

## SOX4 Gene Contributes in Induction of Uterine Adenomyosis

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### Abstract

The objective of this study was to determine the level expression of SOX4 gene and its role in induce adenomyosis. Tissues specimens (30 patients and 10 healthy groups). Histological study of all tissues was carried out to observe the changes in tissues. Thirty biopsies of hysterectomy were concluded in this study. The expression levels of SOX4 gene were estimated by QRT-PCR. This study recorded the mean folding of SOX4 was a highly significant increased ( $p < 0.01$ ) in patients with adenomyosis ( $6.96 \pm 0.27$ ) when compared to the healthy control group ( $1.00 \pm 0.00$ ). We concluded the high expression of SOX4 gene had a role in development the uterine adenomyosis.

**Keywords:** SOX4; Adenomyosis; Gene expression; Gynecological disorders

### Introduction

Adenomyosis is a typical gynecological disorders; it is a myometrial lesion portrayed by the nearness of ectopic endometrial organs and stroma found inside the encompassing myometrium with contiguous myometrial hyperplasia and hypertrophy [1]. Indications are generally announced as menorrhagia, dysmenorrhea, pelvic pain, abnormal uterine bleeding and mass related side effects. In spite of its normal event, the etiology and pathogenesis of adenomyosis stays misty. Past epidemiologic examinations researched chance elements for adenomyosis. Hazard factors include: age between 40 and 50 years, early menarche, short menstrual cycles, a first birth at an early age, multiparity, sharp curettage amid early pregnancy, heftiness and tamoxifen utilize [2]. Adenomyosis, is pseudotumors of several organs, including the uterus and additionally the bile channels and gallbladder [3].

There are many genes that have an important role in induction of adenomyosis including CXCL10, calbindin D-28K, Prostaglandin related factors such as prostaglandin E receptor 3, COX-2, HLA-G, Interleukin-10, Beclin-1 and Histondeastylase [4-9]. These data suggest that the genetic and epigenetic abnormalities contribute to the pathogenesis of adenomyosis.

SRY-related high-motility group box 4 gene SOX4 is overexpressed in a variety of cancers and disease, It has been shown to be involved in the determination of cell fate and the regulation of embryonic development of many organ systems including heart, pancreas, brain and endometrial cancers reported the important role of SOX4 gene in progression of endometrial hyperplasia to endometrial adenocarcinoma [10-14].

SOX4 gene expression is up-regulated in many tumor types, with experimental evidence suggesting that this contributes to cellular transformation, control of apoptosis and or a metastatic phenotype [15-17]. Because of its role in much cellular transformation, this study aimed to evaluate the expression of SOX4 gene and its role in induction of uterine adenomyosis.

### Materials and Methods

#### Subjects

All cases were obtained from Al-Zahra Teaching Hospital in Wasit province/ Iraq

1. Thirty Patients suffering from abnormal uterine bleeding followed by hysterectomy.
2. Ten healthy individuals as a Control group.

#### Histological study

Histological technique of all tissues was carried out to observe the changes in tissues. Thirty biopsies of hysterectomy and curettage were concluded in this study [18].

#### Gene expression

Total Ribonucleic Acid (RNA) of all samples was extracted using the TRIzol® LS Reagent according to the manufacturer's instructions. Total Ribonucleic acid (RNA) was reversely transcribed to complementary Deoxyribonucleic acid (DNA) using WizScript™ RT FDMix Kit. The procedure was carried out in a reaction volume of 20  $\mu$ l 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 SOX4 gene. SOX4 primers sequences are (Forward: 5'-AGGATTCAAACGCAACTCAAAT-3, Reverse 5'-AAAGAAATACGAGGATGGAGCA-3) and sequence of GAPDH (Forward, 5-AACCTTGGCATTGTGGAAGG-3 and Reverse, 5-ACACATTGGGGGTAG GAACA-3).

### Statistical analysis

$\Delta$ CT and  $\Delta\Delta$ CT were calculated according to their equation [19]. This was conducted according to Statistical Analysis System-SAS 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 [20]. 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 Discussion

### Distribution of adenomyosis according to age

This study involved 30 uterine adenomyosis fresh biopsies Iraqi patients women with age ranged between 35 and 60 years. The highest percentage was recorded in age ranged (40-50) years with percentage (53.33%) followed by age group (50-60) years with percentage 26.67%. **Table 1** shows the age and uterine abnormalities.

