DKK4 is an exclusive WNT pathway inhibitor[13].

The main motto of performing microarray is to know differentially expressed m-RNAs in benign and atypical endometrial hyperplasia, which could be the targets of shortlisted miRs (Chp.4). The percent of DE m-RNAs expressed in both the groups involved in different pathways associated with cancer progression is already demonstrated (**Fig.5.3 and Fig.5.4**) and the lists of DE mRNAs targets to corresponding miRs are shown in **Table 5.4, 5.5 and 5.6** for angiogenesis, apoptosis and cancer stem cells respectively. Most of the DE m-RNAs were found to be targets of Up-regulated miRs 205-5p and 509-5p in all hallmarks (**Fig. 5.6, 5.7 and 5.8**) but most in cancer stem cell (**Fig.5.8**) showing the pivotal role of CSCs in endometrial hyperplasia. In CSCs, FOXP1 is validated target of 205-5p and 509-5p, and this act as tumor suppressor as it is lost in several tumor types, is downregulated in endometrial hyperplasia[14]. Down regulation of GATA3 in endometrial hyperplasia indicates aiding to epithelial to mesenchymal transition, as some in vitro[15] and in vivo[16] studies support that GATA3 supress the expression of factors critical to EMT. Similarly, WWC1 is a tumor suppressor gene[17], is downregulated in atypical hyperplasia. On the other hand, YAP gene was proven to accelerate cancerous EMT in endometrial cancer[18], which found up regulated in atypical hyperplasia. ZEB2 is one of the transcription factors in EMT and plays important role in EMT induced processes[19]. It has been reported to be upregulated in benign hyperplasia as well as endometrial cancer (Pawel sadleki 2020), indicating its role towards disease progression[20].

There are certain genes like VEGFA, VIL2, INHBA and PTEN are validated by more than 4 bioinformatics tools as targets of miR 205-5p (**Table 5.3 and 5.4**). In case of apoptotic genes, INHBA is validated as target of miR 205-5p, 509-5p and 875-5p (**Table 5.4**).

Vascular Endothelial Growth Factor A, a well-known angiogenic factor acts though endothelial cell surface receptors VEGFR1 and VEGFR2. The up-regulation of VEGFA in benign endometrial hyperplasia indicates towards hypoxic condition, as cells increase VEGFA production in less oxygen condition and promote angiogenesis[21]. Additionally, hypoxia contributes to Epithelial to mesenchymal transition (EMT) and cancer stem cell like properties[22]. Hypoxia increases TGF-β, which is master regulator to promote EMT[23] and INHBA which is upregulated in both benign and atypical endometrial hyperplasia, is member of TGF-β superfamily. INHBA is demonstrated as

prognostic biomarker and its correlation with immune cell infiltration in cervical cancer by Kaidi Zhao[24].

VIL2 codes for protein Ezrin. In gynaecological oncology, Ezrin found to regulate cell proliferation and invasiveness by modulating EMT in ovarian cancer cell lines SKOV3 and CaOV3[25] while in cervical cancer cell lines, SiHa and CaSki, it plays role in invasion and migration[26]. However, its role in endometrial cancer is still unclear. PTEN (phosphatase and tensin homolog) a known tumor suppressor. It arrests the cell growth and enhances the cellular sensitivity to apoptosis. The PTEN functional loss is one of the causative reasons in different cancers including Endometrial cancer. According to Djordjevic B, Endometrioid tumors had higher PTEN sequence abnormalities and PTEN protein loss than non-endometrioid tumors[27]. This asserts the up-regulation of PTEN in benign hyperplasia and its loss in atypical hyperplasia as it progress, in our study.

Since a microRNA targets 100s of mRNAs, selection of peculiar mRNAs involved in pathogenesis for their prognostic/ diagnostic/ therapeutic practice is tedious. Aiding to this, our microarray study gave enriched microRNA-target data which are differentially expressed in endometrial hyperplasia and has further connection to Endometrioid Adenocarcinoma. This would help in easing creating a panel instead of studying only one or two genes for their aforementioned practice, especially in Indian population.

CONCLUSIONS:

Upon conclusion, we identified the DE mRNA targets of the shortlisted miRs, involved in angiogenesis, apoptosis and cancer stem cell pathways, in BEH and EIN. Considering that most of the differentially expressed mRNAs are involved in epithelial to mesenchymal transition, it could be stated that cancer stem cells are involved in onset and progression of endometrial hyperplasia. Since, the respective DE m-RNA targets to corresponding miRs explain the reasons behind the disease condition, it could be considered as therapeutic targets. The corresponding miRs could also be considered as therapeutic target by using their specific mimics/ inhibitors.

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CHAPTER 6

Role of Itraconazole in Endometrial Hyperplasia

6.1 BACKGROUND

The global burden of cancer has made 'Cancer therapeutics' a scorching area of research in terms of discovering different treatment modalities for survival. Targeted therapy for cancer is one such thriving area in onco research, which could also be effective at precancerous or early stages of cancer inhibiting its further progression.

6.1.1 NEED FOR TARGETED THERAPEUTICS AND REPURPOSED DRUGS

Targeted therapeutics focuses on targeting specific proteins[1] that helps the cancer cells to grow, divide and spread. It majorly includes

1) **small molecule inhibitor-** which could easily enter the cell and attack intracellular target.

2) **monoclonal antibodies-** which either

i) block signal transduction by attaching to the specific target on the surface of cancer cell

ii) flag the cancer cell for better recognition by the host immune system and destroy it

or

iii) deliver the drug/ toxin/ radioactive particles inside the cancer cell to kill it.

3) **immunotoxins**- It is the fusion protein (produced from recombinant DNA) having toxin as additional domain on antibody or growth factor, which on internalization via clathin coated pits, the toxin promotes apoptosis of targeted cell[2,3].

Unlike chemotherapeutics drugs, the targeted therapeutics are harmless to normal cells. Thus proved effective by increasing survival rate of patients, decreased the side effects and improved the quality of life. However, the long-term use of this could make cancer cells resistant. Some targets are difficult in terms of their structure and its function inside the cell which make even more difficult to design a drug against; which gave rise to continuous demand of better drug options with wider target coverage and comparatively lesser side effects4. Discovering such drugs meeting the needs are highly expensive, time consuming and have poor success rate to reach till clinical trials[5].

This paved the way for Repurposing of drugs in Oncology, known as ReDO project[6] aims at reuse of well acknowledged non-cancer drug for its onco-therapy purpose. Some of the common examples of repurposed drugs include statins, angiotensin-receptor blockers (ARBs), metformin, aspirin, and vitamin D[7]. Moreover, some antibiotics have also investigated for their anti-cancer activity, called as Antineoplastic antibiotics having inhibitory effect on the uncontrolled proliferation, aggressive growth and metastasis of malignant cancers[8].

