Iraqi Journal of Biotechnology, September 2018, Vol. 17, No. 2, 82-90

Role of *SOX4* gene in the progression of endometrial hyperplasia to endometrial adenocarcinoma

Mahdi Saber Al-Deresawi [1] , Abdul Hussein Moyet AlFaisal [2] , Salim Rasheed Al- obaidi [3]

[1] College of Science / University of Wasit
[2] Institute of Genetic Engineering and Biotechnology / University of Baghdad
[3] College of medicine / University of Baghdad

Received: July 24, 2018 / **Accepted**: September 12, 2018

Abstract: The objective of this study was to investigate the role of *SOX4* gene in progression of endometrial hyperplasia to endometrial adenocarcinoma .A total of 41 Iraqi patients with abnormal uterine bleeding followed by hysterectomy. Histological findings recorded 36 cases with endometrial hyperplasia and 5 endometrium adenocarcenoma. Cases collected during the period from February 2017 to October 2017. Curettage techniques were used to obtain a ten samples as healthy control group. All cases were obtained from Al-Zahra Teaching Hospital in Wasit Province . Endometrial hyperplasia subjects distributed according to age, the highest percentage ranged (40-50)years recorded (58.33%). To evaluate the *SOX4* gene expression, total RNAs were extracted with TRIzol from each sample then directly converted to cDNA. RT-PCR technique was used to estimate the *SOX4* gene expression and (*GAPDH* gene used as a reference gene). The results revealed that there was a highly significant increase ($p<0.01$) in *SOX4* gene expression in endometrium adenocarcenoma (9.24±0.52) followed by endometrial hyperplasia (6.14±0.17) when compared to the healthy control group (1±0.00).In conclusion, endometrial hyperplasia can be a precursor of endometrium adenocarcenoma when the *SOX4* gene expression elevated gradually.

Keyword: *SOX4* gene, Endometrial hyperplasia, Endometrial adenocarcinoma.

Corresponding author: should be addressed (Email: maladeresawi@uowasit.edu.iq).

Introduction:

Endometrial hyperplasia (EH) form a morphologic continum of abnormal epithelia and stromal prolifration ranging from focal glandular crowding or simple hyperplasia to well differentiated adenocarcenoma(1,2). The international society of Gynecological pathologists (ISGP) agreed on a classification of EH into two main groups; hyperplasia with or without atypia (1).

The lesions were further subdivided into simple and complex hyperplasia. These guidelines were subsequently adopted by the World Health Organization (WHO) as the following : simple cystic without atypia 1%, complex adenomatous without atypia 3%, a typical simple with typia 8% and a typical complex with atypia 29% **(**1,4,5).

A major criterion for hyperplasia is that the endometrium is thickened by an increase in the number and size of proliferating glands. A lesion that does not increase the thickness of the endometrium does not warrant a diagnosis of hyperplasia and best designated as a disorder hyproliferative endometrium or a focal glandular crowding, besides the gross appearance of the endometrium can be confused with a very thick and succulent endometrium removed on day 26 to 28

of normal secretory cycle (3).Approximately 80% of endometrial cancers are endometrium adenocarcinomas. Serous and clear cell carcinomas account for 1-5% and 5-10% of endometrial cancers, respectively. Mucinous, squamous cell, transitional cell, and small cell carcinomas compromise less than 2% of endometrial cancers (6).

Adenocarcinomas arising from epithelial cells account for up to 90% of EC (7). Endometrium adenocarcinoma, the cancer cells raise in patterns reminiscent of normal endometrium, with many new glands formed from columnar epithelium with some abnormal nuclei. The tumor's glands form very close together, without the stromal tissue that normally separates them. There are diverse subtypes of endometrioid adenocarcinoma with identical prognoses , including villoglandular , secretory, and ciliated cell variants. There is also a subtype described by squamous differentiation (8). Endometrial adenocarcinoma found in hysterectomy sample from patients diagnosed with atypical hyperplasia on endometrial specimen is a numerous finding and has been described to occur in up to 42 % of the patients (9). Endometrial carcinomas are characterized by a diversity of genetic changes, the most frequent of which is PTEN (10). They are considered type I endometrial cancers according to the Bokhman classification because of their epidemiologic connotation with estrogen extra (11). The aim of this study was to assess the role of *SOX4* gene in progression the endometrium hyperplasia to endometrium adenocarcinoma.

