Faculty of Medicine
Institutional Review Board (IRB)

Research Proposal Form

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ClinicalTrials.gov ID: NCT04283773

Date of revision: 20/05/2021

Revision 1: ☐

Revision 2: ■

IRB NO: 17100871

Part 1: General

Master Degree ■ **b. MD** ☐ **c. Independent Research/Project** ☐

1.1 Applicant Name (responsible for all correspondences and accuracy of data): **Omar Abdel Aziz Ahmed Ali**	**Department:** **Pathology**
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1.2 English Title of research project:

Immunohistochemical evaluation of p16 protein expression in ovarian germ cell tumors.

1.3 Do you need funding from Assiut Medical School Grants Office?

Yes ■ No ☐

1.4 ID of grant

2019-09-05-001-R1 .Funder: Grant office - faculty of medicine -Assiut university].

Part 2: Research Details

2.1 Background (Research Question, Available Data from the literature, Current strategy for dealing with the problem, Rationale of the research that paves the way to the aim(s) of the work). **(200-250 words max.)**

Ovarian germ cell tumors (OGCTs) constitute 10% of ovarian tumors in Egypt [1] and mainly affect young females [2]. Teratomas are the most common type [3].Most of teratomas is benign. However, it is liable for malignant transformation [4]. Others are malignant including dysgerminoma, immature teratoma, yolk sac tumor,.etc.[5] and accounts 1–1.5% of cancers in young females [6]. The pathogenesis of OGCTs is not clearly understood. Some authors revealed that mature cystic teratoma is a form of human parthenogenesis [7], which makes important concerns about future of fertility and germ stem cell replacement therapy [8].

P16 is a member of cyclin-dependent kinase (CDK) inhibitors. It arrests the cell cycle in G1 phase, so it is known as a tumor suppressor protein [9]. CDK inhibitors now are evolving as novel target therapies under clinical trials such as Palbociclib, Abemaciclib and Ribociclib that offer a hope for different cancers, such as breast cancer [10] and non-small cell lung cancer [11].One member which is recently approved by US FDA is palbociclib for use in postmenopausal women with breast cancer [12].Thus, it is necessary to do further researches on its expression among different neoplasms.

P16 immunohistochemical(IHC) expression has been widely investigated in different cancers. Its IHC expression is either absent [13] or overexpressed [14] .Overexpression of p16 is documented in HPV related endocervical neoplasms and HSILs of the vulvovaginal region [14].Absence of p16 expression is detected in multiple cancers such as Lung cancer, colorectal cancer and lymphoma [15].

P16 IHC expression in OGCTs is poorly investigated. One study suggests that absent p16 is involved in proliferation of malignant OGCTs via molecular assessment[16].Another study suggested that decrease P16 is involved in malignant transformation of MCT to SCC[17].However, Previous studies are still limited and recommended further studies to confirm its results.

As the role of altered P16 protein in OGCTs is not widely investigated, we hypothesized that abnormal P16 expression may be involved in its pathogenesis and germ stem cell proliferation. Abnormalities may be found either in neoplastic or stromal components. The role of stromal component of OGCTs as tumor microenvironment has no published studies yet. This will give more information about molecular pathways of germ stem cell proliferation to give a hope for
CDK inhibitors as novel target therapies in the management of OGCTs and new advances in use of germ stem cell replacement therapy.

2.2 Aim(s) of the Research (100 words max):

In this study , we aim to :

1-Evaluate the immunohistochemical expression of p16 in both neoplastic and stromal components of benign and malignant OGCTs and scoring its immunoreactivity.

2-Compare the immunohistochemical expression of p16 between different histopathological types of OGCTs.

3-Correlate between P16 immunohistochemical expression with clinicopathological parameters (Age, Histopathological type, grading, and staging).

