Heliyon

Received:
14 January 2016
Revised:
6 April 2016
Accepted:
18 May 2016

Heliyon 2 (2016) e00116

FOXP1 forkhead transcription factor is associated with the pathogenesis of endometrial cancer

Makito Mizunuma [a], Yoshihito Yokoyama [a,*], Masayuki Futagami [a], Kayo Horie [b], Jun Watanabe [b], Hideki Mizunuma [a]

[a] *Department of Obstetrics and Gynecology, Hirosaki University Graduate School of Medicine, Hirosaki, Aomori 036-8562, Japan*

[b] *Department of Cellular and Hist-Pathology, Division of Medical Life Science, Hirosaki University Graduate School of Health Science, Hirosaki, Aomori 036-8564, Japan*

* Corresponding author at: Department of Obstetrics and Gynecology, Hirosaki University Graduate School of Medicine, 5-Zaifu-cho, Hirosaki, Aomori 036–8562, Japan.
E-mail address: yokoyama@hirosaki-u.ac.jp (Y. Yokoyama).

Abstract

Endometrial cancers are mostly estrogen-dependent. FOXP1 is a P subfamily of forkhead box (FOX), and known as an estrogen-responsive transcription factor. The aims of this study were to examine histological location of FOXP1 in normal and malignant endometrium, and to investigate a possible association between FOXP1 and other factors considered to be involved in pathogenesis of endometrial cancer. The levels of FOXP1, estrogen receptor (ER)α, and ERβ expression were examined immunohistochemically in normal and malignant endometrium obtained from 75 women (8 normal, 8 atypical endometrial hyperplasia, and 59 endometrial cancers from grade 1 to 3). The effects of estrogen on ERα, FOXP1, KRAS, and PTEN expression were analyzed in telomerase-immortalized human endometrial stromal cells (T HESCs) by Western blotting. Western blotting was also used to examine the effect of FOXP1 plasmid DNA or siRNA transfection on KRAS and PTEN expression in Ishikawa cells (well differentiated endometrioid adenocarcinoma), HEC-50B cells (poorly differentiated endometrioid adenocarcinoma), and T HESCs, respectively. FOXP1 was expressed in normal and malignant

http://dx.doi.org/10.1016/j.heliyon.2016.e00116

endometrium, but the rate of expression was different depending upon menstrual cycle and pathological grade of malignancy. FOXP1 expression in nucleus and cytoplasm of grade 3 endometrioid cancers was significantly lower than that of grade 1 and 2 ones. Estradiol increased levels of FOXP1 and KRAS expression in a dose- and time-dependent manner in T HESCs cells, and FOXP1 transfection or knockdown led to increase or decrease of KRAS expression but not PTEN. KRAS expression level was significantly related to FOXP1 and ERα levels in cancer tissues. Estradiol did not affect KRAS expression in T HESCs cells transfected with FOXP1 siRNA. These results suggest that FOXP1 is involved in estrogen dependent endometrial cancers through KRAS pathway.

Keywords: Medicine, Cell biology

1. Introduction

Endometrial cancer is one of the most common malignancies of female reproductive tract and has been increasing in developed countries [1]. Endometrial cancer is categorized into two types, Type I and Type II based on clinical features and pathogenesis [2]. Type I endometrial cancer is estrogen-dependent and arises in most cases before and at the time of menopause. In addition, Type I endometrial cancer is low grade, shows good response to treatment and is the most prevalent histological type of endometrial cancers.

With regard to pathogenesis of endometrial carcinoma, presence of abnormal PTEN expression [3, 4, 5] and KRAS mutations [5, 6] has been proposed. PTEN plays an important role in cell apoptosis and decreased expression of PTEN has been shown in endometrial carcinoma [3, 4], while KRAS is an oncogene and stimulates proliferation of endometrial cancers [5, 6]. Moreover, Li et al. reported that epithelial cells from normal endometrium showed increased proliferation by co-culturing with stromal cells from endometrial cancer, suggesting that the tumor microenvironment may be involved in the pathogenesis of endometrial carcinoma [7]. Indeed, expression of the oncogene KRAS is increased in epithelial cells cocultured with endometrial cancer stromal cells [7]. Because estrogen synthesized in endometrial cancer stromal cells was found to contribute to cancer cell proliferation [8, 9], it is of importance to clarify the effects of estrogen on PTEN and KRAS.

Recent studies have shown that the forkhead box (FOX) transcription factor family is closely associated with hormone-dependent carcinogenesis through interaction with the steroid receptors [10, 11, 12]. FOXP1, which belongs to the P subfamily of FOX transcription factors, is an estrogen-responsive transcription factor [13]. Rayoo et al. found that FOXP1 expression was positively correlated with estrogen receptor (ER) α expression in breast cancer [14] and Bates et al. found that the pattern of expression of FOXP1 protein was similar to the pattern of expression of

http://dx.doi.org/10.1016/j.heliyon.2016.e00116

ERβ in normal breast tissue [15]. FOXP1 is widely expressed in normal human tissues, including the uterus [16]. Immunostaining for FOXP1 has been observed in the human endometrium, and a shift from nuclear to cytoplasmic staining was noted between the proliferative and secretory phases of the menstrual cycle [17, 18]. The presence of nuclear FOXP1 expression was significantly linked to ERα reactivity in normal endometrium and endometrial endometrioid adenocarcinoma [17].

Previous studies suggested that FOXP1 behaves as an oncogene in B cell lymphoma [19, 20] and hepatocellular carcinoma [21, 22]. Conversely, studies suggested that FOXP1 might be a tumor suppressor gene in breast cancer [23, 24], prostate cancer [25], and epithelial ovarian cancer [10]. Whether FOXP1 behaves as an oncogene or a tumor suppressor gene in the pathogenesis of endometrial conditions such as endometrial hyperplasia and endometrioid adenocarcinoma remains unclear.

The aims of this study are 1) to determine the location and levels of FOXP1, ERα, and ERβ expression in endometrial adenocarcinoma and endometrial hyperplasia, and 2) to investigate a possible role of FOXP1 in pathogenesis of endometrial cancer.

2. Materials and methods

2.1. Subjects and tissue samples

Seventy-five tissue samples were collected from patients seen at Hirosaki University Hospital from 2009 to 2013. Thirty-five patients had grade 1 endometrioid adenocarcinoma, 13 had grade 2 endometrioid adenocarcinoma, 11 had grade 3 endometrioid adenocarcinoma, 8 had atypical endometrial hyperplasia, and 8 had normal endometrium (5 patients were in the proliferative phase and 3 were in the secretory phase). Samples of histologically normal endometrium were obtained from patients who underwent a hysterectomy due to a benign condition such as uterine myoma. Endometrial phases were determined based on hematoxylin and eosin-stained sections assessed in accordance with the histological criteria of Noyes et al. [26]. No patients had received hormone therapy. The histology of all of the samples was determined by 2 gynecologic pathologists (MF and JW). This study involving the use of clinical tissue samples was approved by the Institutional Review Board of the Hirosaki University Graduate School of Medicine (Reference number: 2014–184). All patients were informed of the purposes of this study and results were published on the website of Hirosaki University Hospital. The clinical stage of endometrioid adenocarcinoma was defined in accordance with the staging system of the International Federation of Gynecology and Obstetrics and the grade of endometrioid adenocarcinoma was defined in accordance with the WHO classification.