Age range	Number of patients	Percentage (100%)
30-40	6	20
40-50	16	53.33
50-60	8	26.67
Total	30	100%

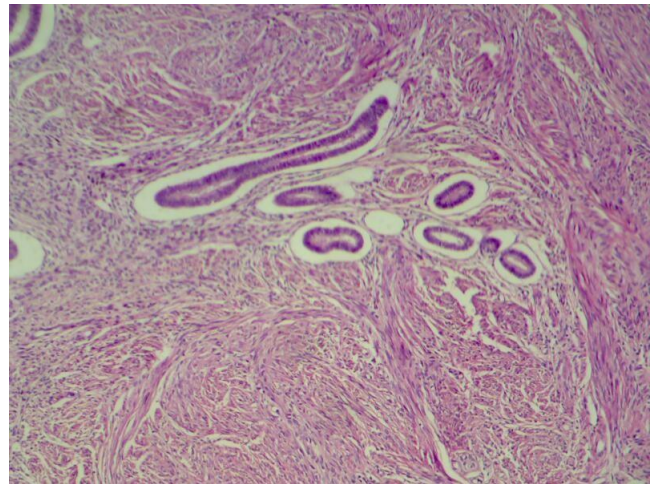
**Table 1:** Weight change comparison of wistar rats at initial and final day of treatment.

Reported adenomyosis to be found in 16.59% of hysterectomy specimen commonly in multiparous women of 31-50 years. Recurrence of adenomyosis increments with age, peaks at 40-50 years and levels-off after menopause. Increment in recurrence is related with multiparity [22]. Menstrual disturbance, smoking, dilatation, and curettage aid to increase the risk of adenomyosis [23].

### Histopathological findings

Adenomyosis refers to endometrial gland and stroma located randomly deep within the myometrium. Adenomyosis may be triggered by either a weakness of the smooth muscle or by increased intrauterine pressure or both [24]. Increased levels of Estadiol-2 (E2) and impaired immune-related growth control in ectopic endometrium are important for maintainance of Adenomyosis [25]. **Figure 1** showed the presence of endometrial

gland and some of the endometrial stroma infiltrated into layers of myometrium.



**Figure 1:** Light microscopic appearance of the uterus showing the endometrial gland penetrating deeply into myometrium.

### Molecular study

Three essential steps to determine the gene expression of SOX4 in patients with adenomyosis:

**Total RNA extraction:** Total Ribonucleic acid (RNA) was successfully extracted from all samples because the tissues were immediately processed and completely homogenized. The concentration of total Ribonucleic acid (RNA) ranged from 63-96 ng/ $\mu$ l and the purity ranged from 1.69-1.97 ng/ $\mu$ l. A high concentration of total Ribonucleic acid (RNA) depends on the extraction conditions whereby strict aseptic techniques must be used

**CDNA synthesis:** Complementary Deoxyribonucleic Acid (cDNA) reverse transcription was carried out directly after the Ribonucleic acid (RNA) extraction processes. The kit was provided with random hexamer primers which coverage to all regions of Ribonucleic acid (RNA) to generate a Complementary Deoxyribonucleic Acid (CDNA). The random primers are unable to distinguished between Messenger Ribonucleic acid (mRNA) and other Ribonucleic acid (RNA) species including micro Ribonucleic acid (RNA) molecules in the reaction.

**Quantitative real-time PCR results:** Real time Polymerase Chain Reaction (PCR) quantification was applied in the present experiment utilizing the EVA green. The fluorescent dye recognized any double stranded Deoxyribonucleic Acid (DNA) including Complementary Deoxyribonucleic Acid (CDNA) and the amplification was recorded as a Ct value (cycle threshold).

The lower Ct value indicates the presence of higher copies of the target and vice versa. In terms of gene expression, high Ct values indicate a low gene expression and a low Ct value indicates a high gene expression [19,26].

**GAPDH gene expression:** There were no significant differences of Ct value of GAPDH in subjects and healthy control group ( $1 \pm 0.00$ ). The housekeeping gene used in the present study is shown in **Table 2**.

The mean fold of GAPDH gene expression in the patients groups was  $(0.98 \pm 0.08)$  and **Figures 2 and 3** show the amplification plots and melting curves for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene.