2.3 Research Area (Faculty Research Plan). Choose one and delete the rest. 7

1. Basic researches that may lead to possible/definite improvement in health services and solving the above problems.

2.4. Research Methods and techniques:

The study protocol was approved by the Institutional Review Board at Faculty of medicine, Assiut University in December, 2019 (IRB No.17100871) and Ethical approval for this study was obtained from the committee of medical ethics, Faculty of medicine, Assiut University.

-**Type of the study:** Matched case-control study.

-**Study Setting:** IHC lab of Pathology department, Faculty of medicine, Assiut University.

-**Tissue sampling:** Sixty-two formalin-fixed, paraffin wax-embedded ovarian specimens were obtained from departments of surgical pathology in Assiut University Hospital (AUH) and South Egypt cancer institute (SECI) from January 2010 to January 2021. These specimens includes:

(**Group A**) 22 malignant ovarian germ cell tumors (MOGCTs),

(**Group B**) 20 apparent normal ovarian tissue as a control group and

(**Group C**) 20 mature cystic teratomas as equal benign comparison group. **Group A** constitutes 5 dysgerminomas, 8 immature teratomas (four of them Grade II and the other four are Grade III, FIGO grading system) and 9 yolk sac tumors. The patients ranged in age from 12 to 23 years (median age 16.5 years). The initial diagnosis of each MOGCTs was re-evaluated according to the WHO Classification of Ovarian Tumors.

Group B includes normally apparent ovaries that surgically resected with specimens of total abdominal hysterectomy and salpingo-opherctomy for non-ovarian, non-malignant causes as multiple fibroid uterus and adenomyosis as a **control** group. The patients ranged in age from 42 to 64 years (median **50** years old).

All available Hematoxylin and eosin stained slides were examined by two independent pathologists by routine light microscopy and the most representative one or two tissue blocks were used for immunohistochemical staining. The clinical and pathological data includes age, grade, stage, recurrence of lesions were evaluated according to pathological reports.

- **Immunohistochemistry**: The tissue samples were formalin-fixed, paraffin embedded tissue blocks, and were stored at room temperature. Thin sections of 4 um thickness were cut from selected representative tissue blocks, and were taken on to coated slides, kept in an oven at 70-C for 25 minutes, deparaffinized by washing in two containers of xylene for 15 minutes for each and rehydrated through serial dilutions (100% , 90% , 80% , 70 %) of alcohol for 5 minutes in each dilution. Then, sections were treated by 3% Hydrogen peroxide for 10 minutes to prevent nonspecific background followed by PBS buffer wash 4 times adequately. For antigen retrieval, the slides were kept in citrate buffer (PH = 6) and autoclaved at 90-C for 15 minutes and left to cool to room temperature. The slides were washed with PBS buffer 4 times adequately and the tissue sections were incubated with primary antibody; **p16INK4a Recombinant Rabbit Monoclonal Antibody (RM267) (MA5-27905**) at a dilution of 1:500 overnight, followed by incubation with secondary antibody; **Polyvalent polymer-HRP** for 10 minutes for each step followed by adequate 4 times of PBS buffer wash. The tissues were then treated with Diaminobenzidine chromogen **(DAP-chromogen)** for brown color development for 10 minutes. Then, sections were treated by **Mayer hematoxylin** as the counter stain for 2 minutes.

For sections received from malignant cases, we add additional **Ki67 IHC** staining as tissue sections after deparffinization and blocking by 3% hydrogen peroxidase were incubated with primary antibody; **Ki67 antibody (DAKO)**, ready to use for 20 minutes, followed by incubation with secondary antibody; **Polyvalent polymer-HRP** for 20 minutes, followed treatment with Diaminobenzidine chromogen **(DAP-chromogen)** for brown color development for 5 minutes. Then, sections were treated by **Mayer hematoxylin** as the counter stain for 2 minutes. The slides were washed by tap water, dehydrated through serial dilutions of alcohol, washed by xylene for a short period, and mounted with DPX.