http://dx.doi.org/10.1016/j.heliyon.2016.e00116

2.2. Immunohistochemistry

All samples obtained for immunohistochemistry were fixed in formaldehyde and embedded in paraffin. As per usual, 6-μm-thick sections were passed through xylene and a graded ethanol series. Sections were incubated with antibodies specific to FOXP1 (Catalog number; ab16645, Abcam, Cambridge, UK), ERα (Catalog number; ab17087, Abcam), ERβ (Catalog number; ab288, Abcam) and KRAS (Catalog number 60309-1-Ig, Proteintech, Chicago, IL) overnight at 4 °C after antigen retrieval in a sodium citrate buffer. Slides were incubated with biotinylated species-specific secondary antibodies for 20 min and then exposed to avidin-biotinylated enzyme complex (VECTASTAIN® Elite ABC KIT, Vector Laboratories, Burlingame, CA). Sections were treated with 0.02% diaminobenzidine as a chromogen and counterstained with hematoxylin. The level of protein expression was graded using staining scores. These scores were calculated by multiplying the staining intensity and the stained areas within the tumors. As shown in Fig. 1, the staining intensity in nuclei was classified as negative (score 0), weakly positive (score 1), moderately positive (score 2), or strongly positive (score 3) [27], and the staining intensity in the cytoplasm and stroma was classified as negative (score 0), weakly positive (score 1), or strongly positive (score 2) [17]. Stained areas were graded as follows: a score of 0 was given to a specimen with an area of 0% staining, a score of 1 was given to a specimen with an area of ≥1% to <25% staining, a score of 2 was given to a specimen with an area of ≥25% to <50% staining, and a score of 3 was given to a specimen with an area of ≥50% staining. Each slide was observed in a 0.75-mm^2 field of vision using a 20 × objective lens. We got the staining score by multiplying staining intensity and stained area scores together, and the sum total from 3 different sites was calculated as the staining score. The staining score was graded in both the nuclei and the cytoplasm of cells in glandular epithelium and stroma. The maximum score of the nuclei staining is 27 points and that of cytoplasm of the cells and stroma is 18 points. The level of protein expression was evaluated by 2 researchers (MM and YY) who were not given any physical and clinical information about the patient. A staining score of 10 points or more in nuclei was defined as positive, and a staining score of 6 points or more in cytoplasm and stroma was defined as positive.

2.3. Cell lines and cell cultures

The telomerase-immortalized human endometrial stromal cells (T HESCs) used were from a cell line of hTERT-immortalized endometrial fibroblasts from a patient with non-malignant myomas (CRL-4003, American Type Culture Collection, Rockville, MD). The Ishikawa cell line established from well differentiated endometrioid adenocarcinoma cells (JCRB1505, the Japanese Collection of Research Bioresources, Osaka, Japan) [28] and the HEC-50B cell line derived from poorly differentiated endometrioid adenocarcinoma (JCRB1145, the Japanese Collection of Research

http://dx.doi.org/10.1016/j.heliyon.2016.e00116

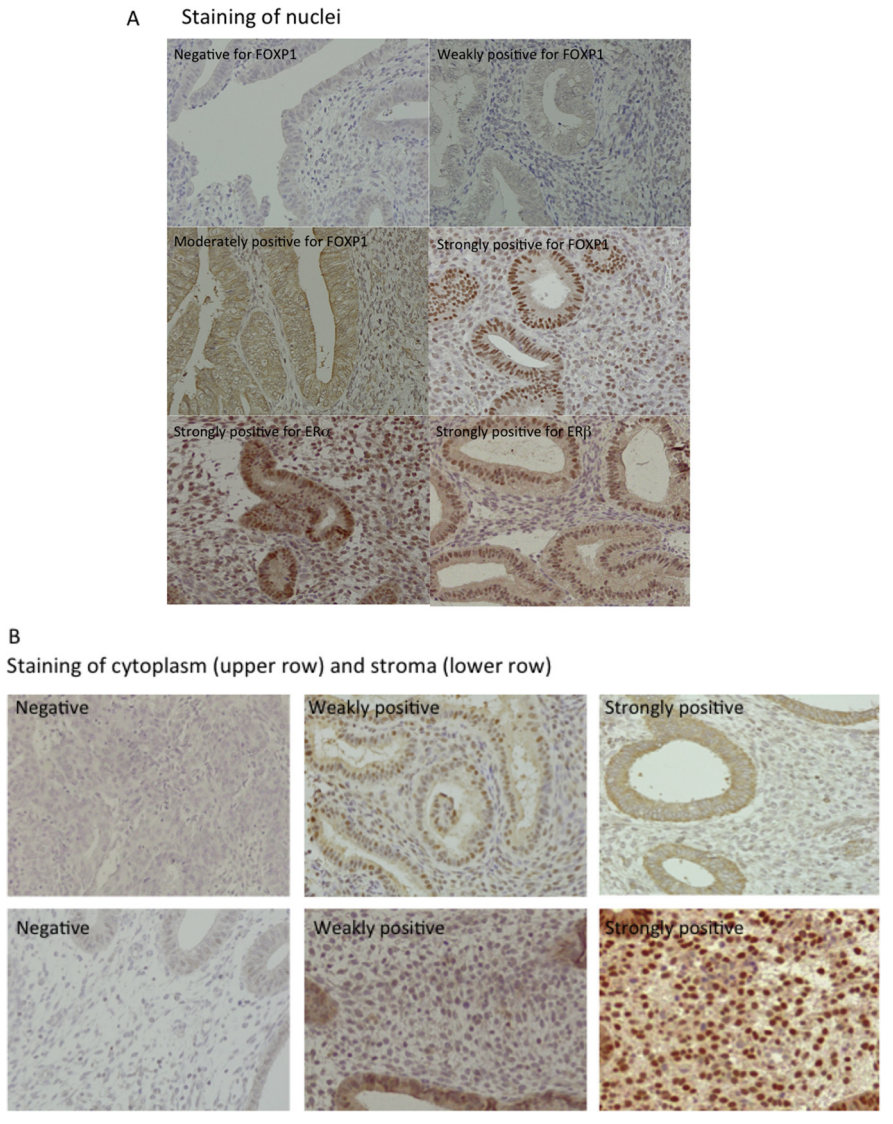


Fig. 1. Staining intensity of FOXP1, ERα and ERβ. **A.** Expression status of FOXP1, ERα and ERβ in nuclei. **B.** Expression status of FOXP1 in cytoplasm and stroma. Evaluation of ERα and ERβ expression was also performed by the same way as that of FOXP1 expression. Magnification of pictures of nuclei and cytoplasm is X200 and that of stroma pictures is X400.

Bioresources) [29] obtained from were also used. T HESCs, Ishikawa cells, and HEC-50B cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) at 37 °C in a water-saturated atmosphere with 5% CO2/95% air. These 3 cell lines were verified in writing as being endometrial in origin.