Itraconazole is also such an example of repurposed drug for cancer therapy.

6.2 ITRACONAZOLE

Itraconazole (ITZ) [Fig.6.1] is a well-known, broad spectrum antifungal drug, belongs to triazole family. Its medicinal use was approved in1992 in U.S and designated as orphan drug by U.S Food and Drug Administration (FDA) and the European Medicines Agency (EMA). The triazoles are inhibitors of cytochome P450 dependent enzyme, lanosterol 14-alpha-demethylase and decreases the synthesis of ergosterol which is essential for membrane integrity of fungal cell. This depletion of ergosterol damages the fungal cell membrane resulting in cell death.

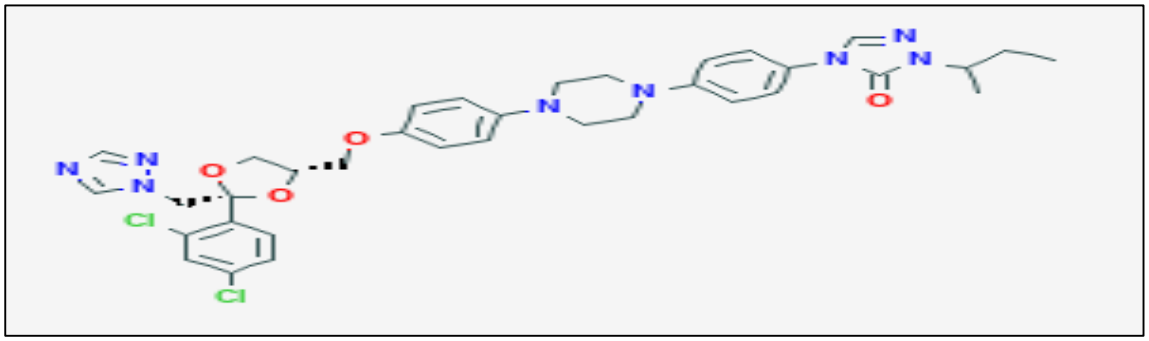

Fig. 6.1: Structure of Itraconazole. Source: pubchem.ncbi.nlm.nih.gov

Hence, it is the most common prescribed drug in fungal infections such as blastomycosis, sporotrichosis, histoplasmosis, and onychomycosis as well as a chosen option to treat systemic fungal infections like aspergillosis, candidiasis, and cryptococcosis[9] Itraconazole as an anti-cancer drug has proven its effectiveness in various cancers following different mechanisms, which has already discussed in review of literature.

The efficacy of this drug in gynaecological oncology, especially Endometrial cancer, pave the way to study its effectiveness in precancerous stage (EIN). Hence, it could be hypothesized that Itraconazole could be an ideal multi-target hope in endometrial hyperplasia, preventing its further transition to Endometrioid Adenocarcinoma.

6.3 METHODS: (Details in chp. 3)

Briefly, histologically confirmed EIN endometrial tissues were selected for primary culture and cells of early passages (P2/ P3) were used for the in-vitro n in-ovo experiments. The experiments are so selected to prove multi-target effect of Itraconazole as inhibitor of angiogenesis, inducer of apoptosis and inhibitor of epithelial to mesenchymal transition.

The in-vitro experiments include CFSE assay, Annexin PI assay (apoptosis), wound scratch assay (angiogenesis), effect on expression of cancer stemness genes (cancer stem cells) i.e. EMT markers and shortlisted microRNAs (chp.4).

In-ovo experiments include, CAM assay, where xenograft model of CAM made by injecting cultured endometrial cells derived from EIN tissue and tested against Itraconazole.

6.4 RESULTS:

6.4.1 CFSE assay and Annexin PI (apoptosis) assay

The analysis of CFSE and Apoptosis assay by flow cytometry shows anti-proliferative effects of ITZ on endometrial hyperplasic cells, showing dose-dependent and time-dependent cessation of the proliferation of these cells in *in vitro* conditions. The percent MFI reduction [**Figure 6.2: (a)]** from day 0 to day 5 is 21.4, 34.8, 41.2, 44.8, and 48.1 for control and 0.2, 0.5, 0.8, and 1 µM concentrations respectively. Additionally, **[Figure 6.2: (b)]** the apoptosis assay revealed the ITZ's ability to induce a dose-dependent increase in apoptotic cell death where the mean percent apoptosis ranges from 3.4 to 11.3.

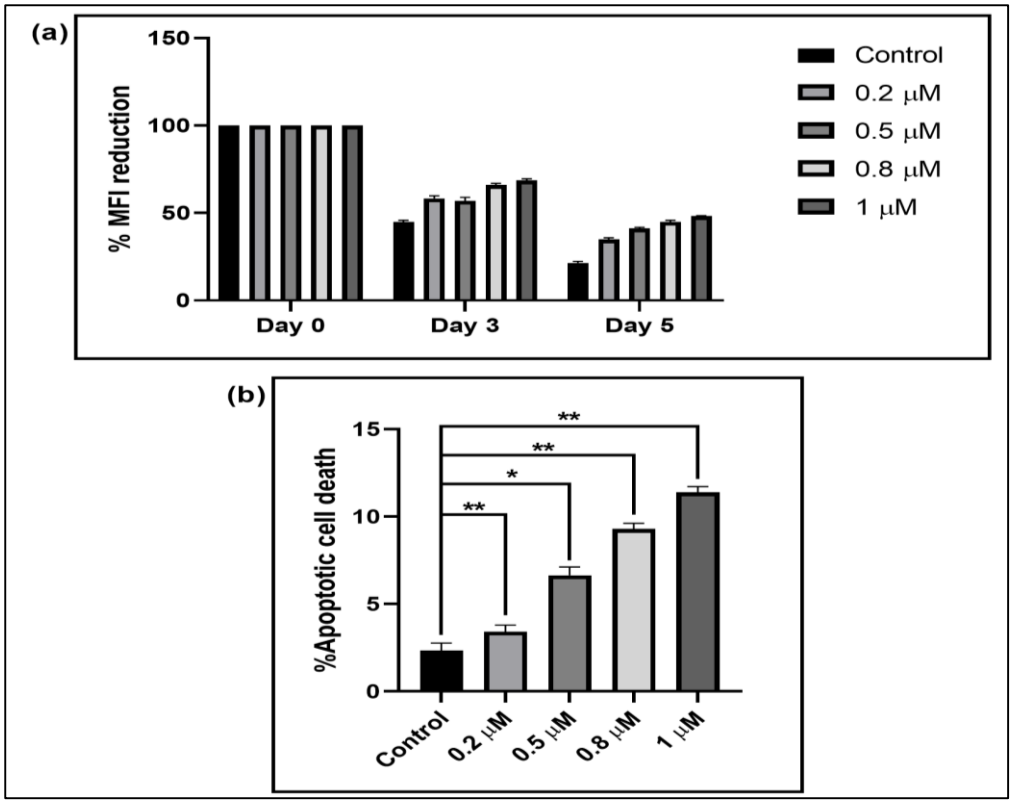

Fig. 6.2: CFSE and annexin PI assay. (a) CFSE assay of ITZ treated at 1, 0.8, 0.5, and 0.2 µM concentrations on endometrial hyperplasic (EIN) cells. (b) Apoptosis assay or Annexin-PI assay of ITZ treated at 1, 0.8, 0.5, and 0.2 µM concentrations on endometrial hyperplasic cells. The highest apoptotic cell death induced was 11.3% (mean) at 1 µM concentration. All the ITZ concentrations show a dose-dependent induction of cell death. For all analyses p-value<0.05 was stated as significant (Significance Levels: *p< 0.05 and **p< 0.01).

