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The Effect of Metformin on Expression of Long Non-coding RNA *H19* in Endometrial Cancer

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Abstract

Background: Endometrial cancer is the fourth most widespread cancer among females, with a growing prevalence in recent years. Management by combined therapies along with surgery, radiotherapy, and chemotherapy have improved patients' prognoses. Besides, the development of new therapies helps preserve fertility and prognosis in aggressive tumors.
The purpose of this research was to identify the efficacy of metformin on the *H19* long non-coding RNA expression in endometrial cancer to provide further insight into the pathogenesis and treatment of the disease.

Methods: A total of 23 patients with endometrial cancer, diagnosed by biopsy or diagnostic curettage, were recruited and divided into three groups, before and after metformin treatment and placebo. Real-time PCR was used to evaluate the *H19* expression in cancer tissue in all patients.

Results: It has been observed that in endometrial tissue of the "after-metformin" treatment group, the *H19* expression level was significantly reduced, compared with the "before-metformin" treatment group, but not in comparison with the placebo. These findings indicate that metformin reduced the *H19* expression in endometrial cancer.

Conclusion: Anti-diabetic drugs, such as metformin, may be beneficial by reducing the *H19* expression in endometrial cancer due to the H19 relation to cancer progression.

Keywords: Endometrial Cancer, Non-Coding RNA, *H19* Gene, Metformin

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Introduction

Endometrial cancer, which encompasses about half of all cancers in females, is considered th e most common gynecological malignancy. It is the fourth more widespread cancer after breast, lung, and colorectal cancers and the eighth primary reason for death due to malignancy in women in the world (1). The highest level of significance regarding the management of this disease belongs to early diagnosis and influential treatment. Endometrial

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↑What is "already known" in this topic:
There is an anti cancer property for metformin and *H19* LncRNA is a cancer biomarker and therapeutic target in many types of cancers. Metfromin reduces endometrial cancer risk and prevents cell proliferation in preoperative endometrial cancer tissue. Also, metformin oppresses *H19* in endometrial cancer tissue samples and decreased expression of *H19* prevents endometrial cancer cells from invading and migrating.

→What this article adds:
This population-based case-control genetic association study, using Real time-PCR rechnique, shows *H19* gene expression can be reduced in endometrial cancer tissue sample as a result of metformin administration which can indicate the efficacy of metformin in control of endometrial cancer progression.

cancer usually occurs after menopause, whereas it is seen in only 3–5% of women younger than 40-years-old (2). According to the data published about the Iranian population, endometrial cancer has been recognized as the fifth most common cancer of females in the last two decades. It is also the third known cause of female death and the third most common malignancy of the female reproductive system (3). Consistent with the first report by the World Health Organization (WHO), the prevalence of endometrial cancer among Iranian women is not significantly lower than that in the Eastern Mediterranean Region (EMR) (4).

Generally, metformin is known as an anti-hyperglycemic medication, and it is used as a type 2 diabetes first line of treatment. However, in recent years, the anti-cancer property of metformin has also become prominent (5, 6). Studies on patients with type 2 diabetes revealed a reduced risk of cancers at many sites, specifically breast, colorectal, ovarian, and endometrial cancers, by using metformin (7). Many clinical studies have demonstrated that metformin decreases cell proliferation, induces apoptosis and cell cycle arrest *in vitro*, as well as decreases the incidence and growth of tumors *in vitro* (8). Therefore, it might be used in combination with chemotherapy and radiation to confront cancer (9). Besides, it plays an important role in targeting cancer stem cells and reverses epithelial-to-mesenchymal transition (EMT), as an implication for the acquisition of metastatic characters of tumor cells (10, 11).

Long non-coding RNAs (LncRNAs) take the lead in gene regulation, maintenance of genome integrity, cell differentiation, and growth. These RNAs are also down-regulated in various human diseases (12). Of the most noticeable is dysregulated LncRNAs, associated with cancer progression (11).

The *H19* LncRNA is an oncofetal gene that is placed adjacent to the telomeric region of chromosome 11p15.5 and binds with the neighboring insulin-like growth factor II (*IGF-2*) gene (13). It is one of the first discovered LncRNAs, which can be considered as a cancer biomarker and therapeutic target (14). Ovarian and endometrial cancers are among many malignancies associated with the *H19* re-expression, in contrast to most normal adult tissues (15, 16). It has also been shown that the *H19* expression is increased in cancer biopsies and plasma of cancerous samples compared to healthy samples; for example, plasma *H19* levels are higher in patients with gastric cancer compared to healthy samples (14). Tumor growth can be attenuated by the *H19* expression blockage *in vivo* (17). It is also shown that *H19* is stimulated by the oncogene *c-myc* (tumorigenesis and metastasis driver), and the expression levels of *H19* and *c-myc* are highly correlated in primary tumors of lung and breast cancer (18). However, the action of *H19* as a tumor suppressor in an SV40 hepatocarcinoma model (12), papillary thyroid carcinoma (19), liver cancer (20), and prostate cancer (21) demonstrates different *H19* expression patterns across different types of cancers and bifunctional effect of H19, emphasizing on its contradictory functions in tumorigenesis (12, 22). Thus, the cancer-specific expression of definite LncRNAs like *H19* gives us an essential incentive to consider this new molecular modifier, particularly in endometrial cancer.