2.4. Culturing in the presence of estradiol

A culturing experiment was performed in the presence of estradiol (17β-E_2) (E7785; Sigma Aldrich, St. Louis, MO). T HESCs cells and normal endometrial stromal cells were stimulated with 2 concentrations of estradiol (100 pg/mL and

http://dx.doi.org/10.1016/j.heliyon.2016.e00116

10,000 pg/mL) for various periods of time (24, 48, and 72 h), and the same types of cells were stimulated with various concentrations of estradiol (10 pg/mL, 100 pg/mL, 1,000 pg/mL and 10,000 pg/mL) for 72 h. T HESCs were cultured for 72 h without estradiol to serve as the control.

2.5. Transfection of FOXP1 plasmid DNA with polyethylenimine

FOXP1Human cDNA ORF Clone (vector; pCMV6-AC-GFP, Tag; C-terminal TurbroGFP, RG216342, OriGene Technologies, Rockville, MD) was selected and amplified, and DNA was extracted using a Hipure Plasmid Filter Maxiprep kit (Invitrogen, Carlsbad, CA). Plasmid DNA was transfected into T HESCs, Ishikawa cells, and HEC-50B cells with polyethylenimine (PEI). PEI and DNA were mixed in a ratio of 3:1 and added to the cells so that the ratio of PEI to DNA would be 7.2 μg of PEI to 2.4 μg of DNA/ml. Before the mixture was added to the cells, it was vortexed and incubated for 15 min at room temperature. Twenty-four hours after the mixture was added, the culture medium was changed to RPMI 1640 medium supplemented with 10% FBS. For the control group, only Tris-EDTA buffer was mixed with PEI to dissolve DNA. The mixture was similarly added to the cells.

2.6. Assessment of green fluorescent protein expression

After incubation for 72 h, transfected cells in 10-cm dishes were evaluated for green fluorescent protein (GFP) expression using a laser scanning confocal microscope (BZ-X700; Keyence, Osaka, Japan), and transfection efficiency was assessed with a digital imaging analyzer (BZ-H3C; Keyence)

2.7. Transfection of FOXP1 siRNA

FOXP1 siRNA (sc-44583, Santa Cruz Biotechnology, Santa Cruz, CA) was transfected into T HESCs, Ishikawa cells, and HEC-50B cells according to the manufacturer's siRNA gene silencing protocol. In brief, 5 μl siRNA and 6 μl siRNA Transfection Regent (sc-29528, Santa Cruz Biotechnology) were mixed and added to the cells in siRNA Transfection Medium (sc-36868, Santa Cruz Biotechnology), and the cells were then cultured for 6 h. RPMI 1640 medium supplemented with 10% FBS was added and the mixture was incubated for 72 h. Control siRNA-A (sc-36869, Santa Cruz Biotechnology) instead of FOXP1 siRNA served as a control.

2.8. Western blot analysis

Protein samples (10 μg) were separated on a 12.5% sodium dodecyl sulfate polyacrylamide gel and loaded and run at 30 mA for 120 min. Samples were then blotted onto 0.45-μm nitrocellulose membranes (Catalog number 162–0114, Bio-Rad, Hercules, CA). The membranes were incubated overnight at 4 °C with the

http://dx.doi.org/10.1016/j.heliyon.2016.e00116

following diluted antibodies: anti-KRAS (Catalog number 60309-1-Ig, Proteintech, Chicago, IL) at 1:500, anti-PTEN (Catalog number 22034-1-AP, Proteintech) at 1:250, anti-FOXP1 (Catalog number ab16645, Abcam) at 1:500, anti-ERα (Catalog number; ab17087, Abcam) at 1:100 and anti-β-actin (Catalog number A5441, Sigma Aldrich) at 1:500. The membranes were then incubated with anti-mouse or rabbit IgG HRP-linked Antibody (Catalog number 7076, Cell Signaling Technology, Beverly, MA). Protein bands on the same membranes were visualized using enhanced chemiluminescence (ChemiDoc XRS, Bio-Rad) according to the manufacturer's protocol. Band intensity was analyzed with Molecular Imager, Image Lab Ver.3.0.1 (Bio-Rad).

2.9. Statistical analysis

All statistical analyses were performed using SPSS (version 21, SPSS Inc., Chicago, IL). Comparisons among the three groups were assessed using the one-way analysis of variance (ANOVA). Statistical significance was set at $P < 0.05$. A correlation between the staining scores for FOXP1, ER α, and ER β in the nuclei of cancer cells and in the glandular epithelium was analyzed using Spearman's correlation coefficient. Correlations were considered significant if $r > 0.4$ and $p < 0.05$.

3. Results

3.1. Patient characteristics

Clinical characteristics of patients are shown in Table 1. Grade 1 and grade 2 endometrioid adenocarcinoma were at a significantly earlier stage compared to grade 3 endometrioid adenocarcinoma ($P < 0.005$).

3.2. FOXP1 expression

Table 2 shows an expression rate of FOXP1 in the nuclei, cytoplasm and stroma of normal endometrium, atypical endometrial hyperplasia and endometrial adenocarcinoma, respectively. In the normal endometrium, the highest expression rate was seen in the nuclei at proliferative phase ($P < 0.05$ vs. secretory phase). In the endometrioid adenocarcinoma, FOXP1 was expressed in nuclei, cytoplasm and stroma, but the expression rate in the nuclei and cytoplasm was significantly reduced as the grade progresses ($P < 0.05$, respectively). FOXP1 expression in the cytoplasm of cells was significantly greater in atypical endometrial hyperplasia than that of endometrioid adenocarcimona at any grade ($P < 0.05$) (Table 2).

http://dx.doi.org/10.1016/j.heliyon.2016.e00116

Table 1. Patient characteristics.

Clinical factor		Normal endometrium		Atypical endometrial hyperplasia (n = 8)	Endometrial adenocarcinoma		
		Proliferative (n = 5)	Secretory (n = 3)		Grade 1 (n = 35)	Grade 2 (n = 13)	Grade 3 (n = 11)
Mean age		38.4 ± 3.9	32.3 ± 1.8	47.6 ± 7.3	54.3 ± 12.2	57.8 ± 11.7	53.9 ± 10.5
FIGO Stage	I/II				32 (94 %)*	10 (77 %)*	5 (45 %)
	III/IV				2 (6 %)*	3 (23 %)*	6 (55 %)

*$p < 0.005$ vs. grade 3 according to one-way ANOVA.

3.3. Correlation between the expression rate of FOXP1 and ERα or ERβ in nuclei

ERα and ERβ expressions in atypical endometrial hyperplasia and endometrioid adenocarcinoma are shown in Table 3. The expression of both receptors in the nuclei and cytoplasm differed significantly in atypical endometrial hyperplasia and all 3 grades of endometrioid adenocarcinoma (Table 3). In particular, ERβ expression decreased or lacked in endometrioid adenocarcinoma of any grade. The staining scores for FOXP1 and ERα in the nuclei were significantly correlated in atypical endometrial hyperplasia and grade 1, grade 2, and grade 3 endometrioid adenocarcinoma (Fig. 2A, $r = 0.501$, $p < 0.0005$; $r = 0.07$, $P < 0.01$). On the other hand, there was no significant correlation between expression of FOXP1 and ERβ in atypical endometrial hyperplasia and grade 1 and grade 2 of endometrioid adenocarcinoma. No significant correlation between expression of FOXP1, ERα, or ERβ was noted in the nuclei of cells from normal endometrium (Fig. 2A and B).