Subjects, Materials and Methods:

Subjects:

All patients suffering from abnormal uterine bleeding followed by hysterectomy. Tissues specimens of 41 patients (36 with EH and 5 with EC) and 10 healthy control group were obtained from Al-Zahra Teaching Hospital in wasit province / Iraq

Histological study:

Histological technique of all tissues were carried out to observe the changes in tissues. Sixty biopsies of hysterectomy and Curettage were enrolled in this study (12).

Gene expression:

Total RNA of all samples was extracted using the TRIzol® LS Reagent according to the manufacturer's instructions .Total RNA was reversely transcribed to complementary DNA (cDNA) using WizScript™ RT FDmix Kit (Alpha DNA Ltd /Canada). The procedure was carried out in a reaction volume of 20 µl according to the manufacturer's instructions. The expression levels of *SOX4* gene were estimated by qRT-PCR . To confirm the expression of target gene, quantitative real time qRT-PCR SYBR Green assay was used. The mRNA levels of endogenous control gene *GAPDH* were amplified and used to normalize the mRNA levels of the *SOX*4 gene. The design process for primers was obtained by Primer3 web version 4.1.0 (online at website http://primer3.ut.ee) for *SOX4* and *GAPDH* genes.The primers sequences are (Forward:5′-AGGATTCAAACG CAACTCAAAT-3,Reverse5′-AAAGA

AATACGAGGATGGAGCA-3) and sequence of *GAPDH* (Forward, 5′-AACTTTGGCATTGTGGAAGG-3′ and Reverse,5′-ACACATTGGG GGTAG AACA-3).

Statistical analysis:

ΔCT and ΔΔCT values were calculated according to their equation (13). This was conducted according to Statistical Analysis System-SAS (14) to measure the effect of different factors in studying the parameters. Least significant difference –LSD test was used to compare between means and Chi-square test between percentages . The means and standard deviations were recorded for each sample (test and control) variables included Ct values and gene expression levels. This included values of housekeeping gene and test gene. P value for all tests was considered significant if <0.05 .

Results and discussions:

1. Distribution of subjects according to age:

This study involved 36 patients with EH ; age ranged between (30 - 70) years , the endometrium adenocarcenoma concluded one case (35) years and others aged (>50) years. Table (1) shows the age and percentage.

Table (1): The age groups of endometrial hyperplasia patients .

Age group	Number of patients	Percentage %
30-40	4	11.11
40-50	21	58.33
50-60	9	25
60-70	2	5.56

Endometrial hyperplasia (EH) can appear between puberty and menopause period (15). Endometrial hyperplasia results agreed with Yuk , (2010) who reported the mean ages of incidence with endometrial hyperplasia were (44.1±0.4) years (16). Endometrial hyperplasia pathophysiology is related to the over and persistent stimulation of the endometrium by estrogen (17). The Endometrial hyperplasia risk factors incorporate constant anovulation, polycystic ovary syndrome, obesity,, tamoxifen treatment, and estrogen-only hormone therapy (17,18,19**).** Endometrial cancer is frequently a disease of ladies who are post-menopausal , the middle age at determination is 62 years and 45 % of cases happen in patients beyond 65 (20).

One of the strongest risk factors for the development of endometrial cancer is unopposed estrogen exposure deficient progesterone to adjust the mitogenic impacts of estrogen (21). This happens either exogenously by means of estrogen-just post-menopausal hormone substitution , or endogenously in fat ladies, abundance of fat tissues leads to increasing peripheral conversion of androgens to estrogens via aromatase enzyme (22). Other risk factors for the development of endometrial cancer include incorporate diabetes, hypertension, tamoxifen utilize, expanding age and hereditary disorders (23).

