Sys Rev Pharm 2021;12(2):280-284
A multifaceted review journal in the field of pharmacy

Receiver Operating Characteristic Analysis Of HIF-1A Mrna Gene Expression Fold As Prognostic Indicator In Different Clinic-Pathological Status Of Breast Cancer

Hamzah H. Kzar[1*], Haider O. Mossa Al-Gazali[2],
Moaed E. Al-Gazally[3], Moshtak A. Wtwt [4]

[1]Veterinary Medicine collage, Al-Qasim Green University, Iraq
[2]Ministry of Health / Al-Qasim General Hospital/Iraq
[3]Collage of Medicine, Babylon university (work now at university of Al-Ameed), Iraq
[4]Collage of Medicine, University of Babylon, Iraq
[*]Author for Correspondence: hamza.hashim@vet.uoqasim.edu.iq

ABSTRACT

Background and objective: The highly prevalent and mortality type of cancer among women worldwide is breast cancer (BC). The mortality cases of BC are probably incidence by inadequate of the benefits of treatment and early detection, moreover the lack of appropriate facilities for diagnosis and detection. The aim of this work is to assess of using of ROC of HIF-1A mRNA GEF as a prognostic indicator in BC. Methods: The Total RNA was extracted from whole blood of both women with BC (n=120) and control (n=120) groups, then converted to cDNA. Amplification of HIF-1A gene (gene of interest, GOI-1) and GAPDH (as housekeeping gene, HKG) were done by quantitative real time polymerase chain reaction (qRT-PCR). ROC analysis was done by using QI Macros for Excel software. Graphs were prepared using Microsoft excel 2010. Results: The results of present study suggested that significant differences between the GER in women with BC (4.58) and GER in control group (1.03) (p-value< 0.05). The ROC curve analysis of GEF of HIF-1A mRNA as prognostic marker in women with BC group showed an area under the curve (AUC) of (0.833)(95% CI, 0.76-0.90) with a sensitivity of (82%) and specificity of (63%) at a cut off value 20.5. Conclusion: HIF-1A mRNA GEF is good prognostic indicator for diagnosis and differentiation between both metastasis and non- metastasis BC groups.

Keywords: Breast cancer, ROC, HIF-1A, gene expression fold, mTOR pathway

Correspondence:
Hamzah H. Kzar
1Veterinary Medicine collage, Al-Qasim Green University, Iraq

*Corresponding author: Hamzah H. Kzar email-address: hamza.hashim@vet.uoqasim.edu.iq

INTRODUCTION

BC is a highly prevalent and mortality type of cancer among women worldwide [1]. The mortality cases are probably caused by an inadequate of the benefits of treatment and early detection, moreover the lack of appropriate facilities for diagnosis and detection [2]. In our country, the BC is the most common among women [3]. HIF-1 was discovered by the identification of a hypoxia response element (HRE; 5'-RCGTG-3') in the 3' enhancer of the gene for erythropoietin (EPO), a hormone that stimulates erythrocyte proliferation and undergoes hypoxia induced transcription [4]. HIF-1 is a heterodimeric protein, composed of HIF-1A and HIF-1B subunits, which modulates the regulation of hundreds of genes according to the cellular O_2 concentration [5]. HIF-1A levels increase dramatically as O_2 concentration declines [6]. Under normoxic conditions, HIF-1A is subjected to ubiquitination and proteasomal degradation [7] due to the binding of the von Hippel-Lindau tumor suppressor protein [8],which is the substrate recognition subunit of an E3 ubiquitin-protein ligase [9]. VHL binds to HIF-1A only when the latter is hydroxylated on proline residue 402 and/or 564 [10]. The hydroxylation reaction is performed by prolyl hydroxylases (PHDs) that utilize O_2 and α-ketoglutarate as substrates and generate carbon dioxide and succinate as byproducts [11]. In normoxia, two proline residues of HIF-1A (P^{402} and P^{564}) and asparagine (N^{803}) are hydroxylated by PHDs and FIH-1, respectively, in an O_2, 2-OG, and Fe^{+2}-dependent manner. N^{803} blocks the recruitment of transcriptional co-activator CBP/p300. In hypoxia, the activities of PHDs and FIH-1 are inhibited due to lack of O_2, resulted in no proline and asparagine hydroxylation. Therefore, there is no VHL binding and HIF-1A is stabilized. Stabilized HIF-1A proteins translocate to the nucleus and bind to HIF-1B. HIF-1B may bind preferentially to the MAPK-induced phosphorylated form of HIF-1A. Non-hydroxylated N^{803} of HIF-1A allows CBP/p300 recruitment to the target genes, resulting in gene transcription. In addition, the expression of ARD1 is decreased under hypoxia, causing less acetylated HIF-1A [12,13]. The sensitivity and specificity of given markers is inversely related. Then, the plot of sensitivity versus 1-Specifity is called receiver operating characteristic (ROC) curve and the area under the curve (AUC), as an effective measure of accuracy has been considered with a meaningful interpretations [14]. The purpose of this study to assessment of using of ROC Analysis of HIF-1A mRNA gene expression fold as prognostic marker in different Clinic-pathological status of BC.