3.4. The effect of sustained stimulation with estradiol on ERα, FOXP1, KRAS, and PTEN expression

In the experiment in which T HESCs were cultured in the presence of estradiol, levels of expression of ERα, FOXP1, and KRAS increased depending on the concentration of estradiol and those levels also increased over time at estradiol concentrations of 100 pg/mL and 10,000 pg/mL (Fig. 3A and B). Although estradiol showed a slight increase in PTEN expression, the effects were not in a dose- or time-dependent manner (Fig. 3A and B).

3.5. Transfection efficiency of FOXP1

The levels of FOXP1 transfection were evaluated by determining the level of GFP expression. FOXP1 DNA was transfected at rates of 48% into T HESCs, 36% into Ishikawa cells, and 36% into HEC-50B cells, respectively.

http://dx.doi.org/10.1016/j.heliyon.2016.e00116

Table 2. The location of FOXP1 positive expression.

Location	Normal endometrium		p value*	Atypical endometrial hyperplasia (n = 8) (%)	Endometrial adenocarcinoma			p value*
	Proliferative phase (n = 5)(%)	Secretory phase (n = 3)(%)			Grade 1 (n = 35)(%)	Grade 2 (n = 13)(%)	Grade 3 (n = 11)(%)	
Nuclei	5 (100)	1 (33)	< 0.05	5 (63)	22 (66)	7 (54)	1 (9)	< 0.05
Cytoplasm	1 (20)	1 (33)	0.34	8 (100)	20 (57)	7 (54)	3 (27)	< 0.05
Stroma	3 (60)	2 (67)	0.44	4 (50)	10 (29)	5 (38)	2 (18)	0.46

*One-way ANOVA.

http://dx.doi.org/10.1016/j.heliyon.2016.e00116

Table 3. The location of ER α and ER β positive expression.

Location		Normal endometrium (n = 8) (%)	Atypical endometrial hyperplasia (n = 8) (%)	Endometrial adenocarcinoma			p value
				Grade 1 (n = 35) (%)	Grade 2 (n = 13) (%)	Grade 3 (n = 11) (%)	
ER α	Nuclei	8 (100)	6 (75)*	14 (40)	4 (31)	1 (9)	< 0.05
	Cytoplasm	8 (100)	7 (88)*	14 (40)	2 (15)	3 (27)	< 0.01
	Stromal	3 (38)	1 (13)	16 (46)	6 (46)	3 (27)	0.27
ER β	Nuclei	3 (38)	3 (38)*	2 (6)	2 (15)	0 (0)	< 0.05
	Cytoplasm	7 (88)	7 (88)*	3 (9)	1 (8)	0 (0)	< 0.01
	Stromal	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	

*p value vs. endometrial adenocarcinoma according to one-way ANOVA.

3.6. Effect of FOXP1 transfection on KRAS and PTEN expression

As shown in Fig. 4, all cell lines expressed FOXP1, KRAS and PTEN at various levels and the transfection of FOXP1 DNA increased the level of expression of FOXP1 protein by 6 to 100 folds by densitometer measurement (Fig. 4A). FOXP1 transfection induced expression of KRAS in all cell lines. However, FOXP1 transfection suppressed the expression of PTEN protein in T HESCs, while FOXP1 transfection showed no significant changes in PTEN expression in Ishikawa and HEC-50B cells (Fig. 4A). Meanwhile, the extra size of FOXP1 protein in Ishikawa cells transfected with FOXP1 gene may be a protein modified by glycosylation or phosphorylation.

The expression of FOXP1 protein decreased in all cells transfected with FOXP1 siRNA in comparison to levels in the controls (Fig. 4B). Expression of KRAS protein decreased in all cells transfected with FOXP1 siRNA in comparison to levels in the controls. However, levels of KRAS protein were slightly altered in T HESCs and HEC-50B cells transfected with FOXP1 siRNA (Fig. 4B). In contrast, the levels of expression of the PTEN protein in all cell lines transfected with FOXP1 siRNA did not change in comparison to levels in the controls (Fig. 4B).

3.7. Positive association between the expression levels of KRAS and FOXP1 as well as ERα in endometrioid adenocarcinoma tissues

As a next stage, association between the expression levels of KRAS and FOXP1 as well as ERα in endometrioid adenocarcinomas was immunohistochemically investigated. Immunohistochemistry showed that KRAS protein was mainly expressed in cytoplasm of cancer cell (Fig. 5). Significantly positive association

