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Hypermethylation of *miR-203* and overexpression of *SOX4* are new methods for prediction of Endometrial adenocarcinoma

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Abstract

Background: Endometrial cancer ranks as the 6th most frequent malignancy among ladies around the world. SOX4 functions include regulation of embryonic development and differentiation to determine cell fate and cellular transformation. It has been reported to be abnormally expressed in a wide assortment of malignancy including endometrial, cervical, esophageal, gastric and breast cancers. The aims of current were evaluating the expression levels of *SOX4* and *miR-203* hypermethylation in patients with abnormal uterine bleeding. In addition, the opportunity of using this method as a marker for diagnosis of endometrial adenocarcinoma will also be investigated.

Methods: A total of 60 fresh biopsies were obtained from Iraqi patients with abnormal uterine bleeding followed by hysterectomy. Curettage techniques were used to obtain ten samples as healthy control group. The expressions of SXO4 and miR-203 genes were investigated using RT-PCR with GAPDH gene as a reference. In addition, quantitative-MSP technique was used for the determination of methylation pattern for *miR-203* promoters.

Results: The result revealed that there was a highly significant increase ($p<0.01$) in *SOX4* gene expression (9.24±0.52) and a hilygh significant decrease ($P<0.01$) in *miR-203* gene expression (0.073±0.2) in endometrium adenocarcinoma patients when compared to the healthy control group (1.00±00). The results also revealed that the highest percentage (100%) of methylation in *miR-203* was displayed in endometrium adenocarcinoma samples.

Conclusion: Current study suggested that promoter hypermethylation of *miR-203* is a common mechanism leading to *SOX4* gene over-expression in endometrial cancer. Also, *miR-203* hypermethylation with *SOX4* over-expression can be useful for the prediction of endometrial cancer in women with abnormal uterine bleeding.

Keyword: *SOX4*, *miR-203,* endometrium adenocarcinoma, promoter hypermethylation, GAPDH gene, quantitative-MSP.

INTRODUCTION

Hysterectomy is an operation to remove the uterus. Hysterectomies might be performed as a result of abnormal uterine bleeding, prolapse, fibroids or other gynecological problems including cancer [1]. Endometrial cancer ranks as the 6th most frequent malignancy among ladies around the world. This disease is ordinarily identified right on time with a generally high general survival rate [2]. Unopposed estrogen therapy, estrogen producing tumors, tamoxifen, obesity, diabetes mellitus, and early onset of menstruation are among the risk factors related to endometrial cancer [3]. Polycystic ovary syndrome (PCOS) predisposes patients to higher risk of developing endometrial and ovarian cancers [4].

Sex-determining region Y-related HMG box (SOX4) is an individual of SOX, a transcription factor family [5]. SOX4 functions include regulation of embryonic development and differentiation to determine cell fate and cellular transformation. It has been reported to be abnormally expressed in a wide assortment of malignancy including endometrial, cervical, esophageal, gastric and breast cancers [7-11].

In silico analysis by using (miRBase database) [12] **,** 13 microRNA loci bond have been identified on 3-UTR of SOX4 and regulate its expression. microRNAs (miRs) are non-coding RNA molecules consisting of 17-25 nucleotides long [13,14]. miRs have been shown to play important roles in regulating gene expression by either repressing the translation or causing the degradation of multiple-target mRNA [15]. miR plays an important role in several cellular processes including proliferation, cell cycle control, apoptosis, differentiation and angiogenesis [16,17,18].

Aberrant DNA hypermethylation also inactivates expression of miRs. Epigenetic gene silencing due to promoter CpG island hypermethylation is one of the most common mechanisms by which tumor suppressor genes are inactivated during tumorigenesis [14,19].The aims of this study were evaluating the expression levels of *SOX4* and *miR-203* hypermethylation in patients with abnormal uterine bleeding. In addition, the opportunity of using this method as a marker for diagnosis of endometrial adenocarcinoma will also be investigated.