Furthermore, reduced expression of *H19* was associated with EMT inhibition in ovarian cancer cell line, A2780 and ARK2 cell line, acquired from human uterine serous carcinoma, which could be due to the molecular mechanism underlying metformin ability (23). In addition, metformin inhibits *c-Myc* by speeding up c-Myc protein degradation (24), and *c-Myc* directly boosts the *H19* transcription (18), suggesting that a positive feedback loop between *c-Myc* and *H19* is disrupted by metformin (23). Given the fact that endometrial cancer is associated with insulin resistance, obesity, and diabetes (25, 26), a therapeutic metformin dose for diabetes has been considered, specifically as an inhibitor of cancer cell proliferation in patients with endometrial cancer (27).

Based on this information, we hypothesized that metformin could have an inhibitory effect on endometrial cancer cells by regulation of *H19* LncRNA expression. Thus, we aimed to show the effect of metformin on endometrial cancer through assessment of the *H19* expression level in cancerous endometrium human tissue samples before and after taking metformin at the therapeutic dosage for patients with diabetes, using real-time PCR.

Methods

Patients

In this study, patients referred to the Firoozgar Hospital were recruited. Patients were selected based on direct observation of endometrial lesions by a surgeon during a biopsy or diagnostic endometrial curettage. The study population comprised 13 patients receiving a placebo and 10 patients in the intervention group treated with metformin. At the time of referral, the clinical information of patients was recorded in a questionnaire.

Patients were selected after completing the metformin sensitivity and excluded from the study if they had a severe hepatic impairment, renal disease, heart disease, uncontrolled hypertension, thromboembolism, and history of metformin or progesterone use. All patients in the intervention group received metformin (initial dose, 750 mg/day, up to 1500 or 2250 mg/day) for 3 to 12 weeks until surgery day. Tissue specimens were genetically examined by endometrial curettage right after initial diagnosis (before treatment), and hysterectomy (after treatment), and the placebo group was given designed metformin tablets prepared by Dr. Abidi factory in the dose of 500 mg. Hereafter, we call the three groups NM (taking a placebo or no metformin group), BM (before taking metformin), and AM groups (after taking metformin).

Tissue samples were placed in a 2 ml cryovial containing 1000 µl RNA Later and stored at -80 ° C.

Ethical approval

In this study, detailed medical history and written informed consent were obtained from all individuals. This research project was conducted and approved under the regulations of the Ethics Committee of the Faculty of Medicine, Iran University of Medical Sciences, according to the Helsinki declaration and based on the submitted documents (IR-IUMS-FMO.REC.1398.388).

RNA extraction by Trizol

For each 50 to 100 mg of sample tissue, 1000 µl of Trizol was added, and the samples were homogenized, then 1.5 µl of 200 µl of chloroform was added before vortexing. The samples were then stored for 5 min on ice or at -20 °C, and then centrifuged for 4 min at 4 °C at 12000 rpm. After centrifugation, the supernatant having RNA was transferred to a new vial, and then isopropanol was added. The sample was then centrifuged at 12,000 rpm, at 4 °C for 30 minutes, and the supernatant was discarded and the precipitate was kept for further experiments. Next, we added 1000 µl of 75% ethanol (equal to Trizol volume) and pipetted several times up and down to immerse the precipitate in alcohol, followed by centrifugation for 8 minutes at 4 °C and 7500 rpm. Alcohol was removed in the next step and then the precipitate was left to dry for 10 minutes, and residual ethanol was removed from the wall. Finally, depending on the amount of sediment formed, 20–70 µl of molecular grade water was added.

To evaluate the quality of the extracted RNA, RNA was quantified using a nano-drop, and a qualitative assay was performed to ensure that there was no DNA contamination by electrophoresis on the agarose gel. The presence of 18S and 28S bands and the absence of DNA confirmed the RNA quality on gel electrophoresis.

Complementary DNA synthesis using the Takara kit

The complementary DNA (cDNA) was synthesized using the Takara kit PrimeScript™ RT reagent Kit (Perfect Real Time), and all procedures were performed according to the standard protocol. The accuracy of cDNA synthesis was confirmed using the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer and polymerase chain reaction (PCR).

