**وه‌زاره‌تی خوێندنی باڵا و تۆێژینه‌وه‌ی زانستی**

**Ministry of Higher Education &**

**Scientific Research**

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| **پرۆپۆزەلى توێژینه‌وه‌ بۆ به‌ده‌ستهێنانی بروانامه‌ی دکتۆرا PhD Research Proposal** | | |
| **Related imageناونيشانی پرۆپۆزه‌لی تۆێژینه‌وه‌ی پێشنیازکراو 1. Title of PhD research proposal**  **Molecular basis of renal cell carcinoma and its relation to hydrogen sulfide signaling** | | |
| **زانیاری گشتی 2. General information** | | |
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| **3. Summary (Abstract) of PhD research proposal**    Renal cell carcinoma (RCC) is the most common type of kidney cancer, accounting for more than 80% of all malignant kidney tumors. Although the exact cause of RCC remains largely unknown, aberrant angiogenesis is considered a hallmark of this disease In the majority of sporadic and hereditary RCC cases, the von Hippel-Lindau (VHL) tumor suppressor gene is functionally disrupted and results in constitutive activation of hypoxia-inducible factor (HIF) and subsequent induction of target genes, such as VEGF. Moreover. The mTOR signaling pathway plays a crucial role in cell growth, survival, proliferation and angiogenesis of this cancer. Mutation of some genes such VHL, VEGFR3, PI3K PI3KCA, AKT1, AKT2, PTEN, and MTOR are frequently observed in general population contribute in developing of breast cancer.  Hence, we study mutations at the level of nucleotide sequences, determine the alteration in the expression of these genes, changes in amino acid sequences in expressed proteins and the activity of these proteins. This will be a unique study in the region to understand the mutations in genes, alteration in expressed RNA proteins, and to answer unrevealed key questions of RCC cancer patients | | |
| **4.Introduction**  RCC (renal cell carcinoma) is the tenth mainly occurring malignant cancer worldwide and extensively a heterogeneous group of cancers which is derived from epithelial cells of renal tubular, representing a comprehensive 80% of all main kidney tumors (Singh, 2021)  The incidence of RCC is generally related to topographical changes and North America and the Czech Republic demonstrating a high incidence of mortality due to the disease. In the United States, 64,000 new cases and 14,000 RCC related mortality cases are seen every year(Thompson et al., 2008) This cancer is more common in male gender as compared to females and a high incidence is generally seen from the sixth to eight decades of its existence. It proves that age, gender and race have an important role in the occurrence of RCC(Siegel et al., 2017)  Universally there are three of RCC: a) clear renal cell carcinoma (80% of individuals with kidney malignancy) b) papillary renal cell carcinoma (10% to 15%) and c) chromophobe renal cell carcinoma (around 5% of cases.)  A renal cell carcinoma examination and treatment strategy has gone through a time span of alteration. An advancement or innovation in the genomics and biological studies will lead to lower mortality rate. This can happen if mutations of the von Hippel-Lindau gene and consequential ballast of the hypoxia response pathway are detected, which is a key channel of the clear cell RCC (Latif et al., 1993)  Production of the targeted therapy on the basis of the molecular level has altered the management archetype for the people who suffer from renal neoplasms  Chemotherapy is the most common management therapy for the cancer, but its efficacy is restricted by the resistance of drug. RCC shows resistance to radiation, exhibiting a slow response to immunotherapeutic agents (interleukin (IL)-12 and interferon (INF)-α).  