METHODS

The target population for current study were all patients suffering from abnormal uterine bleeding followed by hysterectomy. Tissues specimens of 60 removed uterus and control group consists of 10 healthy women of different ages. Curettages were used to collect the control samples. The study was conducted at Al-Zahra Teaching Hospital in Wasit province, Iraq. Histological examinations of all tissues were carried out to observe the changes in tissues.

Gene expression:Total RNA of the examined samples was extracted using the TRIzol® LS Reagent according to the manufacturer's instructions .Total RNA was reversely transcribed to cDNA using WizScript™ RT FDmix Kit. The procedure was carried out in a reaction volume of 20µl 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. *SOX4*, miR-*203* and *GAPDH* primers sequences are listed in Table (1).

***miR-203* methylation pattern**

The most common technique used today remains the bisulfite conversion method. This technique involves treating methylated DNA with bisulfite which converts unmethylated cytosines into uracil [21]. The technique was carried out by EZ DNA Methylation™ Kit (ZYMO RESEARCH /USA). In this study the detection of CpG island methylation was carried out by quantitative methyl specific real-time PCR (QRT-MSP). Primers have been designed in this study depending on the Bioinformatics tools for Q-MSP technique by using *MethPrimer* online at website,table-2 (http://www.urogene.org/cgi-in/methprimer/methprimer.cgi). EpiTect Control® (QIAGEN)

DNAs are ready-to-use, completely methylated or completely unmethylated bisulfite converted DNAs, and untreated, unmethylated genomic DNA, for standardized and reliable control reactions for methylation analysis.

Statistical analysis

ΔCT and ΔΔCT were calculated according to their equations [22]. This was conducted according to Statistical Analysis System-SAS [23] to measure the effects 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

Endometrial adenocarcinoma recorded (60.33%) and one case (35) classified as endometrial adenocarcinoma type I depending on its age, and others aged (>50) years this cancer develops in postmenopausal women and occurs in an estrogen-dependent manner via endometrial hyperplasia and classified as endometrium adenocarcenoma type II

Histological findings

Figure (1) shows microscopic features of a biopsy specimen 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.

Quantitative Real-Time PCR results

***GAPDH* gene expression:** There were no significant differences of Ct value of GAPDH between subjects and healthy controls (1±0.00). The housekeeping gene expression used in current study is shown in Table (3).

***SOX4* gene expression:** Expression of the *SOX4* gene was highly significant ($p<0.01$) in endometrial adenocarcinoma when compared to the healthy controls (Table 4 and Figures 2).

***miR-203* gene expression:** The results obtained from current study showed highly significant differences ($p<0.01$) in the mean fold values of *miR-203* between patients with endometrial carcinoma (0.073±0.02) and healthy controls (1 ± 0.00) (Table 5).

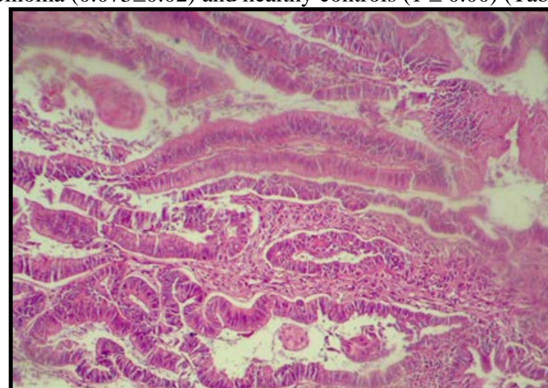

Figure 1 Microscopic features of endometrial adenocarcinoma

Table 1 Amplification primers used in current study

Gene amplified	Primer sequence (5′→3′ direction)	Product size
SOX4	F: AGGATTCAAACGCAACTCAAAT	149 bp
	R: AAAGAAATACGAGGATGGAGCA	
miR-203	F: GCTGGGTCCAGTGGTTCTTA	76 bp
	R: GCCGGGTCTAGTGGTCCTAA	
GAPDH	F: CCCCTTCATTGACCTCAACTAC	135 bp
	R: CGCTCCTGGAAGATGGTGA	

Table 2 Q-MSP primers used in current study

Gene amplified	Sequence (5' → 3')	Size
Methylated MiR-203	F: TGGTTTTTAATAGTTTAATAGTTTTGTAGC R: GTAAACTCCCCTAAATTAATCGC	219
Unmethylated MiR-203	F: TGGTTTTTAATAGTTTAATAGTTTTGTAGT R: CATAAACTCCCCTAAATTAATCAC	220

Table 3 Comparison of *GAPDH* gene fold expression between study groups .