http://dx.doi.org/10.1016/j.heliyon.2016.e00116

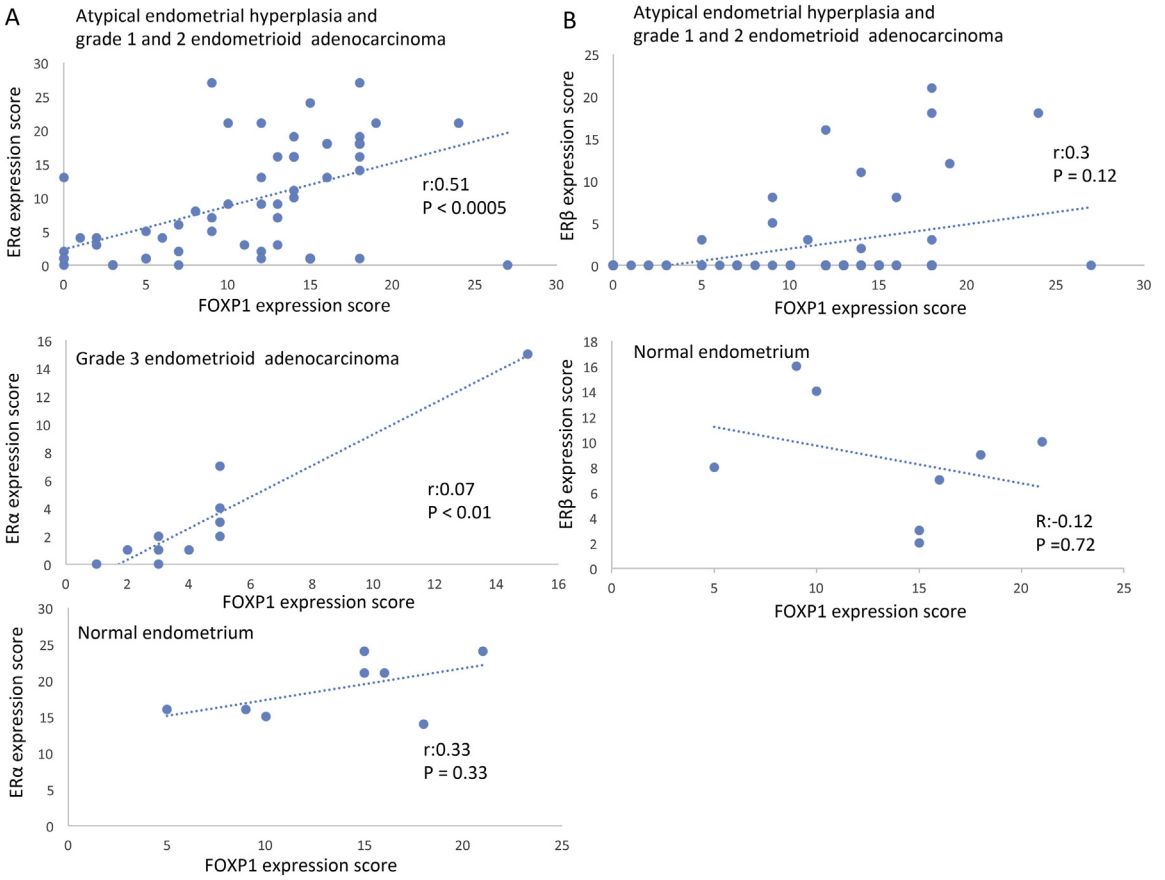


Fig. 2. The correlation between the expressions of FOXP1 and ERα or ERβ in the nuclei. **A.** Correlations between FOXP1 and ERα in grade 1 and grade 2 endometrioid adenocarcinoma and atypical endometrial hyperplasia, in grade 3 endometrioid adenocarcinoma, and in normal endometrium. **B.** Correlations between FOXP1 and ERβ in grade 1 and grade 2 endometrioid adenocarcinoma and atypical endometrial hyperplasia, and in normal endometrium.

was determined in expression levels between KRAS and FOXP1 as well as ERα in cancer tissues (Fig. 5).

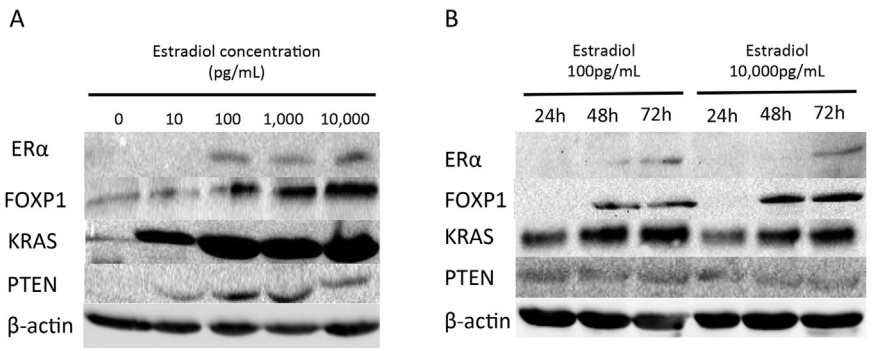
3.8. Effect of estradiol stimulation in FOXP1-knockdown cells on KRAS expression

Finally, effect of estradiol stimulation on KRAS expression level was examined in T HESCs transfected with FOXP1 siRNA. Estradiol did not alter the expression level of KRAS in cells transfected with FOXP1 siRNA in a dose-dependent manner (Fig. 6).

4. Discussion

In this study, levels of FOXP1 expression were significantly correlated with levels of ERα expression in atypical endometrial hyperplasia and all 3 grades of

http://dx.doi.org/10.1016/j.heliyon.2016.e00116



ERα/ β-actin	0.001	0.002	0.082	0.084	0.095
FOXP1/ β-actin	0.012	0.052	0.058	0.098	0.20
KRAS/ β-actin	0.112	1.06	1.88	1.95	2.36
PTEN/ β-actin	0.009	0.041	0.115	0.102	0.082

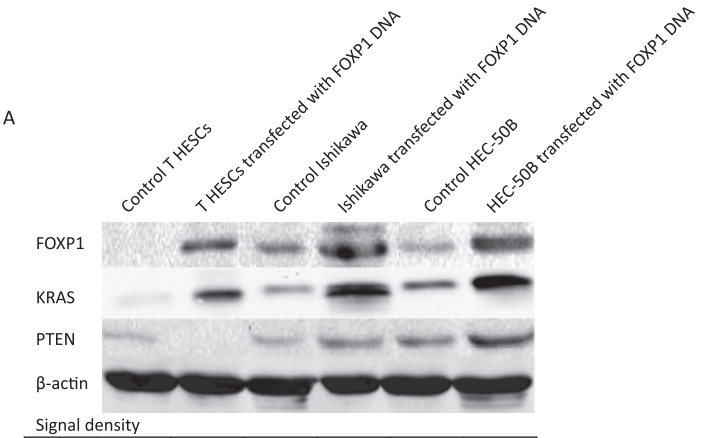
ERα/ β-actin	0.009	0.028	0.075	0.009	0.012	0.091
FOXP1/ β-actin	0.001	0.088	0.092	0.003	0.20	0.25
KRAS/ β-actin	0.52	0.90	1.19	0.48	0.88	1.12
PTEN/ β-actin	0.044	0.043	0.041	0.035	0.027	0.025

Fig. 3. Expression of FOXP1, ERα, KRAS and PTEN in T HESCs cells at being stimulated with estradiol. **A.** Stimulation with different concentrations of estradiol. Levels of expression of ERα, FOXP1, and KRAS increased in accordance with the concentrations of estradiol. **B.** Stimulation with an estradiol concentration of 100 pg/mL and 10,000 pg/mL for different periods of time. Levels of expression of ERα, FOXP1, and KRAS increased over time. Full, unmodified images of this figure are available as Supplementary Material.

endometrioid adenocarcinoma. Moreover, levels of KRAS expression changed in accordance with levels of FOXP1 expression in endometrial adenocarcinoma cells as well as in normal stromal cells. Estradiol increased levels of FOXP1 and KRAS expression in a time- and dose-dependent manner in T HESCs cells that abundantly expressed ERα. These findings indicate that FOXP1 might act as an oncogene through activation of KRAS in atypical glandular epithelium and the microenvironment around the endometrium.

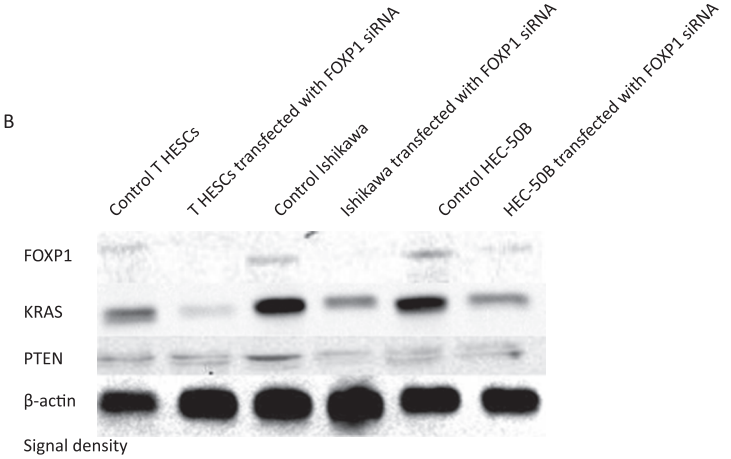
A previous study has shown that loss of expression and nuclear/cytoplasmic localization of FOXP1 expression are common events in early endometrial cancer [17]. FOXP1 expression in the nuclei of cells decreased from 0 to 20% and FOXP1 was highly expressed in the cytoplasm of cells in early endometrial cancer [17]. The current study found that FOXP1 expression in the nuclei of cells was significantly greater in atypical endometrial hyperplasia and in well and moderately differentiated endometrioid adenocarcinoma than that in poorly differentiated endometrioid adenocarcinoma. Expression of FOXP1 in the cytoplasm was greater in atypical endometrial hyperplasia than that in all 3 grades of endometrioid adenocarcinoma. These results suggest that a high level of FOXP1 expression may be an early event in the pathogenesis of an endometrial neoplasm. FOXP1 expression in the nuclei of cells was significantly associated with the expression of ERα. The association between FOXP1 expression in the nuclei of cells and ERα expression is biologically significant since FOXP1 in the nuclei of

http://dx.doi.org/10.1016/j.heliyon.2016.e00116



Signal density

FOXP1/ β-actin	0.001	0.104	0.062	0.398	0.049	0.486
KRAS/ β-actin	0.08	0.48	0.20	0.85	0.33	0.90
PTEN/ β-actin	0.01	0.001	0.031	0.039	0.041	0.112