Many risk factors are linked with the pathophysiologic mechanism of RCC and it includes both genetic and acquired risk factors, the genes which are implicated in the pathogenesis of renal cell carcinoma are PBRM-1 (protein polybromo-1) gene, VHL (Von Hippel–Lindau) gene, Genetic alterations of mTOR pathway-related genes, including mutations of PI3K, AKT, and PTEN (Cao et al., 2012)  Clear cell RCC (ccRCC) accounts for approximately 80% of all RCC and the Von Hippel Lindau (VHL) tumor suppressor gene is mutated, deleted or epigenetically silenced in approximately 85% of sporadic clear cell RCC.[2] Loss of VHL function leads to a constitutionally active Hypoxia Inducible Factor (HIF) pathway. The HIF pathway is considered to be a driver pathway in VHL mutant clear cell RCC. The etiopathogenesis of metastatic RCC, based on our understanding to date of molecular mechanisms involved, is a sequence of events which can be grouped under the following –   1. Loss of VHL activity (germline/ somatic mutation + inactivation of the wild type copy) 2. Constitutive activation of the HIF pathway due to loss of VHL activity and transcription of genes involved in angiogenesis, epithelial mesenchymal transition, invasion, metastasis, survival, anaerobic glycolysis and pentose phosphate pathway. 3. Interactions of the HIF pathway with other oncogenic pathways 4. Genome wide epigenetic changes (likely driven by an overactive HIF pathway) and the influence of epigenetics on various oncogenic, apoptotic, cell cycle regulatory and mismatch repair pathways (inhibition of multiple tumor suppressor genes) 5. Immune evasion, at least partially caused by changes in the epigen   VHL is located at chromosome 3p, and loses its function, owing to point mutations or epigenetic silencing, years after the initial chromothripsis event20. Functional loss of VHL results in impaired ubiquitylation and accumulation of hypoxia-inducible factors (HIFs) within cell nuclei (Fig. 3b). Accumulated HIFs, in turn, increase the production of several growth factors that have key roles in the progression of RCC21(Wiesener et al., 2001)  These mechanisms interact throughout the pathogenesis and progression of disease, and also confer chemoresistance and radioresistance, making it one of the most difficult metastatic cancers to treat(Shenoy and Pagliaro, 2016).  The von Hippel–Lindau (VHL) tumour suppressor has been isolated in 1993 The protein encoded by the VHL gene (pVHL) is the substrate recognition component of a ubiquitin ligase complex that targets a transcription factor, hypoxia inducible factor (HIF), for proteolysis. A biallelic VHL inactivation leads to HIF-1a accumulation and subsequent overexpression of genes, which are critical for tumor angiogenesis, cell proliferation and migration(Patard et al., 2009)  some studies have shown a positive relationship between genetic and epigenetic VHL gene alterations and VEGF tumors overexpression. (Igarashi et al., 2002). Targeting endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) and their receptors have led to significant progress in medical treatment of metastatic RCC(Motzer et al., 2008).  The mTOR signaling pathway plays a crucial role in cell growth, survival, proliferation and angiogenesis. The phosphatidylinositol-3-kinase (PI3K) protein via the several growth factors binds to the cell surface by mTOR–PI3K pathway. Phosphorylated P70SK is produced by the activation of PI3K and it generates mTOR complexes 1 and 2; finally, phosphorylated P70SK moves to the center (nucleus) of the cell and instigates the mRNA code by the process of the transcription and enhances the generation of angiogenic proteins. It also regulates the tumorigenesis, cell expansion and translation of mRNA involved in oncogenic Akt signaling(Santoni et al., 2014)  Considering the critical