Group	Mean Ct of GAPDH	2^{-Ct}	experimental group/ Control group	Mean fold of *GAPDH* expression
Endometrial adenocarcinoma	29.864	1.02 E9	1.02 E9/ 9.7 E10	1.05 ± 0.08 a
Control	29.946	9.7 E10	9.7 E10/ 9.7 E10	1 ± 0.00 a
LSD value	---	---	---	0.217 **NS**
NS: Non-Significant				

Table 4 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/ Control group	Fold of *SOX4* gene expression
Endometrial adenocarcinoma	20.56	29.864	-9.304	632.09	632.09/ 68.40	9.24 ± 0.52
Control	23.85	29.946	-6.096	68.40	68.40/ 68.40	1 ± 0.00
LSD value	---	---	---	---	---	2.073 **

** (P<0.01).

Table 5 Fold of *miR-203* expression depending on $2^{-\Delta Ct}$ method

Groups	Means Ct of miR-203	Means Ct of *GAPDH*	ΔCt (Means Ct of *miR-203* - Means Ct of *GAPDH*	$2^{-\Delta Ct}$	experimental /control group	Mean fold of *miR-203* expression
Endometrial adenocarcinoma	28.3	29.864	-1.564	2.95	2.95/ 40.39	0.073 ± 0.02
Control	24.61	29.946	-5.336	40.39	40.39/ 40.39	1 ± 0.00
LSD value	---	---	---	---	---	0.482 **

** (P<0.01).

Table 6 Effect of *miR-203* methylation on *SOX4* gene expression

Group	*SOX4* Mean fold expression control	*SOX4* Mean fold expression patients	T-Test	*miR-203* % methylation
Endometrial adenocarcinoma	1.00 ± 0.00	9.24±052 a	2.64 **	100%
LSD value				Chi-Square = 9.53 **
** (P<0.01). Means having with the different letters in same column differed significantly				

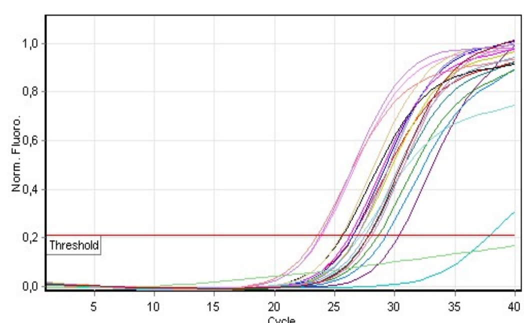

Figure 2 *SOX4* gene amplification plots by qPCR. The photograph was taken directly from Rotor-Gene Software version 2.1.0.9, threshold 0.210.

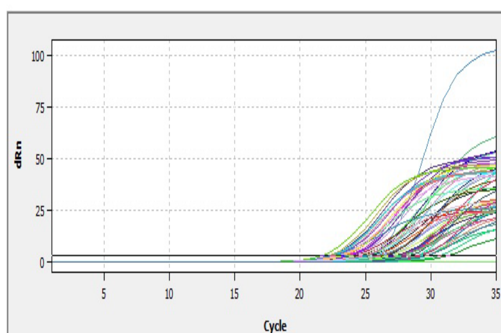

Figure 3 *miR-203* gene amplification plots by qPCR .Ct values ranged from. The photograph was saved directly in Qtower 2.0/2.2 software, threshold 2.176.