Evaluation of the H19 expression using real-time PCR

The *H19* specific primers were applied, using GAPDH primers as an internal control. In each reaction, 250 ng/µL cDNA was used as a template for Real-Time PCR, and 5 picomoles of each primer were added in a final volume of 20 µl. The reaction was performed using Power SYBR Green PCR. Cycling conditions were an initial step of enzyme activation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 58°C for 30 s, and extension at 65°C for 1 min.

The *H19* mRNA expression was performed in triplicate by real-time PCR, using SYBR Green master mix with the primers presented in Table 1. Data analysis was carried out using the $2^{-\Delta\Delta CT}$ method.

Table 1. The primer sequences for the H19 real-time PCR analysis

Genes	Primer pair sequences	Amplicon size
H19-F	5'-TTTCATCCTTCTGTCTCTTTGT-3'	131 bp
H19-R	5'- CAACCAGTG-CAAATGACTTAG-3'	
GAPDH-F	5'-ACACCCACTCCTCCACCTTTG-3'	122 bp
GAPDH-R	5'- TCCACCACCCTGTTGCTG-TAG-3'	

Statistical analysis

Statistical analyses were done using the t-Test and Mann-Whitney test; p-value ≤0.05 was deemed statistically significant.

Results

The LncRNA *H19* expression levels in endometrial cancer samples before and after metformin administration and also in the placebo group are shown in Figure 1. Analysis of expression levels in three groups, including NM, BM, and AM groups, showed that in the NM group, the *H19* expression (1.6±2.5) was lower than the BM group (1.9±2.2). The *H19* expression was significantly decreased in the AM group (0.6±0.7), compared with the BM group (p=0.010).

Discussion

We assessed the *H19* expression level in endometrial cancer cells before and after treatment with metformin to enclose its probable effect on endometrium cancer progression. The *H19* expression level was lower in the AM group compared to the BM group, suggesting the ability of metformin to inhibit the metastasis in endometrial cancer. We observed the metformin effect on human tissue samples rather than *in vitro* conditions or animal models, using the therapeutic dose for diabetic patients, regardless of most preclinical studies, using real-time PCR.

For the first time, the researchers noticed the role of metformin in cancer prevention in studies performed on diabetic patients and cancer. Evans *et al*. reported a lower risk of cancer in metformin-treated diabetic patients and

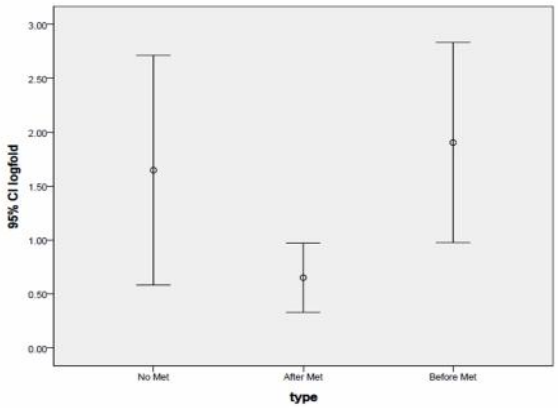

Fig. 1. The real-time PCR analysis. Evaluation of the H19 expression level showed a significant decrease in patients after metformin treatment

observed that its protective effect increases with exposure to a greater dose of metformin (28). Studies of various types of cancers have shown a reduced risk of cancer in metformin-treated diabetic patients compared to those who did not take metformin (29). The highest risk reduction was reported in patients with ovarian/endometrial cancer by metformin use in a retrospective cohort study (30). The anti-tumor effects of metformin have been associated with different mechanisms. These mechanisms include 1) classic (direct or insulin-independent; indirect or insulin-dependent), 2) immune-mediated, 3) impact on cellular metabolism, 4) epigenetic modification effects, and 5) apoptosis. The impact of metformin on epigenetic modification is exerted through DNA methylation (31).

The therapeutic dose of metformin for diabetes was shown to prevent cell proliferation in pre-operative endometrial cancer tissue for the first time prospectively, in 2014. It was confirmed that metformin intake activated adenosine monophosphate-activated protein kinase (AMPK) and inhibited mitogen-activated protein kinases (MAPK) in the endometrium, leading to cell growth inhibition indirectly (27). Before that, the impact of metformin on propagation and expression was the main focus of metformin cell signaling in endometrial cancer cell lines (ECC-1 and Ishikawa), which was evaluated in 2010 by Cantrell *et al.*. They found metformin as a powerful repressor of cell regeneration in endometrial cancer cell lines, intervened with AMPK activation, following the suppression of the mammalian target of rapamycin (mTOR) pathway (32).