role of the pVHL, VEGFA, VEGFR, mTOR pathway genes in RCC, it is possible that SNPs in theses pathways may play an important role in RCC development and treatment resistance , However, no published study has yet addressed this issue in Kurdistan region, for this reason this study is aimed to identify the mutation in each of VHL, VEGFA, VEGFR3, PI3K and mTOR genes and their possibility for development of RCC  Hydrogen sulfide (H2S) is a ubiquitous small gaseous signaling molecule, playing an important role in many physiological processes and joining nitric oxide and carbon monoxide in the group of signaling agents termed gasotransmitters(Powell et al., 2018). Hydrogen sulfide (H2S) and Nitric oxide (NO) have various roles in normal human physiology and regulation of cancer-related events such as proliferation, angiogenesis, cell apoptosis, and metastasis(Ehrenfeld et al., 2019). Endogenous concentrations of H2S are generally low, making it difficult to discern precise biological functions. As such, probing the physiological roles of H2S and NO is aided by exogenous delivery of the gases in cell and animal studies. The second part of this project is aimed to find the impact of H2S, and NO donors or a combination of these donors with Bevacizumab and temsirolimus treatments on HEK 293 cells. | | |
| **5. Research objectives**  1. Genotyping of selected genes polymorphism will perform by using tetra-primer amplification refractory mutation system (T-ARMS), and nucleotide sequencing of the PCR products for the selected genes are detecting using Sanger sequencing analyzer.  2. Examination of VHL and VEGFR3 mutations from peripheral blood samples by Sanger sequencing.  3. Assessment of gene expression changes of the selected genes cDNA fragments by quantitative real-time PCR. | | |
| **6. Methodology and data collection**  This project has been designed to analyze the mutation status of RCC cancer patients and comparing them with the healthy individuals, therefore this study is a case-control study.  The first task is to take a specimen from RCC cancer patients undergoing nephroctomy at hospitals in Erbil, Duhok, Sulaimani and Halabja cities. Also, the blood sample will obtain by phlebotomy under aseptic technique. Venous blood will aspirate into a 5 ml syringe, then place in plain tubes as well as anticoagulant tubes, and maintain at room temperature. The serums of plain tubes preserved in Eppendorf tubes and store at -60oC in the deep freezer until assay. The anticoagulant tubes will further analyze by molecular methods using PCR.  To achieve the goals of this project, we plan to conduct the following experiments:   1. **Mutation and polymorphism analysis using Sanger sequencing technique**   Genotyping of selected genes polymorphism will perform by using tetra-primer amplification refractory mutation system (T-ARMS), which is a rapid and simple technique for recognition of SNP. Polymerase chain reaction (PCR) will perform using commercially available QIAGEN Multiplex PCR Kit (Qiagen, Germany) according to the manufacturer procedure. The PCR products are analyzing by DNA electrophoresis gels stained with SYBR Safe DNA Gel Stain and the imaging, and digital documentation will perform using the ChemiDoc XRS system (Bio-Rad Laboratories, USA).  The nucleotide sequencing of the PCR products for the selected genes is detecting using DNA fragments sequencing with 3130 Genetic Analyzer (Applied Biosystems, Hitachi) with primers used for amplification. The obtained will analyze using Mutation Surveyor software by SoftGenetics (Pennsylvania, USA). Then, the DNA will get from peripheral blood samples, and they are examining by next-generation sequencing with Ion AmpliSeq VHL and PI3K panel using next-generation sequencing.   