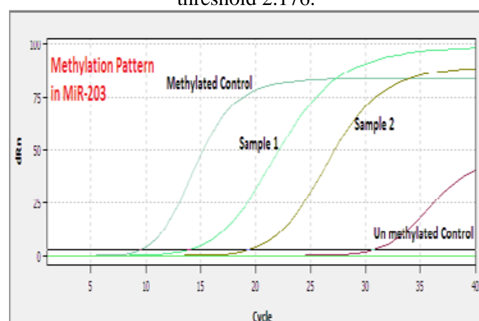

Figure 4 Methylation pattern of *miR-203*. Amplification plots by Q-MSP showed the ct value of samples and unmethylated controls. The photograph was saved directly in Qtower2.0/2.2 software.

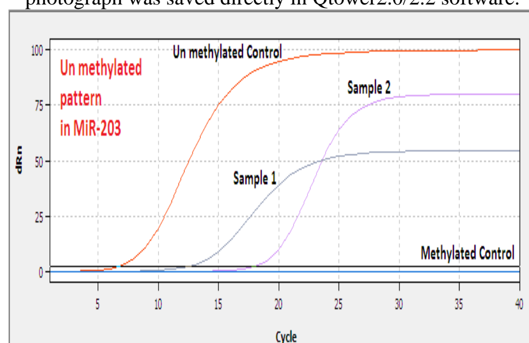

Figure 5 Unmethylated pattern of *miR-203*. Amplification plots by Q-MSP show the ct value of samples and methylated controls. The photograph was saved directly in Qtower 2.0/2.2 software.

Methylation pattern of *miR-203*

Quantitative-MSP analysis showed a significant increase ((P<0.01) in methylation status of *miR-203* promoter when compared to the control group. Promoter methylation was 100% and 0.00% in endometrial adenocarcinoma and control groups, respectively, (Figures 4 and 5).

Effect of *miR-203* down-regulation on *SOX4* expression

To confirm the findings of current study, we studied the relationship between the hypermethylation that occurs in promoter of *miR-203* and its effect on *SOX4* gene expression. The results of current study revealed that the levels of *SOX4* gene expression were significantly (P<0.01) increased in patients with endometrial adenocarcinoma as these was 100% hypermethylation in promoter of *miR-203* (Table 6).

DISCUSSION

Endometrial cancer is frequently a disease of post-menopausal ladies. The average age at determination is 62 years and 45% of cases were reported in women beyond 65 years [24]. One of the strongest risk factors for the development of endometrial cancer is unopposed estrogen exposure and deficient progesterone to adjust the mitogenic impacts of estrogen [25]. This happens either exogenously, by means of estrogen-just post-menopausal hormone substitution, or endogenously in fat ladies as abundance of fat tissues leads to increasing peripheral conversion of androgens to estrogens via aromatase enzyme [26]. Other risk factors for the development of endometrial cancer include incorporate diabetes, hypertension, tamoxifen utilization, advanced age and hereditary disorders [27].

In addition to estrogen, environmnetl factors such as abnormal mismatch repair (MMR), aberrant methylation of DNA and miRs are proposed as major mechanisms of carcinogenesis in endometrial cancer [28]. Mismatch repair system deficiency is the important abnormality in the early stage of endometrial cancer and related with estrogen. Expression of Hmlh and Hmsh2 examined by immuno-staining showed a strong positive correlation with blood levels of estrogen [29]. Many tumor suppressor genes in cancer cells are arrested by aberrant DNA methylation in promoter CpG islands [30]. Muraki et al. [31] reported a hypermethylation rate of 40% of Hmlh1in patients with endometrial cancer.

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]. Robert et al. [34] studied the expression of 1,718 genes using qRT-PCR. They applied the *GAPDH* as a reference gene in 72 kinds of normal human tissues. They found that using *GAPDH* is quite a reliable strategy for the normalization in qRT-PCR when applied in clinical studies.

SOX4 functions include the regulation of embryonic development and differentiation to determine cells fate, cellular transformation [6]. It has been reported to be abnormally expressed in a wide assortment of malignancies including endometrial cancer [37]. The results of current study agreed with Levan et al. [35] who reported the *SOX4* gene was overexpressed in patients with endometrial cancer. On the other hand, miRs plays an important role in carcinogenesis by targeting tumor suppressor gene or by acting as an oncogenes with elevated expression [36]. *miR-203* , *miR-129-2* ,

miR-596 , and *miR-618* identified to be bound to the 3-UTR of *SOX4* gene *insilico* analysis and these miRs keep the levels of *SOX4* by the degradation of its mRNA [37].