MATERIALS AND METHODS

Gene expression study

The Total RNA Mini Kit from Geneaid™ was designed specifically for purifying total RNA from fresh whole human blood. Detergents and chaotropic salt were used to lyse cells and inactivate RNase with an optional in-column DNase treatment. RNA in the chaotropic salt was bound by the glass fiber matrix of the spin column and once any contaminants have been removed, using the washing buffer, the purified total RNA is eluted by RNase-free water. The purified RNA was ready for using in qRT-PCR, and cDNA synthesis. The manufacturer protocol (Geneaid®, Taiwan) was followed for extraction the RNA

from fresh blood sample and this protocol were same for both genes (HIF-1A and GAPDH).

Estimation of RNA purity and concentration

Total yield was obtained by multiplying the RNA concentration by the final total purified sample volume.

-RNA Yield (µg) = RNA Concentration(from device) * Total Sample Volume (ml)

- RNA Yield: 2-3 µg (300 µl blood) in 50 µl elution buffer.

- Only RNA samples with adequate purity ratios (A260/A280= 1.8–2.1) were used for subsequent analyses.

-RNA purity(A260/A280nm) was measured by using nano drop device.

The HiSenScript™ RH(-) cDNA Synthesis Kit was used to synthesis first-strand cDNA from a total RNA preparation.

HIF-1A and GAPDH genes amplification for qRT-PCR analysis

Amplification of HIF-1A gene (gene of interest, GOI-1) and GAPDH (as housekeeping gene, HKG) were done by quantitative real time polymerase chain reaction (qRT-PCR). Amplification was performed in a programmable thermal cycler gradient PCR system .The forward and reverse primers were designed by using NCBI database at the following link:

https://www.ncbi.nlm.nih.gov/tools/primer-blast/

primer pairs for HIF-1A (165 bp) are:

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%
Forward primer	AGAAGCTTCAACCTTGCCCA	Plus	20	61728340	61728359	59.81	50.00
Reverse primer	TGATGTTGTTTGGGGGCAGT	Minus	20	61728504	61728485	60.11	50.00
Product length	165						

Primer pairs for GAPDH (129bp)are:

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%
Forward primer	CATACCCTCACGTATTCCCCC	Plus	21	6536661	6536681	59.65	57.14
Reverse primer	CCTGGAAGATGGTGATGGGATT	Minus	22	6536789	6536768	59.82	50.00
Product length	129						

SYBR Green fluorescence is enormously increased upon binding to double-stranded DNA. During the extension phase, more SYBR Green was binding to the PCR product, resulting in an increased fluorescence. Consequently, during each subsequent PCR cycle more fluorescence signal was detected.

