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Does Pten have an impact on oogenesis of PCOS mouse models?

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Research Article

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Summary

Polycystic ovary syndrome (PCOS) is a complex disorder in which the aetiology is still not explained very well. The PI3K/PTEN (phosphatidylinositol 3-kinase/phosphatase and tensin homolog deleted on chromosome 10) pathway is an important pathway that is involved in many mechanisms, including proliferation, growth and motility. PTEN plays a role in granulosa cell proliferation and regulates the differentiation process. The aim of this study was to investigate the expression levels of *Pten* and *Pik3ca* in PCOS mouse models with and without any treatment procedures. Three groups of mouse models, PCOS, a PCOS group with clomiphene citrate treatment, and a PCOS group with the combination of clomiphene citrate, metformin and pioglitazone treatment, were established. Ovarian tissues, which were obtained from these groups and a control group with no PCOS, were embedded in paraffin and RNA was extracted. cDNA was synthesized and real-time PCR was conducted to evaluate the expression levels of *Pten* and *Pik3ca*. The results of this study showed that both *Pten* and *Pik3ca* genes were expressed in the ovarian tissues from the mouse models. Although one-way analysis of variance results showed that *Pten* was expressed significantly differently in the samples, individual Student's *t*-tests did not show any significantly different expression levels in each group. This study is important as it shows the expression patterns of two genes in PCOS mouse models with different treatment strategies, including clomiphene citrate, metformin and pioglitazone. The results of this study formed the basis of research studies and investigations into different genes within the PTEN pathway, as well as other pathways that are under investigation.

Introduction

The mammalian ovaries are composed of follicles at different developmental stages. The first stage of follicular growth is gonadotropin independent, in which the follicle forms the primary and secondary follicle from primordial follicles. The second stage of follicle development is responsive to gonadotropins, in which the pre-antral follicles develop into the early antral follicles. The final stage of follicular development is gonadotropin dependent, in which one follicle is recruited and completes development, resulting in ovulation (McNatty *et al*., 2007). Follicle-stimulating hormone (FSH) has been reported to stimulate early follicle development with granulosa cell proliferation and differentiation. At this stage, the successful development of the oocyte depends on communication with the granulosa cells (Banerjee *et al*., 2014). The theca cells have a major role in steroidogenesis. The expression of genes involved in apoptosis, survival as well as genes, such as the tumour suppressor genes, are involved in the regulation of the viability of the granulosa cells and therefore the follicles. There are many pathways involved in the regulation of follicular fate, such as the phosphatidylinositol 3-kinase (PI3K) pathway (Reddy *et al*., 2005). The PI3K pathway has been reported to be activated by FSH signalling and is further involved in the regulation of B-cell lymphoma 2 (BCL2) proteins (Alam *et al*., 2004). The PI3K/PTEN (PI3K/phosphatase and tensin homolog deleted on chromosome 10) pathway functions in normal metabolic events, such as cell proliferation, growth and motility. Phophatidylinositol-4,5-bisphosphate (PIP_2) phosphorylation is catalyzed by PI3K and enables the conversion into phophatidylinositol-3,4,5-bisphosphate (PIP_3). PTEN negatively regulates PI3K enabling the conversion into PIP_2 from PIP_3. PI3K/PTEN, as well as the AKT signalling pathway, play an important role in the development of primordial follicles (Markholt *et al*., 2012). One of the underlying mechanisms of premature ovarian failure has been suggested to be through the Pten and PI3K signalling pathways. PTEN has been shown to regulate the proliferation of granulosa cells and, furthermore, to regulate the differentiation process of these cells (Goto *et al*., 2007). Previously published studies have reported that deletion of *Pten* in oocyte-specific patterns led to the overactivation of PI3K signalling, leading to the activation prematurely of the whole group of primordial follicles. This resulted in the depletion of primordial follicles during adulthood, leading to premature ovarian failure (Reddy *et al*., 2008). Although the deletion of *Pten* at the earlier stages resulted in such a drastic outcome,

Pten deletion in oocytes from the primary and more developed follicles did not cause oocyte maturation variations and fertility was not compromised (Jagarlamudi *et al.*, 2009). Therefore, it is possible that PI3K/PTEN functions and affects folliculogenesis in a developmental phase pattern. Inhibitors of PTEN were shown to enable primordial follicle development leading to the production of mature oocytes with fertile mice (Li *et al.*, 2010; Adhikari *et al.*, 2012).