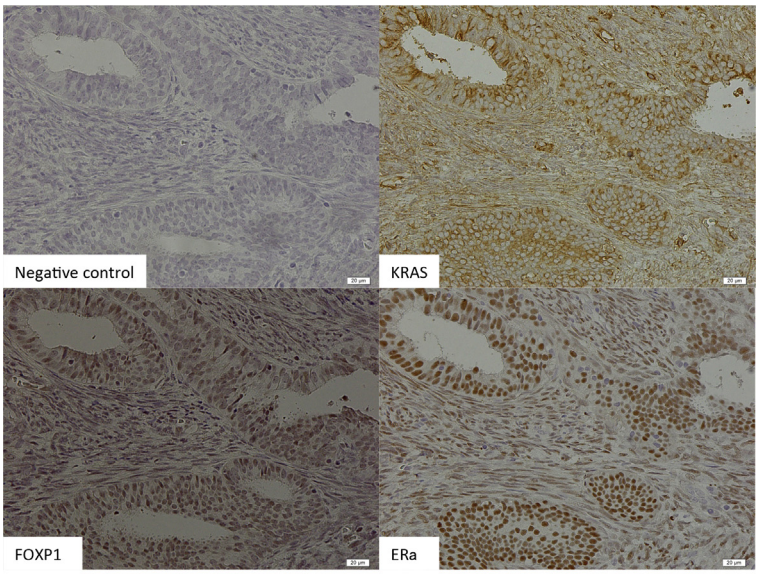
Signal density

FOXP1/ β-actin	0.022	0.001	0.048	0.001	0.054	0.009
KRAS/ β-actin	0.105	0.009	0.42	0.066	0.45	0.082
PTEN/ β-actin	0.031	0.034	0.066	0.030	0.032	0.033

Fig. 4. Altered expression of KRAS in cells transfected with FOXP1 DNA or siRNA. **A.** Up-regulation of KRAS protein in FOXP1-transfected T HESCs, Ishikawa cells and HEC-50B cells. **B.** Down-regulation of KRAS protein in siRNA FOXP1-transfected T HESCs, Ishikawa cells and HEC-50B cells. Full, unmodified images of this figure are available as Supplementary Material.

cells may act as a coregulator of ERα [17] or FOXP1 may be a downstream target of ERα. In the current study, expression levels of ERα in T HESCs cells transfected with FOXP1 DNA or siRNA did not altered (data not shown). On the other hand, cytoplasmic expression of FOXP1 was significantly associated with HIF-1α overexpression and also linked with deep myometrial invasion and with

http://dx.doi.org/10.1016/j.heliyon.2016.e00116



	No. of Patients	KRAS Positive expression	X^2	P value
Nuclear FOXP1				
Positive	30	18 (60.0%)	9.60	0.0019
Negative	29	2 (6.9%)		
Nuclear ERa				
Positive	19	12 (63.2%)	4.84	0.028
Negative	40	8 (20.0%)		

Fig. 5. Relationship in expression levels between KRAS and FOXP1 as well as ERα in endometrioid adenocarcinoma tissues. Note that KRAS protein was mainly expressed in cytoplasm of cancer cell. Magnification of pictures is X200.

poor differentiation in endometrial cancer [17]. Thus, further research is warranted to confirm the exact role of cytoplasmic expression of FOXP1.

Sustained estrogen stimulation of endometrium is considered to be a risk factor of developing type I endometrial cancer [7, 8, 30]. There is sufficient evidence that unopposed estrogen therapy to postmenopausal women with intact uterus significantly increased the risk of endometrial cancer [31]. In general, it is thought that endometrial cancer develops from atypical endometrial hyperplasia, a precursor lesion of endometrial cancer [32]. Genetic abnormalities or mutations such as p53 mutations, KRAS mutations, phosphatidylinositol 3-kinase (PI3 K) mutations, abnormal PTEN expression, and accumulation of β catenin are known to be involved in malignant transformation of normal endometrium [33, 34]. In

http://dx.doi.org/10.1016/j.heliyon.2016.e00116

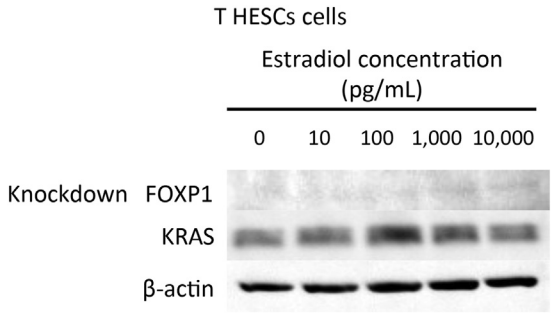


Fig. 6. Effect of estradiol stimulation in FOXP1-knockdown cells on KRAS expression. T HESCs cells transfected with FOXP1 siRNA were stimulated with various concentrations of estradiol for 72 h. Estradiol stimulation did not affect KRAS expression in FOXP1 knockdown T HESCs cells. Full, unmodified images of this figure are available as Supplementary Material.

addition, various abnormalities at the chromosomal level, such as microsatellite instability and chromosomal instability i.e. genomic instability, are known to be present during oncogenesis. These conditions are noted in the early stages of atypical endometrial hyperplasia. In this study, we focused on estrogen-dependent KRAS signaling, since it has been shown that mutant KRAS constantly phosphorylates proteins downstream and activates a signaling cascade [35]. Activation of KRAS induces cell proliferation and growth and inhibits apoptosis [36]. Abnormal activation of the Ras pathway occurs in about 40% of endometrial cancers [37]. KRAS mutations are reported to induce the development of estrogen-dependent tumors [35]. The present study showed that estrogen stimulated KRAS expression and that FOXP1 transfection induced KRAS expression in a dose- and time-dependent manner in in vitro culture system using T HESCs, Ishikawa and HEC-50B cell lines, suggesting that an oncogenic action of estrogen on endometrium is mediated through FOXP1 activation. A high dose of estradiol in FOXP1 induction assay was adopted in this study because 10 to 10,000 pg/ml concentrations of estradiol were used to explore the ability of estradiol to control cAMP and cGMP production by human granulosa cells in culture [38]. Meanwhile, KRAS expression is affected by epidermal growth factor receptor (EGFR) signal transduction. Recent report showed that silencing of FOXP1 inhibits EGFR-dependent tumor growth and, conversely, de-repression of FOXP1 increases tumorigenicity [39], indicating genomic interaction between FOXP1 and KRAS.