6.4.2 Wound scratch assay

The wound scratch assay along with CD326 expression post ITZ treatment shows inhibition of cellular stemness and wound healing potential. Specifically, **[Figure 6.3: (a)]** the wound scratch assay of endometrial hyperplastic cells shows a clear and clean margin after treatment with 0.8 µM ITZ for 30 h. In addition, **[Figure 6.3: (b)]** the cells were assessed for expression of CD326 which shows significant MFI reduction to 2617 post 30 h treatment.

6.4.3 q-PCR

The RT-PCR analysis showed a dose-dependent reduction of stemness-related genes post-treatment with 1, 0.8, 0.5, and 0.2 µM ITZ. **[Figure 6.4: (a)]** At concentrations 0.8 µM and 1 µM the decrease in relative gene expression of SNAIL, SLUG, and

TWIST was 0.8 & 0.6, 0.6 & 0.4, and 0.9 & 0.8 times respectively. **[Figure 6.4: (b)]** The expression of mir 205-5p, mir 509-5p, mir 875-5p & mir 585-3p at 0.8 µM and 1 µM ITZ concentrations. The mir 205-5p and mir 509-5p show 0.8 times downregulation at 0.8 µM ITZ when compared to control or normal tissue, conversely 875-5p and mir 585-3p are upregulated 1.6 and 1.2 times respectively 0.8 µM ITZ in comparison to normal cells.

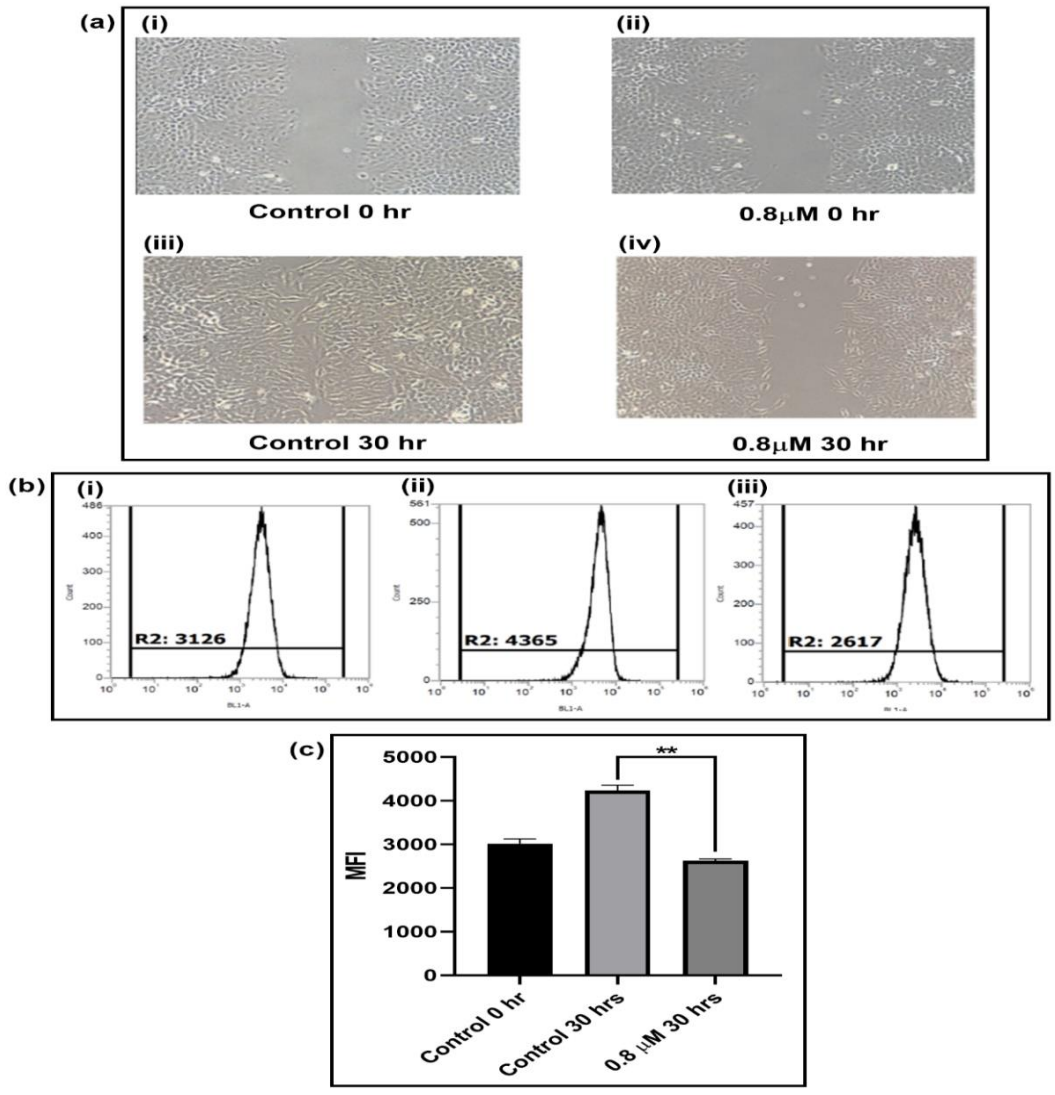

Fig. 6.3: Wound scratch assay. (a) Wound scratch assay of endometrial hyperplastic cells after treatment with 0.8 µM ITZ for 30 h. (b) Expression of CD326 surface marker post-treatment with 0.8 µM ITZ, (i) CD326 expression at 0 h, (ii) CD326 expression at 30 h and (iii) CD326 expression at 30 h after 0.8 µM ITZ treatment. (c) Statistical comparison of CD326 surface expression where analysis p-value<0.05 was stated as significant (Significance Levels: *p< 0.05 and **p< 0.01).