Groups	Means Ct of GAPDH	2-Ct	Experimental group/control group	Mean fold of GAPDH gene expression
Adenomyosis	29.977	9.5 E10	9.5 E10/9.7 E10	$0.98 \pm 0.08$
Control group	29.946	9.7 E10	9.7 E10/9.7 E10	$1 \pm 0.00$
LSD value				0.217 NS

**Note:** NS -Non-significant.

**Table 2:** Comparison of GAPDH fold expression between studygroups.

The inherent assumption in the use of housekeeping genes in molecular studies is that their expression remains constant in the cells [27]. One of the most commonly used housekeeping genes in comparison with the gene expression data is GAPDH [28].

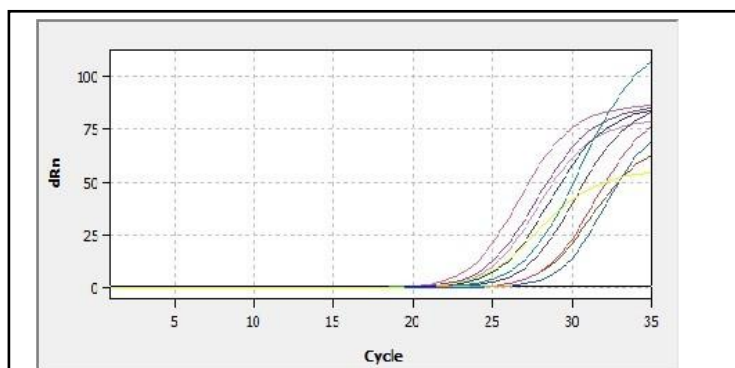
Studied the expression of 1,718 genes using qRT-PCR [29]. They applied the GAPDH as a reference gene in 72 kinds of normal human tissues. They found that using GAPDH is quite a reliable strategy for the normalization in qRT-PCR when applied clinical studies.

**SOX4 gene expression:** Expression of the SOX4 gene was highly significant increased ( $p < 0.01$ ) in patients with adenomyosis when compared to the healthy control group as shown in **Table 3** and **Figures 4 and 5** show the amplification plots and melting curves for SOX4 gene.

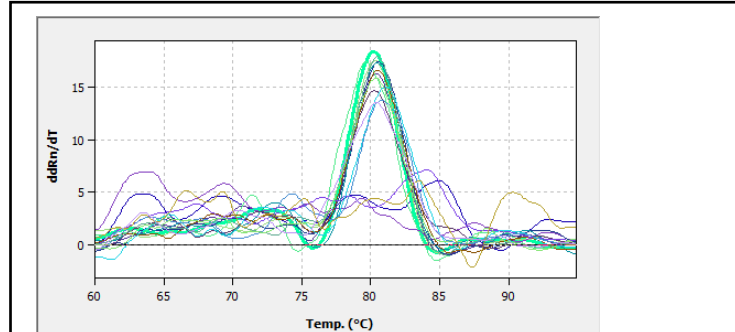
Groups	Means ct of SOX4	Means ct of GAPDH	$\Delta Ct$ (means ct of SOX4-means ct of GAPDH)	2- $\Delta Ct$	Experimen-tal group/control group	Fold of SOX4 gene expression
Adeno myosis	21.08	29.977	-8.897	476.72	476.72/68.40	$6.96 \pm 0.27$
Control	23.85	29.946	-6.096	68.4	68.40/68.40	$1 \pm 0.00$
LSD value	---	---	---	---	---	$2.073^{**}$

**Note:**  $^{**}(P < 0.01)$ .

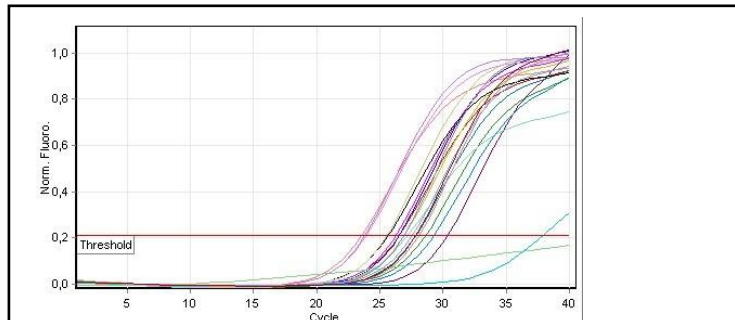
**Table 3:** Fold of SOX4 gene expression. Depending on 2- $\Delta\Delta Ct$  method.