In this study, we aimed to investigate the expression levels of Pten and Pik3ca in mouse models. The mouse models included a control group with normal morphology and normal parameters of ovaries. The study groups included PCOS, the PCOS group with clomiphene citrate treatment and the PCOS group with clomiphene citrate, metformin and pioglitazone treatment.

Materials and methods

Funding was received by Near East University (SAG-2019-1-038). The PCOS mouse models were established at Manisa Celal Bayar University and ethical approval was obtained by the same university (77.637.435-42). The PCOS models were generated as described previously (Tulay *et al.*, 2022). Briefly, female prepubertal BALB/c mice were used to generate the models. In total, four groups were obtained. The first group was the control group consisting of non-PCOS mice. For groups 2, 3 and 4, all mice were induced with dehydroepiandrosterone (DHEA) to develop PCOS (Merck Millipore) (6 mg/100 g) dissolved in 0.01 ml of 96% ethanol and mixed with 0.09 ml sesame oil (total volume: 0.1 ml) for injection on 20 days consecutively. Body weight was determined each day and the administration of DHEA was adjusted accordingly. Group 2 was the PCOS control group and the mice did not receive any treatment. The third group was treated with clomiphene citrate (CC; 1.5 mg/kg), dissolved in 0.2 ml saline, which was administrated by gavage for 5 days consecutively. The final group was treated with clomiphene citrate (0.15 mg/100 g), metformin (1.2 mg/100 g) and pioglitazone (0.02 mg/100 g) dissolved in 0.2 ml saline, which was injected by gavage for 5 days consecutively. Blood glucose levels were determined by glucose tolerance test using a glucometer following fasting of the mice for 6 h (Accu-Check, Roche) prior to intraperitoneal 2 g/kg of body weight glucose injection at 15, 60 and 120 min (Kauffman *et al.*, 2015).

Cytological and histochemical analysis

The oestrous cycle was determined by analysis of vaginal smear samples at the beginning and end of the treatment with DHEA. Haematoxylin and eosin staining was used to evaluate the oestrous cycle under a light microscope (Olympus BX43, Tokyo, Japan). The proestrus cycle was determined by cytological analysis of the smear samples and by identifying the nucleated and some cornified epithelial cells. When the majority of the cells were cornified, this was identified as the oestrous cycle. The metestrus cycle was classified when smears consisted of mostly leukocytes and some cornified epithelial cells. When leukocytes with a small number of nucleated epithelial cells were observed, this was classified as the diestrous cycle. The ovarian samples were also evaluated to analyze the follicles in the cortex and stroma of the medulla using a light microscope (Olympus BX-43, Tokyo, Japan).

Gene expression analysis

Total RNA was extracted from ovarian samples fixed in paraffin blocks using the FFPE RNA Purification kit (Norgen, Biotek Corporation). The quality and concentration of the RNA were analyzed using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA). Reverse transcription was conducted using the Transcriptor First Strand cDNA Synthesis Kit (Roche, USA). cDNA was synthesized using random hexamers as well as anchored-oligo(dT)18 primers. Real-time PCR (SYBR Green The LightCycler® 480, USA) was performed to investigate the expression levels of *Pten*, *Pik3* and *Pik3ca*. The primers were designed using the Primer3 tool to flank two exonic junctions to avoid any DNA amplification (Untergasser *et al.*, 2012) (Table 1). The annealing temperature for all the primers was 55°C. A negative control was used with each run. The C_t values for each gene were obtained using Rotar Gene software (Qiagen) (Table 2). The level of gene expression was normalized against the *Actb* housekeeping gene. The fold changes were calculated using the comparative ΔΔCq (Tulay *et al.*, 2015).