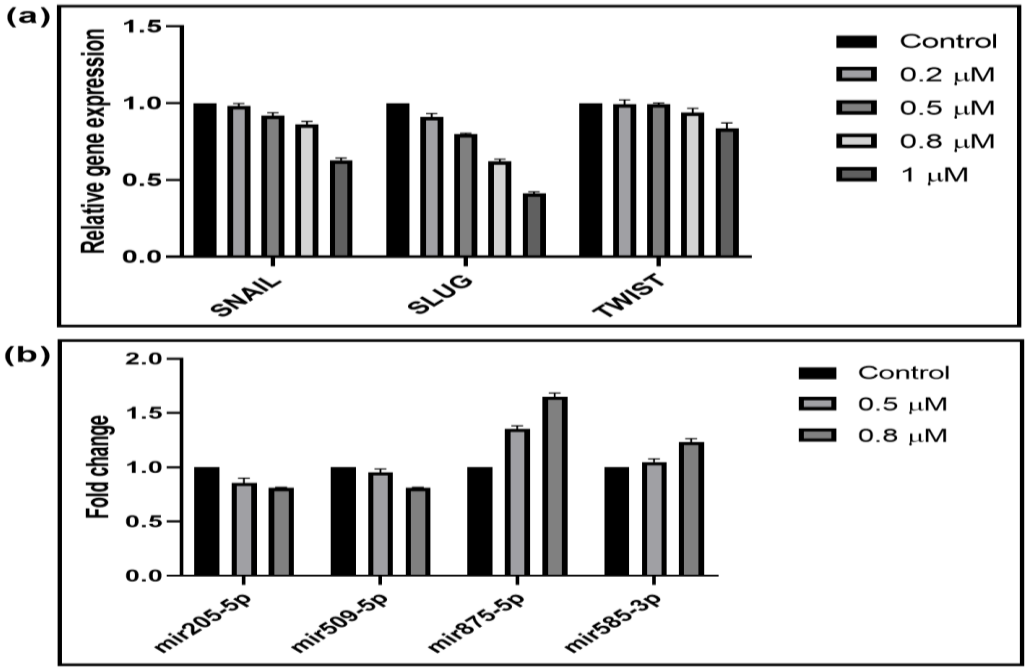

Fig. 6.4: q-PCR. (a) Relative gene expression of stemness-related genes after treatment with 1, 0.8, 0.5, and 0.2 µM ITZ. (b) Fold change of mir205-5p, mir509-5p, mir875-5p, and mir585-3p after treatment with 0.5 and 0.8 µM ITZ.

Chick Chorioallantoic Membrane (CAM) Assay:

The anti-angiogenic potential of ITZ assessed by direct CAM assay (Figure 6.5:b) shows a significant decrease in angiogenesis when compared with positive control endometrial intraepithelial neoplasia (EIN) cells **[Figure 6.5: (a) (ii) and (iii)]** to 0.8 µM ITZ treated CAM **[Figure 6.5: (a) (iv) and (v)]**. [Figure 6.5: (b)] shows the calculated decrease in the number of nodes, junctions, and segments and corroborates the experimental setting with positive control endometrial intraepithelial neoplasia (EIN) cells.

The focal administration method CAM assay where all the ITZ concentrations were used 0.2, 0.5, 0.8, and 1 µM with negative control **[Figure 6.6:(a)],** additionally, **[Figure 6.6:(b)]** is the negative image of the same **[Figure 6.6: (a)]** for precision angiogenic scoring. The angiogenic scores **[Figure 6.6: (c)]** show a graphical representation of the findings which shows a definitive dose-dependent effect of ITZ.

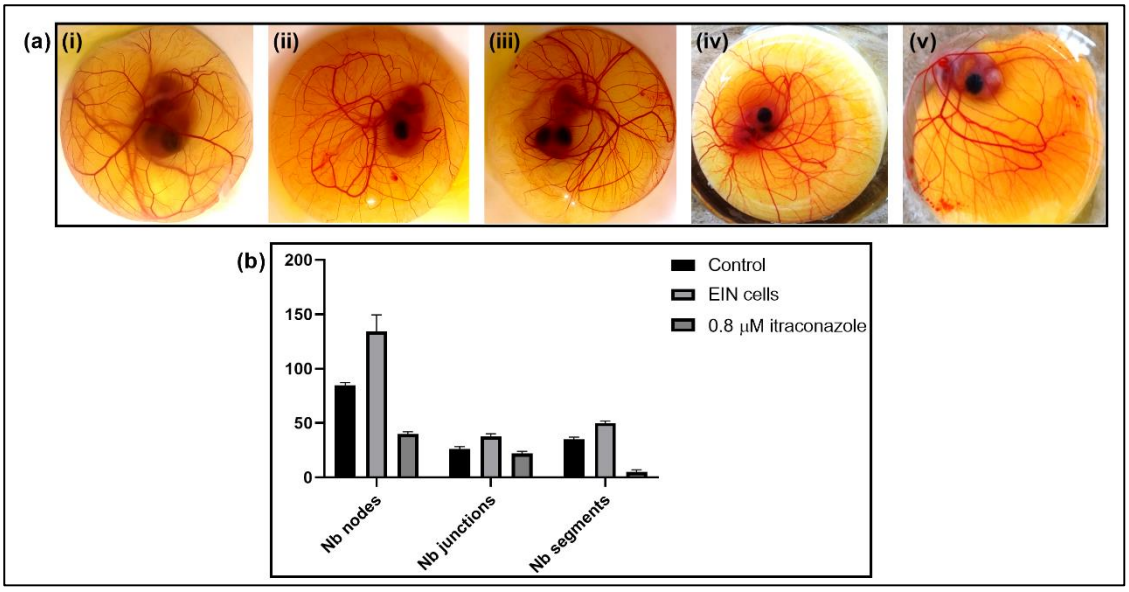

Fig. 6.5: Chick Chorioallantoic Membrane (CAM) Assay (direct method). (a) Effect of ITZ on CAM (direct method), (i) Control, (ii) & (iii) EIN cells (xenograft model), (iv) & (v) ITZ treatment. (b) Image J analysis of angiogenesis of CAM assay (direct method).