Statistical Analysis

ROC analysis was done by using QI Macros for Excel software. Graphs were prepared using Microsoft excel 2010. P-values less than (0.05) was considered significant and less than (0.001) was considered highly significant.

RESULTS

Table -1, showing the Clinic-pathological characteristics of women with BC involved in this study (n=120):

Table (1): Clinic-pathological characteristics of study group

Clinic-pathological Total patients	*Number(%) N=120*
Family history	
Yes	40(33)
No	80(67)
Histological type	
IDC	80(67)
ILC	40(33)
Histological grade	
Grade 1+2	72(60)
Grade 3	48(40)
Menopausal status	
Pre	69(57.5)
Post	51(42.5)
Site of cancer	
Right	57(47.5)
Left	63(52.5)
Metastasis status	
Yes	42(35)
No	78(65)

The gene expression analysis of target and reference genes based on estimation of threshold value (Ct) for real amplification of gene of interest (GOI), HIF-1A (Genbank ID: NM_001530, 2478 nucleotides) and housekeeping gene (HKG), GAPDH (Genbank ID: NM_002046, 1005 nucleotides) in women with BC and control groups. The Ct value was calculated as average of triplicate. The results of present study suggested that significant differences between the GER in women with BC (4.58) and GER in control group (1.03) (p-value< 0.05), as shown in figure-1:

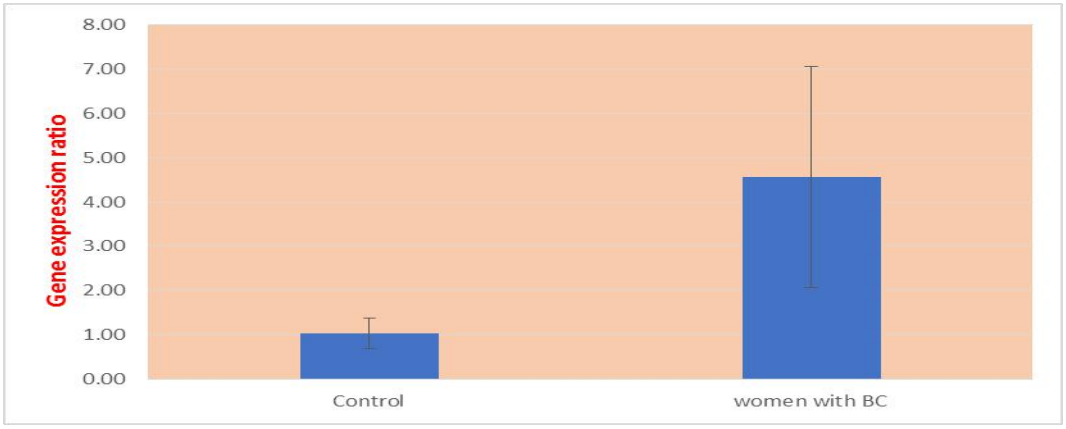


Figure (1): Comparison between GER in patients and control group

The relative quantification results of present study of HIF-1A mRNA gene expression as gene of interest (GOI-1) and GAPDH gene expression as housekeeping gene (HKG) in both women with BC and control group was show the GEF was 21.1 by applying the equation ($2^{-\Delta\Delta Ct}$) when -ΔΔCt was (-4.4). Gene Expression Fold (GEF)= $2^{-\Delta\Delta Ct}$, $\Delta\Delta Ct = (Ct_{HIF\text{-}1A} - Ct_{GAPDH})_{patients} - (Ct_{HIF\text{-}1A} - Ct_{GAPDH})_{control}$.