Histopathological findings:

1. **Endometrial hyperplasia:**

Macroscopically, Hyperplastic endometrium is not distinctive. Focal over growth of glands and stroma produces a diffuse thickening that increases the volume of tissue. The tissue is pale, creamy yellow and spongy, appearing velvety and knobby on the surface, it's often lobulated or with pseudopolypoid appearance. A major criterion for hyperplasia is that the endometrium is thickened by an increase in the number and size of proliferating glands (3,4). Figure (1) shows the irregular shaped proliferative endometrial glands with irregular and shape.

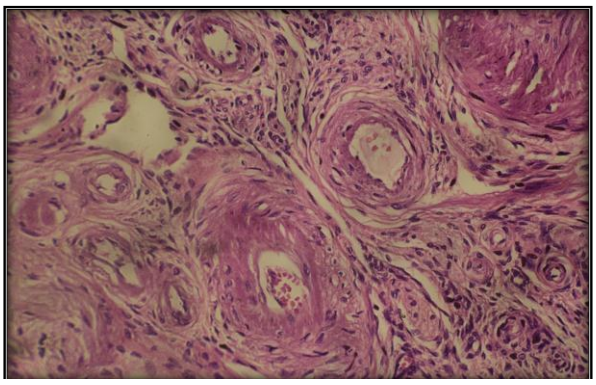

Figure (1): Proliferative endometrial gland with complex endometrial hyperplasia

The most common cause of EH is succession of that omit the periodic differentiating stimulus of progesterone. Therefore, EH is the most commonly seen during the perimenopausal period. Sequential hormone replacement therapy is associated with a prevalence of complex hyperplasia seen in about 5-6% of patients (1). However, it can also be encountered in young patients, even adolescent ones (27)**.** Atypical EH has been related strongly to evolution of endometrial carcinoma (24). The risk of incidence with endometrial carcinoma is about 30% in women with atypical EH (25).

2. **Endometrial adenocarcinoma:**

This study involved 5 cases (18%) of endometrium adenocarcenoma patients with the age ranging between 35 and 72 years. One case was classified as endometrium adenocarcenoma type I depending on the age (35 years). This cancer develops in perimenopausal women and occurs in an estrogen-dependent manner via EH. Another cases (72,50,45 and 63 years were included with endometrium adenocarcenoma type II because it develops in postmenopausal women .Figure (2) shows the microscopic features of the biopsy obtained by total hysterectomy. The section shows a back to back arrangement of pleomorphic malignant cells and glandular structure is observed with a stromal disappearance.

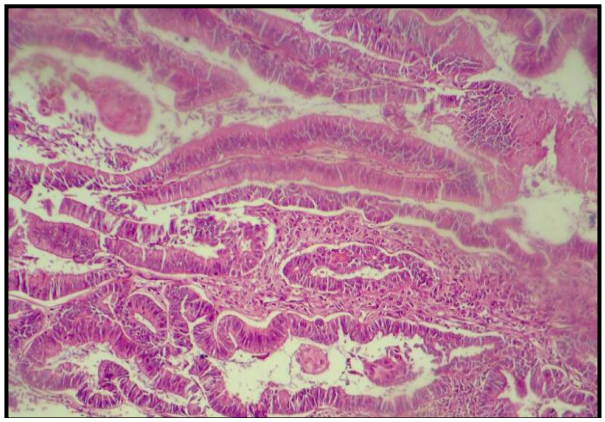

Figure (2): Microscopic feature with high power of endometrium adenocarcenoma .

Environmental factors including estrogen, an abnormal mismatch repair (MMR), aberrant methylation of DNA and miRs are proposed as major mechanisms of carcinogenesis of endometrium cancer (26). Mismatch repair system deficiency is the important abnormality in the early stage of endometrial cancer and related with estrogen, expression of Hmlh1 and Hmsh2 examined by immunohistochemistry show a strong positive correlation with blood estrogen (27). Many tumor suppressor genes in cancer cells are arrested by aberrant DNA methylation in promoter CpG islands (28) . Muraki *et al*., (29) reported a hypermethylation of 40.40% of Hmlh1in patients with endometrial cancer. miRs, short nucleotides that regulate gene expression sometime, act as tumor suppressor, such as *miR-126*, *miR-124*, *miR-152*, *miR-129-2* , *miR-137* and *miR-491* ; therefore the promoters hypermethylation of this miRs lead to activation of oncogenes regulated by these genes (26).