However, PTEN also plays a key role in the development of type I endometrial cancer [32]. PTEN has a variety of cancer-inhibiting capabilities, e.g. it arrests the cell cycle, it induces apoptosis, it increases the stability of P53, and it enhances transcriptional activation by p53 [40]. A dysfunction in PTEN leads to the development and progression of cancer, and a somatic mutation in PTEN is noted in 83% of type I endometrial cancers [41]. Although PTEN was induced by estrogen administration and FOXP1 transfection reduced PTEN expression in T

http://dx.doi.org/10.1016/j.heliyon.2016.e00116

HESCs cells, no such effects were observed in Ishikawa or HEC-50B, suggesting FOXP1 is unlikely involved in the dysfunction in PTEN. Li et al. reported that stromal cells of endometrial carcinoma promoted proliferation of epithelial cells through the HGF/c-Met/Akt signaling pathway [7]. PTEN belongs to Akt signaling pathway [42]. Tumor microenvioronment participates in the endometrial carcinoma pathogenesis [7]. Since T HESCs cells are derived from a normal stromal cell, the discrepant result in this study might be led by biological characterization of the cells between stromal and cancerous tissues. Thus, other factors are involved in inducing abnormalities in PTEN, and these factors need to be studied further.

Neoplastic changes in the endometrium begin with hyperplasia of the endometrium, and they lead to atypical endometrial hyperplasia, and then they progress to the accumulation of abnormalities in various genes such as KRAS and PTEN. Estrogen stimulation is known to be one of putative factors that are responsible for mutations in KRAS. However, the current findings suggest that FOXP1 activates the KRAS pathway, thus playing a role in the pathogenesis of endometrial cancer.

Declarations

Author contribution statement

Makito Mizunuma, Yoshihito Yokoyama: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Masayuki Futagami, Jun Watanabe: Performed the experiments; Analyzed and interpreted the data.

Kayo Horie, Hideki Mizunuma: Conceived and designed the experiments; Analyzed and interpreted the data.

Competing interest statement

The authors declare no conflict of interest.

Funding statement

This work was supported by a Grant-in-Aid for Cancer Research from the Ministry of Education, Culture, Sports, Science and Technology (Tokyo, Japan) to Yoshihito Yokoyama (26462508).

Additional information

Supplementary content related to this article has been published online at http://dx.doi.org/10.1016/j.heliyon.2016.e00116

Acknowledgements

The authors thank Emiko Mizuki, M.T., Tomoko Akaishi, M.T. and Emiko Mikami, M.T. for experimental assistance.

References

[1] R. Siegel, D. Naishadham, A. Jemal, Cancer Statistics, 2013, CA Cancer J. Clin. 63 (2013) 11–30.

[2] J.V. Bokhman, Two pathogenetic types of endometrial carcinoma, Gynecol. Oncol. 15 (1983) 10–17.

[3] H. Sun, T. Enomoto, M. Fujita, H. Wada, K. Yoshino, K. Ozaki, et al., Mutational analysis of the PTEN gene in endometrial carcinoma and hyperplasia, Am. J. Clin. Pathol. 115 (2001) 32–38.

[4] G.E. Hale, C.L. Hughes, J.M. Cline, Endometrial cancer: hormonal factors the perimenopausal 'window of risk', and isoflavones, J. Clin. Endocrinol. Metab. 87 (2002) 3–15.

[5] D.A. Garcia-Dios, D. Lambechts, L. Coenegrachts, I. Vandenput, A. Capoen, P.M. Webb, et al., High-throughput interrogation of PIK3CA, PTEN, KRAS, FBWX7 and TP53 mutations in primary endometrial carcinoma, Gynecol. Oncol. 128 (2013) 327–334.

[6] L.J. Lee, E. Ratner, M. Uduman, K. Winter, M. Boeke, K.M. Greven, et al., The KRAS-Variant and miRNA Expression in RTOG Endometrial Cancer Clinical Trials 9708 and 9905, PloS One 9 (2014) e94167.

[7] M. Li, X. Xin, T. Wu, T. Hua, H. Wang, H. Wang, Stromal cells of endometrial carcinoma promotes proliferation of epithelial cells through the HGF/c-Met/Akt signaling pathway, Tumor Biol. 36 (2015) 6239–6248.

[8] M. Matsumoto, Y. Yamaguchi, Y. Seino, A. Hatakeyama, H. Takei, H. Niikura, et al., Estrogen signaling ability in human endometrial cancer through the cancer-stromal interaction, Endocr. Relat. Cancer 15 (2008) 451–463.

[9] N. Takahashi-Shiga, H. Utsunomiya, Y. Miki, S. Nagase, R. Kobayashi, M. Matsumoto, et al., Local Biosynthesis of Estrogen in Human Endometrial Carcinoma through Tumor-Stromal Cell Interactions, Clin. Cancer Res. 15 (2009) 6028–6034.

[10] Z. Hu, L. Zhu, M. Tan, M. Cai, L. Deng, G. Yu, et al., The expression and correlation between the transcription factor FOXP1 and estrogen receptors in epithelial ovarian cancer, Biochimie 109 (2015) 42–48.

 http://dx.doi.org/10.1016/j.heliyon.2016.e00116

[11] T. Shigekawa, N. Ijichi, K. Ikeda, K. Horie-Inoue, C. Shimizu, S. Saji, et al., FOXP1 an estrogen-inducible transcription factor, modulates cell proliferation in breast cancer cells and 5-year recurrence-free survival of patients with tamoxifen-treated breast cancer, Horm. Cancer 2 (2011) 286–297.

[12] M.A. Augello, T.E. Hickey, K.E. Knudsen, FOXA1: master of steroid receptor function in cancer, EMBO J. 30 (2011) 3885–3894.

[13] N. Ijichi, K. Ikeda, K. Horie-Inoue, S. Inoue, FOXP1 and estrogen signaling in breast cancer, Vitam. Horm. 93 (2013) 203–212.

[14] M. Rayoo, M. Yan, E.A. Takano, G.J. Bates, P.J. Brown, A.H. Banham, et al., Expression of the forkhead box transcription factor FOXP1 is associated with oestrogen receptor alpha, oestrogen receptor beta and improved survival in familial breast cancers, J. Clin. Pathol. 62 (2009) 896–902.

[15] G.J. Bates, S.B. Fox, C. Han, R. Launchbury, R.D. Leek, A.L. Harris, et al., Expression of the forkhead transcription factor FOXP1 is associated with that of estrogen receptor-beta in primary invasive breast carcinomas, Breast Cancer Res. Treat. 111 (2008) 453–459.

[16] A.H. Banham, N. Beasley, E. Campo, P.L. Fernandez, C. Fidler, K. Gatter, et al., The FOXP1 winged helix transcription factor is a novel candidate tumor suppressor gene on chromosome 3p, Cancer Res. 61 (2001) 8820–8829.

[17] A. Giatromanolaki, M.I. Koukourakis, E. Sivridis, K.C. Gatter, A.L. Harris, A.H. Banham, Loss of expression and nuclear/cytoplasmic localization of the FOXP1 forkhead transcription factor are common events in early endometrial cancer: relationship with estrogen receptors and HIF-1α expression, Mod. Pathol. 19 (2006) 9–16.