The amplification and melting curves of HIF-1A mRNA in patients and control groups is shown in figure-2:

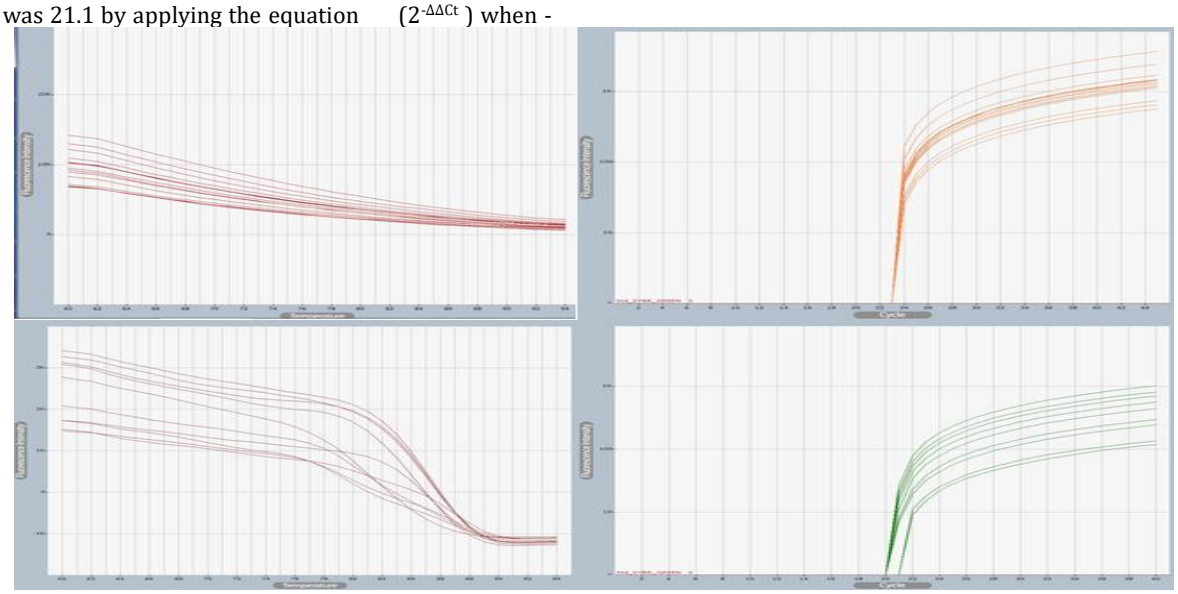
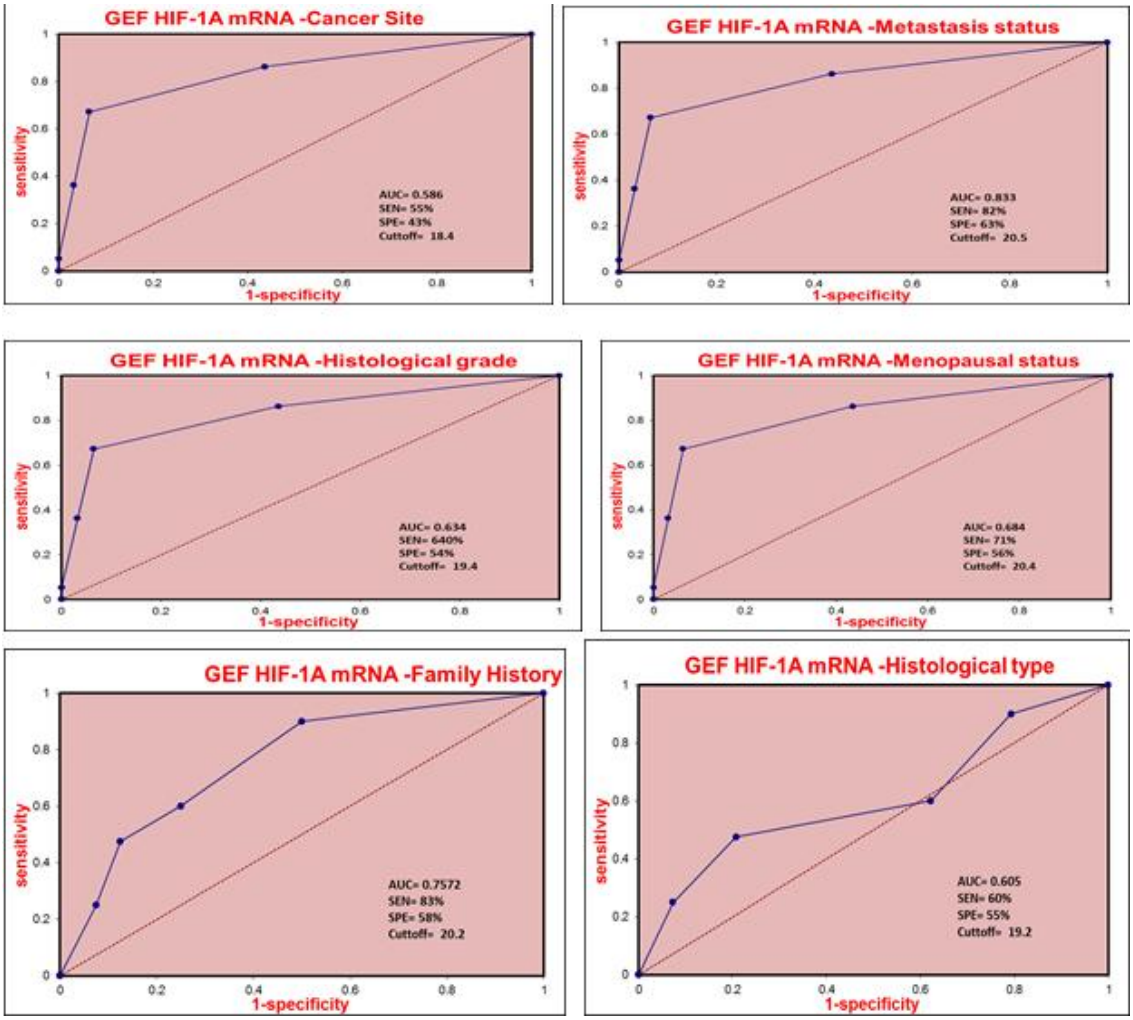