Table 1. Primer sequences. Table shows the forward (F) and reverse (R) sequences of the primers

Gene names	Primer sequences
Pten	F: GATTACAGACCCGTGGCACT
	R: TGGCTGAGGGAACTCAAAGT
Pi3ca	F: GCCATCAGGAAAAAGACTCG
	R: CGGCAGCTGAGAGTATAGGC

Statistical analysis

Statistical analysis was performed to analyze the differences in the expression levels of genes in each mouse model using analysis of variance (ANOVA) test followed by Dunnett's correction. Two-tailed Student's *t*-test was performed to compare the expression level of each gene in the models. GraphPad Prism v8 software was used for all the evaluations.

Results

In this study, PCOS mouse models were successfully generated and different treatment strategies were applied to evaluate the effect on the expression levels of genes involved in the negative feedback mechanism of the PI3K signalling pathway. Overall, it was observed that the ovarian samples obtained from the control group (non-PCOS) preserved follicles were of different sizes as expected. In more detail, follicles at different stages, primordial, primary, secondary and tertiary, presented with expected epithelium and granulosa cells. Furthermore, blood vessels and some loose connective tissue were present in these ovarian samples. The PCOS group presented cysts and follicles of different sizes. In both treatment groups, follicles of different sizes were observed, although groups of cystic follicles were also observed in some places. Irregular oestrus cycles and an increase in weight gain were detected in the DHEA-treated mouse models. Increased levels of basal glucose and abnormalities of glucose tolerance were observed more in the PCOS group compared with the control group. Furthermore, in the PCOS group, immature oocytes within the developing follicles were observed. In these samples, cystic and atretic follicles were also detected. In the larger follicles, the granulosa cells were not as compact as in the control group. Accumulation of lipids was also observed in the granulosa cells. In the treatment groups, the follicles were similar to the control

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Table 2. The average C_t values for each gene analysed

Sample details	Average C_t of *Gapdh*	Average C_t of *PTEN*	Average C_t of *Pik3ca*	Average C_t of *Actb*
Group 1 Sample 1	34.18	34	39.86	29.53
Group 1 Sample 2	32.67	30.78	36.84	28.39
Group 1 Sample 3	32.37	35.63	37.66	39.41
Group 1 Sample 4	31.64	31.84	36.29	25.43
Group 2 Sample 1	41.59	36.39	40.34	37.02
Group 2 Sample 2	35.96	31.98	37.06	38.44
Group 2 Sample 3	31.56	31.46	36.88	28.62
Group 2 Sample 4	40.24	37.97	39.63	38.02
Group 2 Sample 5	38.46	36.15	40.35	34.55
Group 2 Sample 6	36.16	34.83	38.63	37.8
Group 2 Sample 7	34.58	30.85	35.9	34.28
Group 2 Sample 8	40.23	38.65	40.44	38.09
Group 3 Sample 1	30.95	32.46	33.65	30.57
Group 3 Sample 2	29.8	33.89	36.36	31.21
Group 3 Sample 3	30.7	33.32	31.33	29.39
Group 3 Sample 4	33.49	34.77	39.28	30.78
Group 3 Sample 5	35.19	35.13	38.87	32.78
Group 3 Sample 6	35.83	33.71	37.035	32.18
Group 3 Sample 7	40.72	34.29	42.72	40.25
Group 4 Sample 1	40	36.89	37.16	33.155
Group 4 Sample 2	37.18	35.04	41.57	33.12
Group 4 Sample 3	30.72	29	36.145	36.04
Group 4 Sample 4	35.25	35.03	40.53	31.73
Group 4 Sample 5	31.67	36.11	41.25	32.2
Group 4 Sample 6	40.29	36.97	35.26	35.88
Group 4 Sample 7	39.17	35.59	41.07	36.77
Group 4 Sample 8	40.6	35.63	42.08	35.51

group and the granulosa and theca cells were comparable with those of normal follicles.