Quantitative Real-Time PCR results:

Real time PCR quantification was applied in the present experiment utilizing the EVA green.The fluorescent dye recognized any double stranded DNA including cDNA and the amplification was recorded as a Ct value. The lower Ct value indicates the presence of high copies of the target and vice versa. In terms of gene expression, high Ct values indicate a low gene expression and vice versa (30,31).

***GAPDH* gene Expression:**

There were no significant differences of Ct value of *GAPDH* in subjects and healthy group (1.05±0.00). The little variations in gene fold expression between the patents group and healthy group makes *GAPDH* gene useful as a reference gene .The inherent assumption in the use of housekeeping genes in molecular studies is that their expression remains constant in the cells (32). One of the most commonly used housekeeping genes in comparison with the gene expression data is *GAPDH* (33).

***SOX4* gene Expression:**

Expression of the *SOX4* gene was highly significant ($p<0.01$) in endometrial adenocarcenoma and endometrium hyperplasia when compared to the healthy control group as shown in table (2).

Table (2) : Fold of *SOX4* gene expression, depending on $2^{-\Delta\Delta Ct}$ Method

Groups	Means Ct of *SOX4*	Means Ct of *GAPDH*	ΔCt (Means Ct of SOX4 - Means Ct of *GAPDH*)	$2^{-\Delta Ct}$	experimental group/ $2^{-\Delta Ct}$ of Control group	Fold of *SOX4* gene expression
Endometrium hyperplasia	21.10	29.816	-8.716	420.51	420.51/68.40	6.14 ± 0.17 b
Endometrial adenocarcenoma	20.56	29.864	-9.304	632.09	632.09/68.40	9.24 ± 0.52 a
Control	23.85	29.946	-6.096	68.40	68.40/68.40	1 ± 0.00 c
LSD value	---	---	---	---	---	2.073 **

**** (P<0.01).**

**** (P<0.01) means different letters in the same column are significant at 0.01 level.**

Figures (3) and (4) show the amplification plots and melting curves for *SOX4* gene .

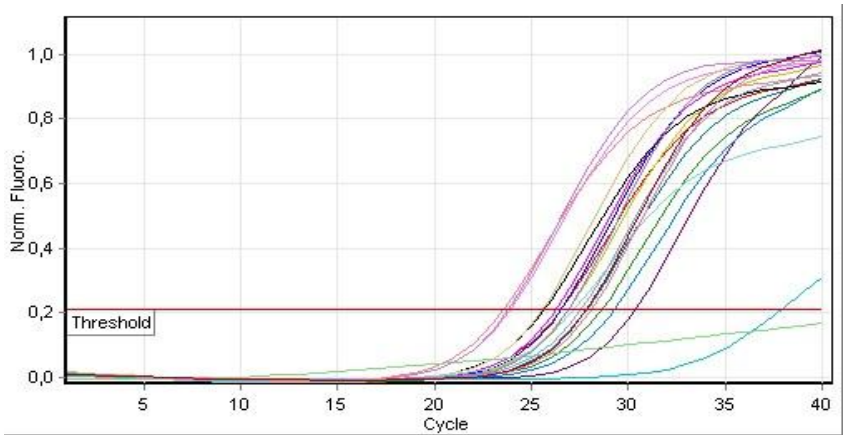

Figure (3): *SOX4* gene amplification plots by qPCR .Ct values ranged from 23.58 to 28.63. this plot for samples(1-36). The photograph was taken directly from Rotor-Gene Software Version 2.1.0.9 , Threshold (0.210).