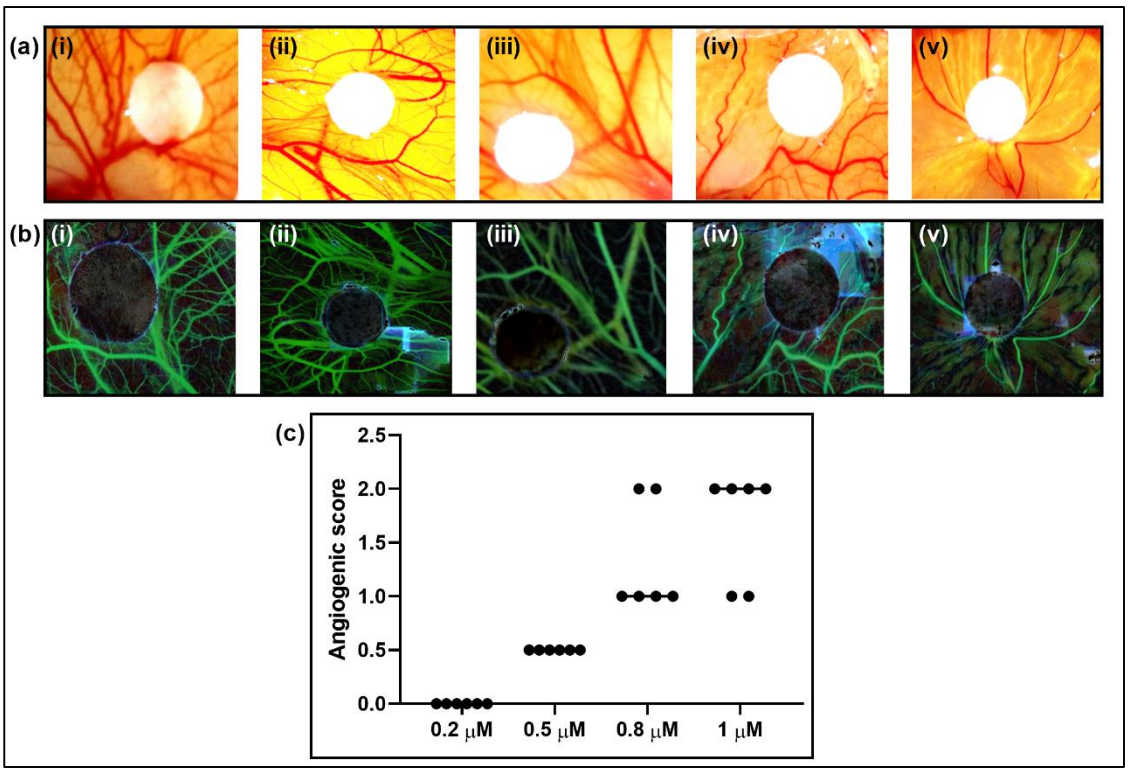

Fig. 6.6: Chick Chorioallantoic Membrane (CAM) Assay (focal method). (a) Effect of ITZ on CAM (focal administration), (i) Negative control, (ii) 0.2 µM, (iii) 0.5 µM, (iv) 0.8 µM & (v) 1 µM. (b) Negative image effect of CAM (focal administration), (i) Negative control, (ii) 0.2 µM, (iii) 0.5 µM, (iv) 0.8 µM & (v) 1 µM. (c) Dot-plot graphical representation of angiogenic scoring.

Discussion

The well-established role of ITZ as an anti-fungal agent along with its FDA-approved safety has led many researchers to repurpose this drug for other diseases and disorders. Evident reports of the anti-cancer potential of this drug in various oncologies including gynecological oncologies, we aimed to demonstrate its efficacy in pre-cancrous stage of Endometrioid Adenocarcinoma i.e. EIN (Endometrial atypical hyperplasia) as an additional treatment option where conventional methods fail. For any cancer to flourish from precursor stage to well differentiated carcinoma, basic hallmarks of cancer are responsible which primely includes, replicative immortality (stemness aquirance), failed apoptosis, angiogenesis induction and invasion as well as metastasis activation. ITZ showed its profound effect on the primary culture of EIN cells in terms of aforementioned hallmarks. ITZ induced anti-proliferative effect on EIN cells, assessed by CFSE assay which showed dose-dependent and time-dependent inhibition of the proliferation of endometrial hyperplasia cells at day 5 as compared to day 3 and day 0 **(Fig. 6.1:a)**. It also induced dose-dependent apoptosis demonstrated by Annexin V- PI assay **(Fig. 6.1:b).**

Additionally, metastasis is the primary mechanism by which tumor cells proliferate in the tumor microenvironment and evade immune surveillance [10,11].

Furthermore, the epithelial cell adhesion molecule CD326 (EpCAM) is the key molecule in cell proliferation and migration which is elevated during metastasis and characteristic of many neoplasms[12,13]. Therefore, **(Fig.2:a)** shows inhibition of wound cell migration after 30 h of treatment with 0.8 µM ITZ **(Fig. 2:b and 2:c).**

The role of ITZ as a therapeutic agent in endometrial hyperplasia is further advocated by its effect on the modulation of gene and miRNA expression. The stemness-associated genes SNAIL, SLUG, and TWIST show decreased relative gene expression upon ITZ treatment evidently showing a dose-dependent decrease for all thee genes (Figure 3:a). The lowered expression of stemness-related genes is necessary to halt the proliferation of cancer stem cell proliferation in endometrial hyperplasia.

The role of miRNA in cancers has been investigated extensively, their down regulation and upregulation are closely associated with cancer proliferation and tumor suppression[14] Therefore, the assessment of the expression of miRNAs is imperative in evaluating the anti-cancer potential of therapeutic interventions. The shortlisted miRs

(Chpt. 4) had shown their connection in cancerous transition, and their target genes too have reports of their participation in carcinogenesis (Chpt.5). Hence, the expression of miR 205-5p, miR 509-5p, miR 875-5p & miR 585-3p at 0.8 µM and 1 µM ITZ concentrations were studied. The miR 205-5p and miR 509-5p show 0.8 times down regulation at 0.8 µM ITZ when compared to control or normal tissue, conversely miR875-5p and miR 585-3p are upregulated 1.6 and 1.2 times respectively0.8 µM ITZ in comparison to normal cells **(Fig. 3:b)**. The down regulation of miR 205-5p and miR 509-5p correlates with decreased angiogenesis which was observed in CAM assay **(Fig. 4 and 5)** and directly correlates with tumor proliferation and growth via desmocolin-2 and MDM2[15]. In addition, the upregulated mir 875-5p and mir 585-3p corroborate with decreased tumorigenicity and suppression of TGF-β1[16,17]. The immune escape mechanisms employed by tumor cells for metastasis require the neogenesis of blood vessels though a process of epithelial-mesenchymal transition[18,19]. EIN cells attested to this by increased angiogenesis (Fig.4 a (ii) and (iii)), while ITZ exerted anti-angiogenic effect by decreasing capillary density **(Fig. 4: a (iv) and (v))** supporting literature of ITZ as anti-angiogenic agent. Additionally, the anti-angiogenic score 2.0 affirms ITZ a good anti-angiogenic agent.