[18] L. Fu, J.E. Girling, P.A. Rogers, Expression of Fox Head Protein 1 in Human Eutopic Endometrium and Endometriosis, Reprod. Sci. 15 (2008) 243–252.

[19] L. Wlodarska, E. Veyt, P. De Paepe, P. Vandenberghe, P. Nooijen, I. Theate, et al., FOXP1, a gene highly expressed in a subset of diffuse large B-cell lymphoma, is recurrently targeted by genomic aberrations, Leukemia 19 (2005) 1299–1305.

[20] S. Hoeller, A. Schneider, E. Haralambieva, S. Dirnhofer, A. Tzankov, FOXP1 protein overexpression is associated with inferior outcome in nodal diffuse large B-cell lymphomas with non-germinal centre phenotype independent of gains and structural aberrations at 3p14.1, Histopathology 57 (2010) 73–80.

 http://dx.doi.org/10.1016/j.heliyon.2016.e00116

[21] Y. Zhang, S. Zhang, X. Wang, I.J. Liu, L. Yang, S. He, et al., Prognostic significance of FOXP1 as an oncogene in hepatocellular carcinoma, J. Clin. Pathol. 65 (2012) 528–533.

[22] J. Qin, Y. Xu, X. Li, Y. Wu, J. Zhou, G. Wang, et al., Effects of lentiviral-mediated Foxp1 and Foxq1 RNAi on the hepatocarcinoma cell, Exp. Mol. Pathol. 96 (2014) 1–8.

[23] H.B. Koon, G.C. Ippolito, A.H. Banham, P.W. Tucker, FOXP1: a potential therapeutic target in cancer, Expert Opin. Ther. Targets 11 (2007) 955–965.

[24] S.B. Fox, P. Brown, C. Han, S. Ashe, R.D. Leek, A.L. Harris, et al., Expression of the forkhead transcription factor FOXP1 is associated with estrogen receptor α and improved survival in primary human breast carcinomas, Clin. Cancer Res. 10 (2004) 3521–3527.

[25] K. Takayama, K. Horie-Inoue, K. Ikeda, T. Urano, K. Murakami, Y. Hayashizaki, et al., FOXP1 is an androgen-responsive transcription factor that negatively regulates androgen receptor signaling in prostate cancer cells, Biochem. Biophys. Res. Commun. 374 (2008) 388–393.

[26] R.W. Noyes, A.T. Hertig, J. Rock, Dating the endometrial biopsy, Am. J. Obstet. Gynecol. 122 (1975) 262–263.

[27] Y. Zhang, S. Zhang, X. Wang, J. Liu, L. Yang, S. He, et al., Prognostic significance of FOXP1 as an oncogene in hepatocellular carcinoma, J. Clin. Pathol. 65 (2012) 528–533.

[28] M. Nishida, K. Kasahara, A. Oki, T. Satoh, Y. Arai, T. Kubo, Establishment of eighteen clones of Ishikawa cells, Hum. Cell 9 (1996) 109–116.

[29] H. Kuramoto, M. Nishida, T. Morisawa, M. Hamano, H. Hata, Y. Kato, et al., Establishment and characterization of human endometrial cancer cell lines, Ann N.Y. Acad. Sci 622 (1991) 402–421.

[30] H.I. Kim, T.H. Kim, J.M. Lim, J.W. Jeong, Steroid hormone intervenes in the endometrial tumorigenesis of PTEN ablation, J. Cancer Prev. 18 (2013) 313–321.

[31] D. Grady, T. Gebretsadik, K. Kerlikowske, V. Ernster, D. Petitti, Hormone replacement therapy and endometrial cancer risk: a meta-analysis, Obstet. Gynecol. 85 (1995) 304–313.

[32] H. Gbelcová, P. Bakeš, P. Priščáková, V. Šišovský, I. Hojsíková, Ľ. Straka, et al., PTEN sequence analysis in endometrial hyperplasia and endometrial carcinoma in Slovak women, Anal. Cell Pathol. (2015) 746856.

 http://dx.doi.org/10.1016/j.heliyon.2016.e00116

[33] V. Chandra, I. Fatima, M. Manohar, P. Popli, V.K. Sirohi, M.K. Hussain, et al., Inhibitory effect of 2- (piperidinoethoxyphenyl)- 3- (4-hydroxyphenyl)- 2H- benzo(b)pyran(K-1) on human primary endometrial hyperplasial cells mediated via combined suppression of Wnt/β-catenin signaling and PI3 K/Akt survival pathway, Cell Death Dis. 5 (2014) e1380.

[34] Y.P. Geels, L.J. van der Putten, A.A. van Tilborg, I. Lurkin, E.C. Zwarthoff, J.M. Pijnenborg, et al., Immunohistochemical and genetic profiles of endometrioid endometrial carcinoma arising from atrophic endometrium, Gynecol. Oncol. 137 (2015) 245–251.

[35] K. Kato, T. Sakamoto, N. Wake, Requirement of estrogen receptor expression and function for [12Val] K-Ras-mediated NIH3T3 cell transformation, Oncology 55 (Suppl 1) (1998) 45–52.

[36] T. Enomoto, M. Inoue, A.O. Perantoni, G.S. Buzard, H. Miki, O. Tanizawa, et al., K-ras activation in premalignant and malignant epithelial lesions of the human uterus, Cancer Res. 51 (1991) 5308–5314.

[37] D.M. Boruta , P.A. Gehrig, A.N. Fader, A.B. Olawaiye, Management of women with uterine papillary serous cancer: a Society of Gynecologic Oncology (SGO) review, Gynecol. Oncol. 115 (2009) 142–153.

[38] A.V. Sirotkin, J. Mlyncek, J. Lavrincik, J. Bulla, L. Hetenyi, The ability of steroid hormones to control cAMP and cGMP production by human granulosa cells in culture, Cell Signal. 7 (1995) 61–65.

[39] G.G. Gomez, S. Volinia, C.M. Croce, C. Zanca, M. Li, et al., Emnett RSuppression of microRNA-9 by mutant EGFR signaling upregulates FOXP1 to enhance glioblastoma tumorigenicity, Cancer Res. 74 (2014) 1429–1439.

[40] A. Doll, M. Abal, M. Rigau, M. Monge, M. Gonzalez, S. Demajo, et al., Novel molecular profiles of endometrial cancer-new light through old windows, J. Steroid Biochem. Mol. Biol. 108 (2008) 221–229.

[41] G.L. Mutter, M.C. Lin, J.T. Fitzgerald, J.B. Kum, J.P. Baak, J.A. Lees, et al., Altered PTEN expression as a diagnostic marker for the earliest endometrial precancers, J. Natl. Cancer Inst. 92 (2000) 924–930.

[42] A. Carnero, C. Blanco-Aparicio, O. Renner, W. Link, J.F. Leal, The PTEN/PI3 K/AKT signalling pathway in cancer: therapeutic implications, Curr. Cancer Drug Targets 8 (2008) 187–198.

 http://dx.doi.org/10.1016/j.heliyon.2016.e00116