The expression levels of two genes, *Pten* and *Pik3ca*, were investigated in ovarian tissue samples obtained from the four groups. *Gapdh* and *Actb* were used as housekeeping genes and normalization was performed for each analysis against *Gapdh* and *Actb*. The results of this study showed that all these genes were expressed in mouse ovaries. The C_t values for each gene are shown in Table 2. The results of the one-way ANOVA showed that the expression levels of *Pten* were significantly different in the study groups. However, statistical analysis using Student's *t*-test for each group individually showed that there was no significant difference.

Discussion

In the present study, the expression levels of *Pten* and *Pik3ca* were investigated in PCOS mouse models treated with clomiphene citrate only or a combination of clomiphene citrate, metformin and pioglitazone. The results of this study showed that *Pten* expression was significantly different in the PCOS mouse models and the treatment groups. Therefore, it is suggested that Pten regulates PCOS development possibly through the PI3K/AKT pathway.

The PI3K/AKT pathway is important in many cellular functions such as the cell cycle, proliferation and cancer. The negative regulatory role of PTEN in the PI3K/AKT pathway has been well established. The roles of PTEN as a tumour suppressor have long been established. However, the PI3K/AKT signalling pathway, as well as the regulation of PTEN through this pathway, have been proposed to be involved in the pathogenesis of PCOS that may be involved in the adjustment of insulin resistance and hyperandrogenism. PTEN has been shown to be associated with insulin concentrations in the follicular fluid (Iwase *et al.*, 2009). Expression levels of PTEN have been reported to increase during follicular development. Simultaneously, phospho-AKT has been shown to be reduced, implying a regulatory role for PTEN in granulosa cell development by regulating the PI3K/AKT pathway (Goto *et al.*, 2007). In the PI3K/AKT pathway, the levels of PIP_3 are regulated through the PI3K and PTEN balance. PIP_3 is involved in regulating the signalling of many downstream genes, including Akt. Akt has been shown to regulate folliculogenesis (Iwase *et al.*, 2009). PTEN has also been suggested to be involved in the development and atresia of primordial follicles (Ouyang *et al.*, 2013). PTEN has also been shown to be present in granulosa cells (Iwase *et al.*, 2009). Furthermore, *PTEN* was shown to be upregulated in PCOS patients and mouse models, respectively. Therefore, it has been suggested that *PTEN* regulates the PI3K/AKT signalling pathway and is involved in PCOS development (Gao *et al.*, 2021). Furthermore, PTEN expression was shown to be induced with insulin in human granulosa cells leading to reduced Akt phosphorylation. This had a negative effect on the proliferation of granulosa cells. The results of this study showed that Pten was expressed significantly differently in the PCOS and treatment groups. Therefore, it is a possibility that Pten has suppressive effects on the development and function of granulosa cells. The exact mechanism of how PTEN regulates granulosa cell functioning and therefore oogenesis is not clear. Even though some studies have shown decreased expression of PTEN in granulosa cells of PCOS (He *et al.*, 2019), others have reported higher expression of PTEN in the granulosa cells of PCOS patients (Iwase *et al.*, 2009). However, it has also been reported that PTEN is downregulated when granulosa cells are proliferating and differentiating in human ovaries and therefore regulating oogenesis through the PI3K

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pathway (Goto *et al.*, 2009). The studies in mice showed that, in the absence of Pten, higher levels of androgens and high numbers of antral follicles with larger ovaries and fertility issues are observed that resemble the PCOS profile in humans (He *et al.*, 2019).

In conclusion, together with previously published studies, this study suggests that Pten is involved in the regulation of PCOS. Treatment strategies can have an effect on the pathogenesis of PCOS by regulating Pten expression. Further studies are ongoing in investigating gene expression levels of other pathways in the pathogenesis of PCOS.

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Conflict of interest. None to declare.

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