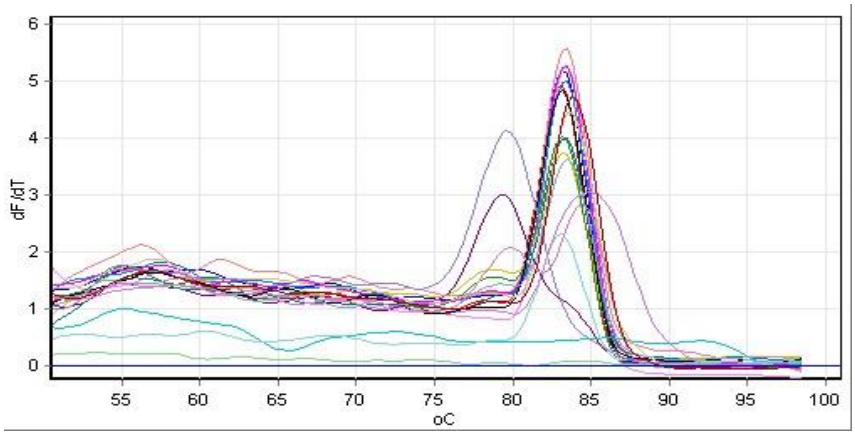

Figure (4): *SOX4* melting curves by qPCR Samples included all study groups. Melting temperature ranged from 82°C to 86°C, No primer dimer could be seen. The photograph was taken directly from Rotor-Gene qPCR machine Rotor-Gene Software Version 2.1.0.9.

The gene expression of *SOX4* in EH subjects recorded a high significance fold (6.14± 0.17) when compared to the control group . The diagnosis of EH is made typical by endometrium biopsy or curettage after a woman coming to a gynecologist with unusual uterine bleeding , including intermenstrual or postmenopausal bleeding (24). A relative abundance of estrogen, be it exogenous or endogenous, contrasted and progesterone, is thought to be one of the essential etiological factors in both endometrial hyperplasia and endometrial carcinomas (34). It is clear that postmenopausal ladies treated with supplemental estrogens are at a risk of EH and carcinoma if a progestin is not utilized to restrict the proliferative activities of estrogen on the endometrium. The level of hazard increments with measurements and span of therapy, with an around 10-fold increases risk related with every time of use (35). A high expression of *SOX4* gene has been reported in many types of tumor and there is evidence of its contributes in cellular transformation (36) ; therefore, the role of SOX4 protien in inducing EH and endometrial adenocarcenoma is suggested, because the high expression of *SOX4* leads to the transformation of cells lining the endometrium.

Endometrial adenocarcenoma recorded a high significance (9.24 ± 0.52) when compared to other groups and the control group (1±0.00). The result of this study agreed with Levan *et al*., (37) who reported the *SOX4* gene was overexpressed in patients with endometrial cancer. miRs play an important role in carcinogenesis by targeting tumor suppressor gene or by act as oncogenes with elevated expression (38) . *miR-203* , *miR-129-2*, *miR-596* , and *miR-618* identified to bound to the 3-UTR of *SOX4* gene *in silico* analysis and these miRs keep the levels of SOX4 protein by the degradation of its mRNA. Huang *et al*., (39,40) reported that the hypermethylated promoters of *miR-129-2* and *miR-203* lead to *SOX4* gene overexpression . In conclusion , the gradually elevated of *SOX4* gene in patients with endometrium hyperplasia can be lead to endometrium adenocarcinoma

References:

1. Armstrong, A.J.; Hurd, W.W.; Elguero, S.; Barker, N.M. and Zanotti, K.M. (2012). Diagnosis and management of endometrial hyperplasia. *Journal of Minimally Invasive Gynecology*. 19:562-571.
2. Bancroft, J. D. and Stevens, A. (1999). Theory and Practice of Histological Techniques. 4th edition. Churchill Livingstone. Pp:127-129.
3. Banno, M.; Yanokura, I.; Kisu, W.; Yamagami,N.; Susumu, and Aoki,D . (2013). MicroRNAs in endometrial cancer. *International Journal of Clinical Oncology*, 18(2); 186–192.
4. Barber, D. (2005). GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. Physiological Genomics. 21 (3): 389-395.
5. Boruta, D.M.; Gehrig, P.A.; Groben, P.A.; Bae-Jump, V.; Boggess, J.F.; Fowler, W.C. *et al* . (2004). "Uterine serous and grade 3 endometrioid carcinoma is there a survival difference? *J Cancer*. 101(10): 2214-2221.
6. Carlson, M.J.; Thiel, K.W.; Yang, S. and Leslie, K.K.(2012). Catch it before it kills: progesterone, obesity, and the prevention of endometrial cancer. *Disc Med*. 14:215-222.
7. Chen, X.; Xiang, Y.B.; Long, J.R.; Cai ,H.; Cai, Q. and Cheng, J. (2012).Genetic polymorphisms in obesity-related genes and endometrial cancer risk. Cancer. 118:3356–3364.