Figure (2): A,B-Amplification curves(HIF-1A and GAPDH) , C,D-Melting curve measured as -dF/dT versus temperature (°C) of both genes in cases and control.

Table 2 and figure 3 showing the AUC for ROC analysis corresponding to the diagnostic value of GEF of HIF-1A mRNA depending on clinic-pathological variables:

Table (2): AUC for ROC curve corresponding to the diagnostic value of GEF of HIF-1A mRNA

Variables	AUC	ST. ER	95%CI
Family history	0.7572	0.04	0.66-0.85
Histological type	0.605	0.05	0.49-0.71
Histological grade	0.634	0.05	0.52-0.74
Menopausal status	0.684	0.04	0.59-0.77
Site of cancer	0.586	0.05	0.48-0.68
Metastasis status	0.833	0.03	0.76-0.90



Figure(3): AUC of ROC analysis for HIF-1A mRNA GEF corresponding to Family history, Histological type Histological grade, Menopausal status, Site of cancer, and Metastasis status of women with BC

DISCUSSION

HIF-1A function is considered to be amplified by the PI3K–AKT–mTOR signal transduction pathway, but there may be unknown mediators that are not regulated in this pathway. To date, no selective HIF-1A inhibitor has been clinically approved, partially due to the requirements of targeting protein-protein interactions without affecting other pathways [15]. Clinically, HIF-1A overexpression has been shown to be a marker of highly aggressive disease and has been associated with poor prognosis and treatment failure in a number of cancers including ovarian, cervical, esophageal, and oropharyngeal cancer. Thus, mTOR as an upstream activator of HIF-1 function, is highly expected to become a prime target molecule for anticancer therapeutic strategies [16]. The results revealed that the sensitivity and specificity for diagnostic of BC by evaluation of GEF of HIF-1A mRNA of patient compare to control group were 87% and 62%, respectively. *Warwick et al., (2014)* were using ROC analysis and showed the AUC was 0.62 by assessing the body mass index and age to evaluation the risk factors of BC, lower than the AUC of present results [17]. *Chen et al., (2008)* reported that by using ROC analysis that miR-199a is involved in tumor progression and chemo-resistance in ovarian cancer by regulating IKKβ expression [18]. To the best of the present study knowledge, this is the first study reporting the association of HIF-1A mRNA expression (GEF) with the clinic-pathological profile in Iraqi women with BC, the expression of HIF-1A mRNA of metastasis status yielded a significant AUC of 0.833 (95 % CI 0.76–0.90) with a sensitivity of 82 % and specificity of 63 % from an optimal cutoff value of 20.5. The results of this study showed that the GEF of HIF-1A mRNA may be had higher sensitivity, specificity, and diagnostic value of metastasis status subgroup than other types of Iraqi women with BC. Therefore, this result may be useful in diagnosis and following up of chemotherapy treatment similar to other studies have specific prognositic markers [19-22]. ROC curve analysis is the most commonly used method for assessing the accuracy of diagnostic tests and this analysis is based on a plot of sensitivity as a function of 1-specificity [23,24]. The AUC is a measure of diagnostic accuracy such that values between 0.5 and 0.7 indicate low accuracy, values between 0.7 and 0.8 indicate moderate accuracy and values greater than 0.8 indicate high accuracy. With respect to diagnosis of metastatic BC, HIF-1A mRNA exhibited the highest level of accuracy (AUC= 0.833).

CONCLUSION

The expression of HIF-1A can be used as predictive marker for prognosis as the reduction of its expression may indicate in different clinic-pathological status such as metastasis. It can be also be targeted as treatment for metastasis status in the therapy of BC.

Authors contribution

All authors contributed equally to this manuscript.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

Acknowledgement

We thank all staff of oncology center in Merjan teaching hospital for their supported of this work.