Moreover, Huang et al. [7] reported that the hypermethylated promoters of *miR-203* lead to SOX4 overexpression. miRs, which are 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, promoters' hypermethylation of these miRs leads to activation of oncogenes regulated by these genes [28].

miRs are processed and exported from the nucleus to the cytoplasm. The defects of these machineries can lead to degradation of functional miRs [38]. The XPO5 is a protein that transports miRs from the nucleus to the cytoplasm, inactivating mutations in *XPO5* gene reported in human carcinomas leading to a reset of the pre-miRs in nucleus and deregulating the mature miRs in cancers cells [39]. Germ-line mutations in the DICER1gene have been described in ovarian neoplasms. Dicer protein targeted by *miR-103* and down-regulation of its translation into protein and impact on global miRs [40,41]. DICER1 gene arrested was determined by promoter hypermethylation, the lower of DICER1 transcrip has been related with incidence in endometrial adenocarcinoma [42].

Aberration of DNA hypermethylation inactivates gene expression including miRs and loss of its tumor suppressor in human cancers by silencing their transcripts [43]. DNA methylation is one of the heritable epigenetic signs of the genome connected to gene expression/regulation and developmental processes in various eukaryotes. This DNA alteration is accomplished through the addition of a methyl group to cytosine, bringing about the arrangement of 5-methylcytosine [44]. In mammals, methylated cytosine are principally framed on CpG dinucleotides (CG) by the action of the DNA methyltransferase DNMT1 and DNMTs. CG sites are under-represented in mammals and tend to cluster in regions that are frequently located next to gene promoters and show atypically high CG recurrence. These areas are known as CpG islands [45,49].

The hypermethylation status of *miR-203* has not been broadly written about in cervical, endometrial and ovarian malignancies. The investigation of the present study added to the present literature on *miR-203*. In current study, *miR-203* hypermethylation was found in endometrial adenocarcinoma. In the cervix, past reports exhibited that the declaration of *miR-203* was down-regulated in high-review cervical intraepithelial neoplasia (CIN) and carcinoma [46,47]. miRs have been observed to be dysregulated in tissue-specific manners in different malignancies [48]. One of the most widely recognized reasons for the loss of tumor-silencer miRs in human malignancy is the silencing of their primary transcripts by CpG island promoter hypermethylation [49,50].

SOX4 gene is an individual of the SOX family transcription factors. Its known functions include control of embryonic growth and differentiation to determine cell fate [11]. *SOX4* gene expression appeared to increase in a wide range of tumors, including those of endometrium [37] recommending an essential part in tumorigenesis. The functions of *SOX4* in tumor development and progression could be dependent upon tumor origin. *SOX4* gene acts as a pro-oncogene and is related to the increased cells proliferation, cells survival, epithelial-to-mesenchymal transition, metastasis and with reduced apoptosis [11]. Kozomara et al. [12], depending on miRBase database, they identified the *SOX4* gene expression that may be regulated by at least 13 putative miRs including *miR-203*. Our results agreed with [7] who reported that hypermethylation of *miR-203* had led to an increase in *SOX4* gene expression in endometrial carcinoma cell line and the transfection of *miR-203* mimic had decreased *SOX4* gene expression.

CONCLUSIONS

Current study suggested that promoter hypermethylation of *miR-203* is a common mechanism leading to *SOX4* gene over-expression in endometrial cancer. *miR-203* hypermethylation with *SOX4* over-expression can be useful for the prediction of endometrial cancer in women with abnormal uterine bleeding.

Ethical Clearance: Permissions for carrying out the study were obtained from the Research Ethics Committee at Al-Zahra Teaching Hospital in Wasit province, Iraq.

Financial Disclosure: There is no financial disclosure.

Conflict of Interest: None to declare.

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