1. **Quantitative real-time PCR**   To assess the changes in gene expression of the selected genes cDNA fragments are amplifying by RT-PCR Rotor Gene Q (Qiagen, Germany) by using the primers and a Rotor-Gene Multiplex RT-PCR kit (Qiagen, Germany) against the housekeeping gene, ACTB (Beta Actin). In which, the extraction of total RNA is done using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and according to supplier’s instructions, and RNA from each sample is retrotranscribed (RT) using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Gene expression variations are evaluating in term of fold induction respect to the control by both the 2-∆∆CT method and ‘Comparative Quantitation’ tool of the Rotor Gene Series software. Expression stability values of the different housekeeping genes were calculated by Norm Finder software to choose the best reference gene for normalization.  The concentration of expressed proteins is determining according to Sandwich-enzyme linked immunoassay (ELISA) method. The micro ELISA plate is pre-coating with an antibody specific to each protein. Then a biotinylated detection antibody specific for proteins and Avidin-Horseradish Peroxidase conjugates are adding to each microplate well successively. Only those wells that contain expressed proteins, biotinylated detection antibody and Avidin-HRP conjugate, appears in colour. The optical density is measuring spectrophotometrically at a specified wavelength.   1. **Cell culture**   Human embryonic kidney cells (HEK-293) (ATCC) is cultured in DMEM with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. For assessment of in vitro proliferation, the reduction of (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) is a method to measure and monitor cell proliferation. MTT assays is performed using the Cytoselect MTT Cell Proliferation Assay. Furthermore The terminal deoxynucleotidyl transferase (dUTP)-nick end labelling (TUNEL) method that determines DNA-strand breaks throughout the apoptosis process is used to confirm (HEK-293) cells undergoing apoptosis after treatment with different compounds for different time of period | | |
| **7. Scope and limit to the research**  A small group of scientists tried to identify the molecular bases, genetics and pathophysiology of breast cancer in the middle east, Iraq and Kurdistan region. To date, there is no study and direct evidence to identify the molecular bases, and pathophysiology of renal cell carcinoma in the Kurdistan region of Iraq and PubMed search found no previous reports on this direction. | | |
| **8. Duration and timeline**   |  |  |  |  |  |  |  |  |  |  |  |  |  | | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | | **Task** | **First Year** | | | | | | **Second Year** | | | | | | | Collection of samples, mutation and polymorphism analysis |  |  |  |  |  |  |  |  |  |  |  |  | | Sanger sequencing |  |  |  |  |  |  |  |  |  |  |  |  | | Next-Generation sequencing |  |  |  |  |  |  |  |  |  |  |  |  | | Quantitative real-time PCR |  |  |  |  |  |  |  |  |  |  |  |  | | Western blotting |  |  |  |  |  |  |  |  |  |  |  |  | | Enzyme-linked immunoassay |  |  |  |  |  |  |  |  |  |  |  |  | | Data analysis |  |  |  |  |  |  |  |  |  |  |  |  | | | |
| **9. Conclusions**  Renal cell carcinoma is a very aggressive with poor prognosis and high cancer mortality due to lack of targeted medicines applied in this type of cancer. This is the beginning of an exciting period in genetic oncology, but much more remains to be learned. The ultimate outcome of the study for the identification of the molecular pathways underlying the development of breast cancers, allowing for prevention and treatment, and the pathogenic molecular genetic defects will be repaired. | | |