In summary, it could be stated that, a repurposed anti-cancer drug Itraconazole was found to be effective in targeting angiogenesis, enhancing apoptosis and reducing cancer stemness genes expression in pre-cancerous cells of endometrial intraepithelial Neoplasia. Moreover, the expression of shortlisted miRs were reversed as compared to original, proving the efficacy of Itraconazole at molecular level.

Conclusions

In conclusion, the study demonstrated that ITZ has anti-cancer effects on endometrial hyperplasia cells, as shown by the inhibition of cell proliferation and induction of apoptosis in a dose-dependent and time-dependent manner. Furthermore, ITZ was found to inhibit wound cell migration, which is crucial for tumor metastasis and to modulate the expression of stemness-associated genes and miRNAs. The downregulation of miR-205-5p and miR-509-5p, and the upregulation of miR-875-5p and miR-585-3p, correlated with decreased angiogenesis, tumorigenicity, and suppression of TGF-β. Moreover, ITZ was found to have a significant anti-angiogenic effect as demonstrated by the direct CAM assay and focal administration method CAM

assay. These findings suggest that ITZ may be a promising therapeutic agent for endometrial hyperplasia.

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CHAPTER 7

SUMMARY AND CONCLUSIONS

Endometrial Intraepithelial Neoplasia (EIN)/ Endometrial atypical hyperplasia is a precancerous stage, arose from benign hyperplasia and has higher risk to progress into Endometrioid Adenocarcinoma if neglected/ left untreated. This condition has a chance to be reversed if treated at right time. Unlike other gynaecological cancers including Endometrial cancer, the research literature with respect to treatment modalities other than conventional, molecular basis of the etiology, altered pathways, ways for better prognosis in endometrial hyperplasia are fewer. So, unlocking some molecular mechanisms involved in pathogenesis of endometrial hyperplasia and its progression is much needed. Additionally, finding other treatment options which could be used synergistically with conventional ones could help in cases where surgical option fails.

Good prognosis the key to decide best treatment options and get rid of the disease before its establishment. MicroRNAs plays the vital role here. Globally, numerous research on miRs as prognostic markers were demonstrated in diverse range of cancers. However, such data of patients belongs to Indian ethnicity is lacking. The diversity in geographical condition, lifestyle (including nature of work) and food habits etc. have direct impact on the menstrual health of women. Considering this, this research aim to profile microRNAs, mRNAs, in endometrial hyperplasia. Further target enrichment of culprit miRs may add the prognostic/diagnostic/therapeutic value to it.

Thus, the research described in this thesis is divided into six chapters and explained in short as follows:

7.1 Summary:

Chapter 1: It describes the overview of research problem i.e. Endometrial Hyperplasia and the important key words of the thesis i.e. microRNAs their biogenesis, role in etiology as well as significance. It also describes the concept of repurposed drug in targeted therapeutics and the possible alternative/ synergistic way to conventional treatment to endometrial hyperplasia.

Chapter 2: It describes the review of literature on physiology endometrium, its monthly regeneration cycle with hormone synchrony and its connection to pathophysiology of endometrial hyperplasia. It also includes the role of microRNAs in hallmarks of cancer, deregulated microRNAs and mRNA expression in EH and the

potential of repurposed drug – Itraconazole in gynaecological oncology suggesting its use in treatment of EH. Additionally it includes the aim and objectives of the thesis.

Chapter 3: It describes the materials and methods with information of the reagents, experimental kits, buffers, chemicals, culture media etc. used for the experiments. It also includes the detailed protocols of the *in silico* (Next Generation Sequencing, Microarray and target enrichment using bioinformatics tools), *in vitro* and *in ovo* (CAM assay) experiment.

Chapter 4: It deals with microRNA profiling of human endometrium from physiologic stage to precancerous stage i.e endometrial hyperplasia, Endometrial Intraepithelial Neoplasia and cancerous stage i.e. Endometrioid Adenocarcinoma. Since microRNAs are vital for the normal development of animals and are involved in various biological processes, their dysregulation invites different kinds of pathologies. Unlike Endometrial cancer, there is lacking of differential expression profile of microRNAs in endometrial hyperplasia and its further progression especially in patients of Indian ethnicity. This forms the basis of this chapter. Next generation sequencing with Ion torrent s5 platform used for the profiling of histologically confirmed, QC passed endometrial tissue samples. Following the protocol, taking secretory phase as a control, 6 oncomiRs and 8 tumor suppressor miRs were identified in EIN, an actual precursor and its progression. Depending on the p value, log2 fold change as well as the literature survey, two up (hsa-mir-205-5p and hsa-mir-509-5p) and two down (hsa-mir-875-5p and hsa-mir-585-3p) regulated miRs were shortlisted and further retrospect in triplicate (FFPE blocks) which showed similar expression.

Chapter 5: It deals with mRNA profiling of human endometrium from physiologic stage to precancerous stage i.e. endometrial hyperplasia and Endometrial Intraepithelial Neoplasia. Single MicroRNA targets 100s of mRNA, hence target enrichment becomes essential post miR identification. This forms the basis of this chapter. Here, microarray was done to profile mRNAs of histologically confirmed, QC passed endometrial tissue samples. Microarray analysis revealed DE mRNAs involved in basic hallmark of cancer (angiogenesis, failed apoptosis and cancer stem cells in terms of EMT), other oncogenic factors and abberent pathways involved in pathogenesis (cytokines and chemokines, stem cells, endometrial cancer, MAP kinase, P53, PI3K-AKT, VEGF signalling and WNT signalling). Further, target enrichment of the shortlisted miRs of

previous chapter to the DE mRNAs of aforementioned basic hallmarks of cancer was done using MIRSYSTEM- a bioinformatics tool and discovered more contribution of cancer stem cells in the etiology of the EH and its progression.

Chapter 6: Translation of microRNA research in terms of diagnosis and therapeutics is a time consuming process. Hence, looking for an effective alternative in meantime is always beneficial. So, this chapter sheds the light on the area of repurposed drug – Itraconazole, for its use in the treatment of Endometrial Hyperplasia. As Itraconazole proved its efficacy in various cancers including endometrial cancer, its use in EIN condition is hypothesised. Hence, to prove it, in-vitro cell proliferation assay, apoptosis assay, wound scratch assay and expression of EMT markers (SNAIL, SLUG, TWIST) was done on the Itraconazole treated primary culture of endometrium of EIN stage and found effective at 1uM concentration. Since neo-angiogenesis is an important factor in metastasis, anti-angiogenic property of Itraconazole was validated in In-Ovo approach using CAM assay with anti-angiogenic score of 2.0. Additionally, Itraconazole altered the expression of shortlisted miRs. Decrease in the expression of shortlisted oncomiRs (hsa-mir-205-5p and hsa-mir-509-5p) and increase in expression of tumor suppressor miRs (hsa-mir-875-5p and hsa-mir-585-3p) was recorded. Thus proving the hypothesis of use of Itraconazole in EIN condition might be fruitful.