The normal apparent ovarian tissues were used as controls. Sections from cervical non keratinized Squamous cell carcinoma with block p16 expression, were used as positive controls; and the tissue slides without the primary antibody treatment served as the negative controls.

-**Immunohistochemical evaluation**: **For P16 IHC staining**, the percentage of P16 positive cells and the location of positive signals (nuclear or cytoplasmic) were visually estimated for neoplastic components of all lesions. **German Semi-quantitative scoring** system were used to evaluate P16 expression as every tumor will be given a score according to the intensity of the cytoplasmic and nucleic staining (no staining = 0, weak staining = 1, moderate staining = 2, strong staining = 3) And the extent of stained cells (0% = 0, 1–10% = 1, 11–50% =2, 51–80% = 3, 81–100% = 4). The final immunoreactive score will be determined by multiplying the intensity scores with the extent of positivity scores of stained cells, with the minimum score of 0 and a maximum score of 12 (score 0, 1,2,3,4,6,8,9 and 12). **For KI67 IHC staining for malignant cases**, the labelling index for Ki-67 antigen was calculated as the percentage of tumor cells with distinct nuclear staining in the most evenly and distinctly labelled tumor areas in each section. At least 1000 tumor cells were counted for each section.

2.5-Data management and analysis:

Data were analysed using IBM-SPSS version 24. Numerical data were presented as mean and standard deviation, while categorical data were presented as number and percentage. Descriptive statistics: Means, standard deviations, medians, ranges, and percentages were calculated. **Test of significances**: for continuous variables with more than two categories; **ANOVA test** was calculated to test the mean differences of the data that follow normal distribution and **independent sample Kruskal-Wallis** was used to compare the median difference between groups that do not follow normal distribution, post-hoc test was calculated using Bonferroni corrections. Correlation analysis was used (**Spearman' Ranked correlation, 2-tailed**). **A p-value < 0.05 was considered significant**.

2.6-References (max. 15) and written in Vancouver style:

1-Saber M.M., A.A. Zeeneldin, M.M. El Gammal, et al., Treatment outcomes of female germ cell tumors: The Egyptian National Cancer Institute experience. Journal of the Egyptian National Cancer Institute, 2014. 26(2): p. 103-108.

2-Smith H.O., M. Berwick, C.F. Verschraegen, et al., Incidence and survival rates for female malignant germ cell tumors. Obstetrics & Gynecology, 2006. 107(5): p. 1075-1085.

3-Outwater E.K., E.S. Siegelman and J.L. Hunt, Ovarian teratomas: tumor types and imaging characteristics. Radiographics, 2001. 21(2): p. 475-490.

4-Takagi H., S. Ichigo, T. Murase, et al., Early diagnosis of malignant-transformed ovarian mature cystic teratoma: fat-suppressed MRI findings. Journal of gynecologic oncology, 2012. 23(2): p. 125-128.

5-Gershenson D.M., Update on malignant ovarian germ cell tumors. Cancer, 1993. 71(S4): p. 1581-1590.

6-Ali A., H. Sayed, M. Salem, et al., Clinicopathological pattern and outcome of pediatric malignant ovarian germ cell tumors: South Egypt Cancer Institute experience. Journal of pediatric surgery, 2018. 53(4): p. 837-840.

7- Whittingham, D. (1980). "Parthenogenesis in mammals." Oxford Reviews of Reproductive Biology 2: 205-231.

8-Lee, S. T., M. H. Choi, et al. (2008). "Establishment of autologous embryonic stem cells derived from preantral follicle culture and oocyte parthenogenesis." Fertility and sterility 90(5): 1910-1920.

9-Romagosa C., S. Simonetti, L. Lopez-Vicente, et al., p16 Ink4a overexpression in cancer: a tumor suppressor gene associated with senescence and high-grade tumors. Oncogene, 2011. 30(18): p. 2087.