| **10. References**  CAO, Q., JU, X., LI, P., MENG, X., SHAO, P., CAI, H., WANG, M., ZHANG, Z., QIN, C. & YIN, C. 2012. A functional variant in the MTOR promoter modulates its expression and is associated with renal cell cancer risk. *PLoS One,* 7**,** e50302.  EHRENFELD, P., CORDOVA, F., DURAN, W. N. & SANCHEZ, F. A. 2019. S-nitrosylation and its role in breast cancer angiogenesis and metastasis. *Nitric Oxide,* 87**,** 52-59.  IGARASHI, H., ESUMI, M., ISHIDA, H. & OKADA, K. 2002. Vascular endothelial growth factor overexpression is correlated with von Hippel-Lindau tumor suppressor gene inactivation in patients with sporadic renal cell carcinoma. *Cancer,* 95**,** 47-53.  LATIF, F., TORY, K., GNARRA, J., YAO, M., DUH, F., ORCUTT, M., STACKHOUSE, T., KUZMIN, I., MODI, W., GEIL, L. & ET, A. 1993. Identification of the von Hippel-Lindau disease tumor suppressor gene. *Science,* 260**,** 1317-1320.  MOTZER, R. J., ESCUDIER, B., OUDARD, S., HUTSON, T. E., PORTA, C., BRACARDA, S., GRÜNWALD, V., THOMPSON, J. A., FIGLIN, R. A., HOLLAENDER, N., URBANOWITZ, G., BERG, W. J., KAY, A., LEBWOHL, D. & RAVAUD, A. 2008. Efficacy of everolimus in advanced renal cell carcinoma: a double-blind, randomised, placebo-controlled phase III trial. *Lancet,* 372**,** 449-56.  PATARD, J. J., RIOUX-LECLERCQ, N., MASSON, D., ZERROUKI, S., JOUAN, F., COLLET, N., DUBOURG, C., LOBEL, B., DENIS, M. & FERGELOT, P. 2009. Absence of VHL gene alteration and high VEGF expression are associated with tumour aggressiveness and poor survival of renal-cell carcinoma. *Br J Cancer,* 101**,** 1417-24.  POWELL, C. R., DILLON, K. M. & MATSON, J. B. 2018. A review of hydrogen sulfide (H(2)S) donors: Chemistry and potential therapeutic applications. *Biochem Pharmacol,* 149**,** 110-123.  SANTONI, M., BERARDI, R., AMANTINI, C., BURATTINI, L., SANTINI, D., SANTONI, G. & CASCINU, S. 2014. Role of natural and adaptive immunity in renal cell carcinoma response to VEGFR-TKIs and mTOR inhibitor. *International Journal of Cancer,* 134**,** 2772-2777.  SHENOY, N. & PAGLIARO, L. 2016. Sequential pathogenesis of metastatic VHL mutant clear cell renal cell carcinoma: putting it together with a translational perspective. *Ann Oncol,* 27**,** 1685-95.  SIEGEL, R. L., MILLER, K. D. & JEMAL, A. 2017. Cancer statistics, 2017. *CA: A Cancer Journal for Clinicians,* 67**,** 7-30.  SINGH, D. 2021. Current updates and future perspectives on the management of renal cell carcinoma. *Life Sci,* 264**,** 118632.  THOMPSON, R. H., ORDONEZ, M. A., IASONOS, A., SECIN, F. P., GUILLONNEAU, B., RUSSO, P. & TOUIJER, K. 2008. Renal cell carcinoma in young and old patients--is there a difference? *J Urol,* 180**,** 1262-6; discussion 1266.  WIESENER, M. S., MÜNCHENHAGEN, P. M., BERGER, I., MORGAN, N. V., ROIGAS, J., SCHWIERTZ, A., JÜRGENSEN, J. S., GRUBER, G., MAXWELL, P. H., LÖNING, S. A., FREI, U., MAHER, E. R., GRÖNE, H. J. & ECKARDT, K. U. 2001. Constitutive activation of hypoxia-inducible genes related to overexpression of hypoxia-inducible factor-1alpha in clear cell renal carcinomas. *Cancer Res,* 61**,** 5215-22. | | |
| **11. General notes:** هەر زانیارییەکی گشتی دیکە کە سەرپەرشتیار بە گرنگی بزانێت | | |
| **12.**  **په‌سه‌ندكردنی پرۆپۆزەل له‌ لایه‌ن لیژنه‌ی زانستی به‌ش**  ژماره‌ی كۆنووسی كۆبوونه‌وه‌:  رێكه‌وتی كۆبوونه‌وه‌:  بریار: په‌سه‌ند كرا په‌سه‌ند نه‌كرا    ناوی سیانی و واژووی لیژنه‌ی زانستی به‌ش  واژوو:  ناوى سه‌رۆكی لیژنەى‌ زانستی به‌ش مۆری به‌ش  واژوو:  ناوى سه‌رۆكی به‌ش: | | |
| **13.**  **په‌سه‌ندكردنی پرۆپۆزەل له‌ لایه‌ن ئه‌نجومه‌نی كۆلێژ/فاکەڵتى**  ژماره‌ی كۆنوسی كۆبوونه‌وه‌:  رێكه‌وتی كۆبوونه‌وه‌:  بریار: په‌سه‌ند كرا په‌سه‌ند نه‌كرا  واژوو:  ناو راگری كۆلێژ: مۆری كۆلێژ | | |

**تێبینی:** تكایه‌ فۆرمه‌كه‌ ته‌نها به‌ یه‌ك زمان (زمانی توێژینه‌وه‌) پڕ بكرێته‌وه‌.