7.2 Conclusions

1. Hsa-miR-205-5p could be consider as a global prognostic/diagnostic marker in EIN condition.
2. Shortlisted miRs (Hsa-miR-205-5p, 509-5p, 875-5p, 585-3p) could be used as prognostic/ diagnostic panel of 4 miRs in endometrial hyperplasia.
3. DE mRNAs of cancer stem cells to aforementioned shortlisted miRs could be considered as therapeutic targets to avoid relapse of endometrial hyperplasia.
4. Attested to in-vitro and in-ovo studies, Itraconazole could be considered as a multitarget promising drug in treating endometrial hyperplasia.

RECOMMENDATION

8.1 Recommendations:

The ultimate goal of this research is to help doctors/clinicians to take a better call on treatment modality in endometrial hyperplasia, especially EIN condition; through the aid of biomarkers (microRNAs) for prognosis/ diagnosis and an alternative to conventional therapy.

As microRNAs had come long way since their discovery and proved their involvement in pathology due to aberrant expression and resultant downstream pathways, it is essential to identify the culprit microRNAs having prognostic/diagnostic/ therapeutic value and are involved in the pathogenesis of endometrial hyperplasia and its progression. Taking this in account, the panel of 4miRs (Hsa-miR-205-5p, 509-5p, 875-5p and 585-3p) and their corresponding targets, identified in endometrial hyperplasia will surely support in prognosis/ diagnosis of the disease condition.

Hormonal therapy (progestin/ GnRH) and/or hysterectomy are the widely used conventional treatment strategies in endometrial hyperplasia. Still there is a scope for new treatment strategies in the form of targeted therapeutics using repurposed drugs. This research investigated the efficacy of Itraconazole in terms of its multitarget ability against angiogenesis, apoptosis and cancer stem cells which are the basic hallmarks of cancer are involved in the pathogenesis and the progression of endometrial hyperplasia to type-I endometrial cancer. It also studied the effect of Itraconazole on the expression of panel of 4 culprit miRs and proven effective in EIN condition in all experiments, thereby, opening new / synergistic way in treatment strategy.

It is recommended that the use of panel of 4 miRs would aid in better prognosis/diagnosis of endometrial hyperplasia and its progression. Additionally, Itraconazole might be a promising drug in cases of recurrence and no surgical option in endometrial hyperplasia.

8.2 FUTURE PROSPECTUS

The present study can be interpreted as a base or pilot study in identifying microRNAs and their respective m-RNA targets involved in the transition of endometrial hyperplasia to Endometrioid Adenocarcinoma, in women of Indian ethnicity. However,

the results of this study should be treated with caution due to small size and is confined to a specific parts of the state of India.

So, it can be taken to the broader level by –

- Retrospect the common miRs in patients from all parts of India, creating a database of DE miRs in Indian population.
- Transfecting miR mimics/inhibitors to study gain-of –function/ loss –of – function of selected miR. This study could validate mRNA targets.
- Itraconazole showed its effectiveness in primary culture of EIN i.e. endometrial atypical hyperplasia, which can be further explored in animal model in synergistic way with conventional method to prove regression of the disease.

ANNEXURE

D. Y. PATIL
MEDICAL COLLEGE
KOLHAPUR

D. Y. Patil Education Society (Deemed to be University), Kolhapur.
Re-accredited by NAAC with 'A+' Grade CGPA 3.48

Dr. Rakesh Kumar Sharma
MD, FMASG, MAMS
Dean & Professor
Member, Governing Body
Indian Council of Medical Research
(ICMR), New Delhi

Dr. D. Y. Patil
Padmashree Awardee
Founder President

Dr. Sanjay D. Patil
Chancellor
President, DYP Group

Ref No: DYPMCK/ 7 / 2017

Date : 14 / Mar / 2017

INSTITUTIONAL ETHICS COMMITTEE, D. Y. PATIL MEDICAL COLLEGE, KOLHAPUR.

This is to certify that the research project titled,

"Role of key microRNAs, mRNAs and Itraconazole in Endometrial Hyperplasia."

Submitted by : **Mrs. Apurava R. Birajdar**

Under the supervision of appointed Guide (if any): **Dr. Indumati Somasundaram**

Has been studied by the Institutional Ethics Committee (IEC) at its meeting held on **14/03/2017** and granted approval for the study with due effect with the following caveats:

1. If you desire any change in the protocol or standard recording document at any time, please submit the same to the IEC for information and approval before the change is implemented.
2. All serious and/or unexpected adverse events due to the drug/procedures tested in the study must be informed to the IEC within 24 hours and steps for appropriate treatment must be immediately instituted.
3. In case of injury/disability/death of any participant attributable to the drug/procedure under study, all compensation is to be made by the sponsor of the study.
4. The Chief investigator/Researcher must inform the IEC immediately if the study is terminated earlier than planned with the reasons for the same.
5. The final results of the study must be communicated to the IEC within 3 months of the completion of data collection.
6. The researcher must take all precautions to safeguard the rights, safety, dignity and wellbeing of the participants in the study.
7. The researcher must be up to date about all information regarding the risk/benefit ratio of any drug/procedure being used and any new information must be conveyed to the IEC immediately. The IEC reserves the right to change a decision on the project in the light of any new knowledge.
8. Before publishing the results of the study, the researcher must take permission from the Dean of the Institution.
9. Annual progress report should be submitted for all sponsored projects to the committee.
10. Unethical conduct of research in non-sponsored projects will result in withdrawal of the ethics approval and negation of all data collected till that date.

Dr. Mrs. Shimpa R. Sharma
(Member Secretary, IEC)

Institutional Ethics Committee
D. Y. Patil Medical College, Kolhapur.
(DGHS, CDSCO No : ECR/1[illegible]/Inst/MH/2015/RR-21)

INFORMED CONSENT

NAME OF THE DST PROJECT: MicroRNA profiling of human endometrium at tissue and cellular level: Identifying the microRNA regime regulating stem cell proliferation and differentiation in endometrial hyperplasia condition

NAME OF THE HOSPITAL (Sample collection)

PURPOSE OF THE PROJECT:

- Identification of differentially regulated microRNAs affecting endometrial hyperplasia (simple and complex).
- Targeted therapeutic mechanism of treating vascular diseases/ endometrial dysfunction

SELECTION CRIETERIA OF PATIENT:

Female patient with mean age of 38±1 & BMI of 25.4±0.766, undergoing surgery of hysterectomy /dilation and curettage for fibroids/ prolapse/ endometriosis/dysfunctional uterine bleeding etc**.** Patients who are not under hormonal treatment for at least 3 months prior to the surgery are preferred.