Metformin has been shown to suppress the *H19* expression and increase *miR-29b-3p* in HTOG (ovarian-derived cell tumor), using real-time PCR. It has been also demonstrated that *miR-29b-3p* could attach to *H19*, matrix metalloproteinase (*MMP*)-9, and *MMP-2*, respectively, suggesting a regulatory relationship of *H19*/*miR-29b-3p*/*MMP*-9/*MMP*-2 (33). *MMP*-9 and *MMP*-2 are known to be related to tumor cell migration (34). Besides, as metformin could increase the AMPK level and decrease the level of mTOR and Akt in HTOG cells, leading to inhibition of cell cycle progression, angiogenesis, cell proliferation, and protein synthesis (35). Thus, it can be predicted that metformin implies its therapeutic effect through two diverse signaling pathways, AMPK, and H19. The same findings in a rat model of Poly Cystic Ovary Syndrome (PCOS), validated the therapeutic impact of metformin on PCOS *in vivo* (33).

On the other hand, metformin stimulates changes in DNA methylation across the genome by adjusting the function of S-adenosyl homocysteine hydrolase (SAHH). Cancer cells that are exposed to metformin indicate hypermethylation of genes that are part of pathways, boosting tumor and simultaneous reticence of cell proliferation (36). Zhong *et al.* demonstrated that metformin provokes the *H19* oppression and revises gene methylation in tissue samples of endometrial cancer taken from patients who received antidiabetic doses of metformin. It has been explained that metformin can increase the expression of microRNA *let-7* by activating AMPK, causing the *H19* LncRNA breakdown, which usually joins to and cancels SAHH hydrolytic activity (36). SAHH is the only eukaryotic enzyme able to hydrolyze S-adenosyl homocysteine (SAH), a powerful feedback inhibitor of S-adenosyl-L-methionine-dependent methyltransferases (SAM MTase), including DNA methyltransferases (DNMTs) (37).

Furthermore, decreased expression of *H19* prevents endometrial and ovarian cancer cells from invading and migrating. Yan *et al.* confirmed that metformin greatened DNA methylation in the promoter region of *H19* to downregulate the *H19* expression and thus, hold back tumor cell migration and invasion (23, 31). This is orchestrated by *Let-7*, which is less available by *H19*. *Let-7* as a tumor suppressor microRNA represses the expression of factors needed for cell growth and mobility and can inhibit tumor cell migration and invasion when *H19* is methylated in its promoter region and is expressed less (23). These established mechanistic links between metformin and *H19* promoter hypermethylation, leading to its chronic inhibition, is consistent with the reduced *H19* level in endometrial cancer cells after metformin administration in the current study.

Other investigations support the antitumor effect of metformin through *H19* regulation as well. *H19* was confirmed to be downregulated, making it a key component of gastric cancer cell invasion suppressor induced by metformin (22). Moreover, a study of phenformin, the analog of metformin, on the stemness, apoptosis, and proliferation of glioma stem cells (GSCs) in glioblastoma (GBM), concluded that phenformin exerts its impacts on GSC stemness through the upregulation of *let-7* expression and downregulation of *H19* that further elevate the *let-7* bioavailability *in vitro*, as well as indicating its inhibitory impact on GSC-derived xenografts' growth (38).

Generally, *H19* seems to have the potential as a biomarker of endometrial cancer via predicting cancer metastasis, recurrence, or as a predictor of medicinal reaction because biomarkers selected according to DNA methylation have located promising usages at the practice in recent years. To achieve this goal, it should be able to define risk and identify the early stages of carcinogenesis. Moreover, it can be used to develop drugs more rationally and as a result, improving preclinical and clinical endeavors for patients' benefit. Here, the direct effect of metformin on *H19* is suggested in endometrial cancer, along with other investigations (23, 36). Hence, metformin's potential therapeutic and chemopreventative action in endometrial cancer is justifiable. The limitation of this study is the small sample size which necessitates the replication of this study with a bigger sample size and application of more complex analysis to reach a definite and more comprehensive conclusion.

Conclusion

In this study, the placebo group showed little effect on the *H19* expression level, but in the BM group, the *H19* expression level was significantly higher compared to the AM group. The results after metformin administration, in general, indicate that metformin reduced the *H19* expression.

Based on previous research and the current study, it can

be concluded that anti-diabetic drugs such as metformin can prevent the migration and invasion of cancer cells and directly affect the gene expression in cancer cells by altering DNA. Our results emphasized the impact of metformin on the inhibition of cancer progression in human endometrial tissue and at the therapeutic dose for diabetic patients.

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Conflict of Interests

The authors declare that they have no competing interests.

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