8. Chen, Y.; Cheng, W.; Lin,M.; Huang, C.; Hsiech, C. and Chen, C. (2009). Concurrent endometrial carcinoma in patients with a curettage diagnosis of endometrial hyperplasia .*J Formos Med Assoc*.108 (6):502-507.
9. Cohen, B.J. and Wood, D.L. (2000). Endometrial cancer. The measured female reproductive system. The human body in healthy disease chapter 23. 9th edition.Lippincott Williams, Wilkins ; 433-434.
10. Colombo, N.; Preti, E.; Landoni, F.; Carinelli, S.; Colombo, A.; Marini, C. and Sessa, C. (2011). Endometrial cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up . *J Annal Onco.* 22 (6): 35–39.
11. Devor, E.J.; Hovey, A.M.; Goodheart, M.J.; Ramachandran, S. and Leslie, K.K. (2011). microRNA expression profiling of endometrial endometrioid adenocarcinomas and serous adenocarcinomas reveals profiles containing shared, unique and differentiating groups of microRNAs. *Oncol. Rep. 26*, 995–1002.
12. Elkholi, D.G.; and Nagy, H.M. (2015). Unexplained postmenopausal uterine bleeding from atrophic endometrium :histopathological and hormonal studies .*Mid Eset Fert Soci*.20:262-270.
13. Epplein, M.; Reed, S.D.; Voigt, L.F.; Newton, K.M.; Holt, V.L. and Weiss , N.S. (2008) . Risk of complex and atypical endometrial hyperplasia in relation to anthropometric measures and reproductive history. *American Journal of Epidemiology* 168:563-570.
14. Ling F.W. and Duff, P. (2000). Endometrial carcinoma, uterine cancer, obstetrics and Gynecology principles for practice ch (65):1281 –1293.
15. Gao, J.; Yang, G.; Wen, W; Cai, Q.Y.; Zheng, W.; Shu, X.O. *et al*. (2015). Impact of known risk factors on endometrial cancer burden in Chinese women. *Eur J. Cancer Prev* .31:387-394.
16. Hoffman, B.L.; Schorge, J.O.; Schaffer, J.I.; Halvorson, L.M.; Bradshaw, K.D. and Cunningham, F.G. (2012). Endometrial Cancer Williams Gynecology (2nd ed.) editors. McGraw-Hill. Pp 827.
17. Huang, Y.W.; Liu, J.C.; Deatherage , D.E.; Luo, J.; Mutch, D.G.; Goodfellow, P.J *et al*. (2009). Epigenetic repression of microRNA-129-2 leads to overexpression of SOX4 oncogene in endometrial cancer. *Cancer Res*. 69:9038–9046.
18. Huang, Y.; Kuob, C .; Chenb, J.; Paul, J.; Huangd, T.; Radera, J.S. and Uyara, D.S. (2014). Hypermethylation of *miR-203* in endometrial carcinomas. *Gynecol Oncol*. 133(2):340–345.
19. Jones, P.A. and Baylin, S.B. (2007). The epigenomics of cancer. *Cell* 128: 683–692.
20. Leitao, J. M.; Han, G. and Lee, L.X. (2010). Complex atypical hyperplasia of the uterus: characteristics and prediction of underlying carcinoma risk. *Am J Obstet Gynecol*. 203(4): 349–349.
21. Levan , K.; Partheen, K.; Osterberg, L.; Helou K.; and Horvath, G. (2006). Chromosomal alterations in 98 endometrioid adenocarcinomas analyzed with comparative genomic hybridization. *Cytogenet Genome Res*. 115:16–22.
22. Liu, P.; Ramachandran, S.; Al- Seyed, M.; Scharer, C.D.; Laycock, N.; and Dalton, W.B. *et al*. (2006). Sex-determining region Y box 4 is a transforming oncogene in human prostate cancer cells. *Cancer Res.* 15; 66(8): 4011-4019.
23. Livak, K.J. and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔ CT *Method. Methods.* 25, 402–408.
24. Livak, K.J. and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔ CT Method. Methods. Vol. 25, 402–408.
25. MacKenzie, A.R. (2014). Endometrial Cancer in the Elderly. *Curr Geri Rep* . 3:220–227.
26. Miyamoto, T.; Shiozawa, T. and Kashima, H. (2006). Estrogen up-regulates mismatch repair activity in normal and malignant endometrial glandular cells. *Endocrinol*. 147: 4863–4870.
27. Muggia F. and Oliva, E. (2009). Uterine Cancer Screening, Diagnosis and Treatment Springer Dordrecht Heidelberg London New York. P :296-302.
28. Muraki, Y.; Banno, K. and Yanokura, M. (2009). Epigenetic DNA Hypermethylation: Clinical applications in endometrial cancer. *Oncol Rep*. 22: 967–972.