SAMPLE TYPE & STUDY OF SAMPLE

Sample: Endometrium tissue collected during operation/ post operation of both normal and diseased conditions

The collected sample will be characterized at histological, cellular and molecular level.

RESPONSIBILITY OF THE PATIENT

To help doctors and researchers associated with the project

PROBABLE SIDE EFFECT

Nil (As we are dealing with biological waste)

SECRECY

The medical information regarding respective patient will be secret, only limited to related doctor and researcher. The results of the study will get published in scientific journals, but patient's identity will not be revealed.

Name of patient	
Patient's ID	
Age	
Gender	
Address	
Mobile no.	

I,

Hereby declare that, I went through above information about project and agree. I know that my participation in this project is voluntary and won't get any fund for participating. I am sure about secrecy of my identity. I know, the results coming out of this project will be published in scientific journals and there is no objection for me to participate in this project as voluntary donor of study sample (Endometrial tissue collected during operation/ post operation).

Patient's sign/ thumb print Date	
Unbiased witness sign (Doctor's sign) Date	

PATIENT HISTORY

PATIENT DETAILS

NAME OF PATIENT	
PATIENT ID (IPD NO.)	
AGE	

PATIENT DETAILS (Please tick the right ones)

NAME OF THE PROCEDURE	• EB • Hysterectomy • D & C • Hysteroscopy • Any other (_____)
MENSTRUAL PHASE	• Pre menopausal • Post menopausal
LMP	
CLINICAL FINDINGS	• White PV discharge Consistency – • Number of pads a day: _____ • How long periods last? _____ • Abdominal pain – mild/ severe • Bleeding or spotting between menstrual periods • Post menopausal bleeding • Menorrhagia • Disturbed Menstruation period
ANY OTHER FINDINGS	• Diabetes • Obesity • PCOD • Anemia • Others (Pl specify)_____

USG DETAILS

UTERUS APPEARANCE	• Normal • Bulky • Uterus thickness:_____
PRESENCE OF	• Fibroid (dimension -)

	• Cyst (dimension -) • Any other (_____)
THICKNESS OF	• Endometrium - mm • Myometrium - mm
Impression	

MEDICATION DETAILS

HORMONAL THERAPY	• Yes • No
DRUG DETAILS (hormonal)	

HISTOPATHOLOGICAL STUDY

CONCLUSION	• Normal physiologic endometrium • Proliferative phase • Secretary phase • Simple hyperplasia • Complex hyperplasia • Complex atypical hyperplasia

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Anti-Angiogenic Potential of Itraconazole and Its Reversal by Endometrial Stem Cells Using Chick Embryo Model

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Abstract

Vascular diseases are the disease of arteries and veins, mediated by endothelial cell dysfunctions, leading to blockage of vessels. Although bone marrow mononuclear cells and endothelial progenitor cells are currently used for treating vascular diseases, stem cells derived from endometrium are far superior in treating vascular diseases as it undergoes dynamic proliferation and has inherent angiogenic ability. However, taking these endometrial stem cells into clinic for treating vascular diseases is a challenge. We hypothesise herein, an easy and reliable method to study the anti-angiogenesis and its reversal using a chick embryo model. Briefly, we propose herein that, anti-angiogenesis of itraconazole, an anti-fungal drug and its reversal by forming neoangiogenesis using endometrial stem cells can be best studied on chick embryo model. A preliminary study was done to justify our hypothesis. In-depth investigations will be carried out to justify the proposed hypothesis effectively. This opens new avenues to unravel the mechanism of itraconazole in blocking CAM vasculature and the mechanism of EnScs in reversing the blocked vessels, therebypostulating the use of endometrial stem cells for vascular regeneration, after in-depth investigation in pre-clinical and clinical model.

Keywords: Chick embryo, itraconazole, endometrial stem cells, angiogenesis, vascular disease

Introduction

Vascular disorders such as peripheral vascular disease and cardiovascular disease is a disease of blood vessels, affecting arteries and veins. Ischemic heart disease, myocardial infarction & other vascular diseases are the leading cause of death & constitute a major epidemiological challenge. On the other hand Acute Hind Limb Ischemia (ALI) is a medical condition caused by a sudden lack of blood flow to hind limb. Critical Limb Ischemia (CLI) is an advanced form of peripheral artery disease with

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Review Article

Check for updates

The Role of MicroRNAs in Development of Endometrial Cancer: A Literature Review

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Abstract

Endometrial cancer (EC) ranks as the second most common gynaecological cancer worldwide. EC patients are diagnosed at an early clinical stage and generally have a good prognosis. Therefore, there is a dire need for development of a specific marker for early detection of endometrial adenocarcinoma. The development of EC is conditioned by a multistep process of oncogenic upregulation and tumor suppressor downregulation as shown by molecular genetic evidence. In this setting, microRNAs appear as significant regulators of gene expression and several variations in the expression of microRNAs have been implicated in normal endometrium, endometrial tissue, metrorrhagia, and endometrial cancer. Furthermore, microRNAs act as highly precise, sensitive, and robust molecules, making them potential markers for diagnosing specific cancers and their progression. With the rising incidence of EC, its management remains a vexing challenge and diagnostic methods for the disease are limited to invasive, expensive, and inaccurate tools. Therefore, the prospect of exploiting the utility of microRNAs as potential candidates for diagnosis and therapeutic use in EC seems promising.

Keywords: Biomarkers, Endometrial neoplasms, Gene therapy, MicroRNAs, Signal transduction.
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Introduction

Endometrial cancer (EC), a common female genital cancer, is also known as uterine corpus cancer or corpus cancer (1). World wide, it is the second most common gynaecological cancer, with an estimating 417,367 new cases and 97,370 deaths annually (2). Essentially, EC is one of only two common cancers that does not follow the general inclination of progress in morbidity and death with a worse survival rate today than in the 1970s when it hit a peak in 1975 in parts of the US (3, 4). Risk factors include aging, hormonal imbalance, and obesity and these are some of the factors that cause a rise in the incidence of endometrial cancer. Yet, genetic factors significantly influence EC ranging from the inheritance of various low-risk cancer susceptibility variants to single and very high-risk variants (5). Based on histologic and pathologic characteristics, EC is divided into two subtypes, namely endometrioid (Type I) and non-endometrioid (Type II). Patients with Type I are usually diagnosed with early-stage disease and generally have a good prognosis. However, patients with Type II succumb to poorly differentiated or high-grade cancer with the worst prognosis (6, 7). Endometrioid adenocarcinoma (EAC) has emerged as the most common subtype, amounting to approximately 75% of EC cases (8). In patients diagnosed with advanced-stage or recurrent EAC, the survival rate is low due to the inefficacy of treatments (9). These subtypes exhibit distinctive mutations, amplification, and

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