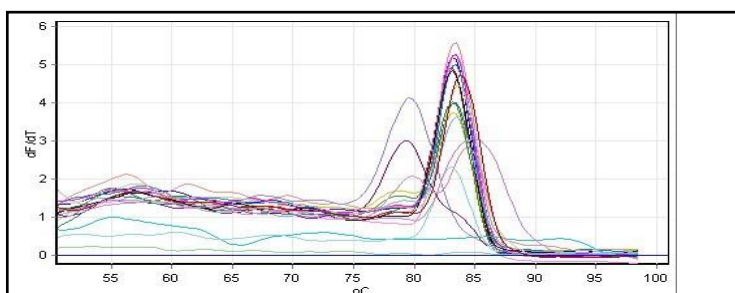
**Figure 2:** GAPDH amplification plots by qPCR .Ct values ranged from. 23.58-28.63 .The photograph was saved directly in Qtower2.0/2.2 software . Threshold (0.210), gain Green: 9.77.



**Figure 3:** GAPDH melting curves by qPCR Samples included all study groups. Melting temperature ranged from 83°C to 87°C, No primer dimer could be seen. The photograph was saved directly in Qtower 2.0/2.2 software.



**Figure 4:** SOX4 gene amplification plots by qPCR Ct values ranged from 16.81 to 30.15. The photograph was taken directly from Rotor-Gene Software Version 2.1.0.9, threshold (0.210) , gain Green: 10.0.



**Figure 5:** 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/Software Version 2.1.0.9.

Although the functional contributions of smooth muscle cells to adenomyosis development are poorly understood, reported that epithelial and stromal cells in endometriosis develop from persistent coelomic epithelial cells by metaplasia [30].

In pelvic endometriosis, eutopic endometrial cells arriving through retrograde transplantation at ectopic sites could induce the surrounding tissue to undergo smooth muscle metaplasia. Similar to endometriosis, adenomyosis associated smooth muscle cells may be of metaplastic origin. Myofibroblastic metaplasia with formation of an abnormal myometrial hypertrophy may contribute to an impaired uterine function.

Immunohistochemical study in adenomyosis tissue revealed expression of specific uterine marker molecules, including the essential components characterizing uterine myometrial cells such as Oxytocin Receptor (OTR), Vasopressin Receptors (VPR), Estrogen Receptors (ER) and Progesterone Receptors (PR) [31]. Oxytocin Receptor (OTR), Estrogen Receptors (ER) and Progesterone Receptors (PR) are also expressed in peritoneal endometrial smooth muscle cells [32].

On the other hand, reported that the presence of adenomyotic nodules in deeply infiltrating endometriosis lesions is accounted for by a reaction of the local environment to the presence of ectopic endometrium [33]. Myofibroblasts play an important role in the development of adenomyosis by their expression of Extracellular Matrix (ECM) proteins. These data suggest that myometrial hypertrophy is a response/reaction of the ectopic endometrial cells to the surrounding tissue, which shares characteristics with physiological and pathological mechanisms of wound healing (tissue injury and repair) [32-35].

Adenomyosis is a benign pathology represented by the presence of endometrial gland and stroma within the myometrium and it has been associated with endometrial cancer [36]. A high expression of SOX4 gene was reported with endometrial cancer incidence by epigenetic manner [13]. Confirm that the adenomyosis can be a precursor of some carcinoma but the exact molecular mechanisms that lead to malignancy are poorly understood. Therefore, this study suggested there are relationships between high expression of SOX4 gene and adenomyosis incidence [37,38].

## Conclusion

MiRs play an important role in carcinogenesis by targeting tumor suppressor gene or by act as Oncogenes with elevated expression. MiR-203, miR-129-2, miR-596, and miR-618 identified to bound to the 3-UTR of SOX4 gene insilico analysis and these miRs keep the levels of SOX4 by the degradation of its mRNA. Reported that the hypermethylated promoters of miR-129-2 and miR-203 lead to SOX4 overexpression. In conclusions the SOX4 gene participate in induction of uterine

adenomyosis as well as in many histological changes especially when in overexpression status.

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