REFERENCES

1. National Institutes of Health; National Cancer Institute (2018). Surveillance, Epidemiology, and End Results Program. Cancer stat facts: female breast cancer.
2. Matsuda T and Okuyama A (2018).Cancer incidence rates in the world from the cancer incidence in Five Continents XI. Japanese Journal of Clinical Oncology,48(2), 202-203.
3. Alwan N, Tawfeeq F, Maallah M et al (2017). The Stage of Breast Cancer at the Time of Diagnosis: Correlation with the Cinicopathological Findings among Iraqi Patients. Journal of Neoplasm; Vol. 2 (3:22); 1-10.
4. Hu Y, Liu J, Huang H (2013). Recent agents targeting HIF-1α for cancer therapy. J Cell Biochem 114:498–509.
5. Wigerup C, Påhlman S, Bexell D (2016). Therapeutic targeting of hypoxia and hypoxia-inducible factors in cancer. Pharmacol Ther. ;164:152–169.
6. Luo D, Wang Z, Wu J, Jiang C, Wu J (2014). The role of hypoxia inducible factor-1 in hepatocellular carcinoma. Biomed Res Int. ;2014:409272.
7. Warfel N.A., Dolloff N, Dicker D, Malysz J, El-Deiry W (2013). CDK1 stabilizes HIF-1alpha via direct phosphorylation of Ser668 to promote tumor growth. Cell Cycle.; 12:3689–3701.
8. Dengler V, Galbraith M, Espinosa J (2014). Transcriptional regulation by hypoxia inducible factors. Crit Rev Biochem Mol Biol 49:1–15.
9. Geng H, Liu Q, Xue C, David L, Beer T, Thomas G, Dai M, Qian D (2012). HIF1alpha protein stability is increased by acetylation at lysine 709. J. Biol. Chem. 287:35496–35505..
10. Kim Y, Nam H, Park D, Kim C, Yu Y, Kim D, Park S, Bhin J, Hwang D et al (2016). Methylation-dependent regulation of HIF-1alpha stability restricts retinal and tumour angiogenesis. Nat. Commun. 7:10347..
11. Liu X, Chen Z, Xu C, Leng X, Cao H, Ouyang G, Xiao W (2015). Repression of hypoxia-inducible factor alpha signaling by Set7-mediated methylation. Nucleic Acids Res. 43:5081–5098.
12. Lee J, Park J, Choi H, Won H, Joo H, Shin D, Park M et al (2017). LSD1 demethylates HIF1alpha to inhibit hydroxylation and ubiquitin-mediated degradation in tumor angiogenesis. Oncogene. 2017; 36:5512–5521.
13. Lee J, Kim Y, Bhin J, Shin H, Nam H, Lee S, Yoon J (2011). et al. Hypoxia-induced methylation of a pontin chromatin remodeling factor. Proc. Natl. Acad. Sci. U.S.A. 108:13510–13515.
14. Hajian-Tilaki K. (2013). Receiver Operating Characteristic (ROC) Curve Analysis for Medical Diagnostic Test Evaluation. Caspian journal of internal medicine, *4*(2), 627–635.
15. Rankin, EB and Giaccia A (2016). Hypoxic control of metastasis. Science. 352, 175–180.
16. Georgina N and Wei Li (2015). HIF-1α pathway: role, regulation and intervention for cancer therapy. Acta Pharm Sin B. 5(5): 378–389.
17. Warwick J, Birke H, Stone J et al (2014). Mammographic breast density refines Tyrer-Cuzick estimates of breast cancer risk in high-risk women: Findings from the placebo arm of the International Breast Cancer Intervention Study I. Breast Cancer Res. 16(5): 451.
18. Chen R, Alvero AB, Silasi DA, et al (2008). Regulation of IKK beta by miR-199a affects NF-kappaB activity in ovarian cancer cells. Oncogene. 27:4712–23.
19. Farah A. Ashour, Moaed E. Al-Gazally, Monem M.Alshok. Association between Lipoprotein Lipase Polymorphism and the Myocardial Infarction in Patients with Diabetes Mellitus Type 2. IJPHRD, 2018, 10(2).653-658.
20. Al-Hussainy, A.D.O., Al-Gazally, M.E., Ewadh, W. Oxidative stress, Anti-melanocyte and anti tyrosinase antibody in vitiligo and response to treatment, Annals of Tropical Medicine and Public Health, 2020, 23(5), 500-507.
21. Al-Gazally, M.E., Al-Awad, A.S., Kzar, H.H. Study some of the risk factors on total antioxidant capacity in Iraqi patients with sporadic colorectal cancer, Journal of Global Pharma Technology, 2018, 10(3), 48-55.
22. Raghad, N.A., Al-Gazally, M.E., Ewahd, W.A. The effect of dihydrotestosterone hormone on the sulfotransferase 1A1 enzyme level in the scalp hair follicle in patients with androgenic alopecia and its effect on response to minoxidil, Journal of Global Pharma Technology, 2017, 9(12), 179-183.
23. Hamzah H. Kzar , Moshtak A. wtwt , Moaed E. Al-Gazally (2020). Study the Glucose Transport, Angiogenesis and Apoptosis Behavioral through Chemotherapy Treatment According to Receptors Status in Women with Breast Cancer. Indian Journal of Forensic Medicine & Toxicology, 14(3), 2555-2559.
24. Ali SH, AL-Azawi RSA, Kzar HH. Study the IL6 (C174G) Promoter SNP and Correlation with Physiological Growth Hormone and TNFA levels in Iraqi Subjects with Psoriasis. SRP. 2020; 11(9): 272-276.