29. Murali, R.; Soslow, R.A. and Weigelt, B. (2014). Classification of endometrial carcinoma: more than two types. *J. Lancet Oncol* .15(7): 268-278.
30. Nolan T.; Hands R. and Bustin A. (2006). Quantification of mRNA using real-time RT-PC Nat Protoc; vol. 1 (3), pp. 1559–1582.
31. Palmer, A.J.; Perunovic, B.; and Tidy, J.A. (2008). Review endometrial hyperplasia. *Obestet Gynaecol*. 10:211-216.
32. Philip, J.; Disaia, M.D.; William, T. and Creasman, M.D. (1989). Endoemtrial hyperplasia chapter 4, Clinical Gynecologic oncology. Third edition. The C.V. Mosby Company (133 –158).
33. Reboucas, E.; Costa, J.; Passos M.; Passos, J.; Hurk, R. and Silva, J. (2013). Real Time PCR and Importance of Housekeepings Genes for Normalization and Quantification of mRNA Expression in Different Tissues. *Brazil Arch Biol Technol*. 56: 143-154.
34. SAS. (2012). Statistical Analysis System, User's Guide. Statistical. Version 9.1th ed. SAS. Inst. Inc. Cary. N.C. USA.
35. Trimble, C.L.; Kauderer, A.J.; Zaino, R.; Silverbereg, S.; Lim, P.C. and Burke, J.J. (2006). Concurrent endometrial carcinoma in women with a biobsy diagnosis of atypical endometrial hyperplasia: a Gynecologic Oncology Group study. *Cancer* . 106:812-819.
36. Trope, C.G.; Marth, C.; Scheistroen, M.; and Abeler, V.M.(1999). Endometrial hyperplasia-Diagnosis and treatment. *Tidsskr-Nor-Laegeforen.* 119(14):2030 – 2034.
37. Wan, Y.L.; Beverley-Stevenson, R.; Carlisle, D.; Clarke, S.; Edmondson, R.J.; Glover, S. *et al*., (2016). Working together to shape the endometrium cancer research agenda: the top ten unanswered research questions. Gynecol Oncol.143:287-293.
38. Wang, S.; Wang, Z.; and Mittal, K. (2015). Concurrent endometrial carcinoma and endometrial hyperplasia. *Hum Path Cas Rep*.(2):1-4.
39. Williams and Wilkins. (1995). Endometrial Hyperplasia and cancer Obstetrics and gynecology chapter (44) second edition. Lippincott Williams, Wilkins. 447 –452.
40. Yuk, J. (2016). The incidence rates of endometrial hyperplasia and endometrial cancer: a four-year population-based study. *Peer J*., 1-9.