10-Finn R.S., J. Dering, D. Conklin, et al., PD 0332991, a selective cyclin D kinase 4/6 inhibitor, preferentially inhibits proliferation of luminal estrogen receptor-positive human breast cancer cell lines in vitro. Breast Cancer Research, 2009. 11(5): p. R77.

11-Gopalan P.K., M.C. Pinder, A. Chiappori, et al., A phase II clinical trial of the CDK 4/6 inhibitor palbociclib (PD 0332991) in previously treated, advanced non-small cell lung cancer (NSCLC) patients with inactivated CDKN2A, 2014, American Society of Clinical Oncology.

12- Raedler, L. A. "Ibrance (Palbociclib): First CDK4 and CDK6 Inhibitor FDA Approved for the Treatment of Postmenopausal Women with Metastatic Breast Cancer."

13-Ayhan S., A. Isisag, M. Saruc, et al., The role of pRB, p16 and cyclin D1 in colonic carcinogenesis. Hepato-gastroenterology, 2010. 57(98): p. 251-256.

14-Klaes R., T. Friedrich, D. Spitkovsky, et al., Overexpression of p16INK4A as a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri. International journal of cancer, 2001. 92(2): p. 276-284.

15-Cheung H.H., T.L. Lee, O.M. Rennert, et al., DNA methylation of cancer genome. Birth Defects Research Part C: Embryo Today: Reviews, 2009. 87(4): p. 335-350.

16-Kawauchi S., X.P. Liu, K. Kawasaki, et al., Significance of β-catenin and pRB pathway components in malignant ovarian germ cell tumours: INK4A promoter CpG island methylation is associated with cell proliferation. The Journal of Pathology: A Journal of the Pathological Society of Great Britain and Ireland, 2004. 204(3): p. 268-276.

17-Iwasa A., Y. Oda, S. Kurihara, et al., Malignant transformation of mature cystic teratoma to squamous cell carcinoma involves altered expression of p53-and p16/Rb-dependent cell cycle regulator proteins. Pathology international, 2008. 58(12): p. 757-764.

Part 3: Ethical Considerations *(Written in details taking in consideration the items below):*

3.1. Risk – benefit assessment.

All research work will be conducted on formalin fixed paraffin embedded tissue blocks collected from surgical pathology Lab of Assiut University hospital (AUH) from January 2010 to January 2021.

3.2. Confidentiality (dealing with data and data dissemination should be confidential).

Dealing with data and data dissemination will be confidential and limited to investigators participating in this research only.

3.3. Statement describing the research procedure to be given to the participants.

- The research will be conducted only by scientifically qualified and trained personnel.

- It is our responsibility to safeguard the rights and welfare of human subjects involved in our research in accordance with the appropriate ethics, in the form of the rights and welfare of the subjects involved in the research are adequately

protected.

3.4. Informed consent.

All research work will be conducted on formalin fixed paraffin embedded tissue blocks tissue blocks collected from surgical pathology Lab of Assiut University hospital (AUH) from January 2010 to January 2021.

3.5. Other ethical concerns:

- The research should be conducted only by scientifically qualified and trained personnel.
- The research should be based on relevant pre-clinical investigations in animals.
- **The consent form must be provided with the proposal.**

INFORMED CONSET:

I am Dr / Omar Abd El Aziz Ahmed Ali, preparing for a research titled by:

Immunohistochemical evaluation of p16 protein expression in ovarian germ cell tumors.

Convinced that:

1- All research work will be conducted on formalin fixed paraffin embedded tissue blocks collected from surgical pathology Lab of Assiut University hospital (AUH) from January 2010 to January 2021.

2- Dealing with data and data dissemination will be confidential and limited to investigators participating in this research only.

3- The research was conducted only by scientifically qualified and trained personnel.

4- It is our responsibility to safeguard the rights and welfare of human subjects involved in our research in accordance with the appropriate ethics, in the form of the rights and welfare of the subjects involved in the research are adequately protected.