BJMHR
British Journal of Medical and Health Research
Journal home page: www.bjmhr.com

HIF-1α, MMP-1 & MMP-9: A prognostic tool for early breast cancer detection

El Khatib G[1], Antoun S[1], Salloum E[1, 2], Irani J. [2,3], Anastasiades E. [2,3], Ghandour F[2], El Hajj I[2, 3], Chalhoub E[1*]

1.University Of Balamand, Faculty of Health Sciences, Medical Laboratory Sciences Program Achrafieh, Beirut 1100-2807, Lebanon
2.Saint George Hospital – University Medical Center
3.University Of Balamand, Faculty Of Medicine

ABSTRACT

Breast cancer is one of the most malignant cancers responsible for women death worldwide. Approximately half of them will eventually develop metastases. With early detection, chances of cure increases with a long term survival over 95%. Tumor cells secrete chemotactic factors capable of attracting monocytes to the site of inflammation. Once recruited, monocytes become under direct influence of malignant cells and differentiate into Tumor-Associated Macrophages able to secrete different pro-angiogenic factors, an important extracellular matrix change that enables tumor growth. The purpose of this study is to investigate the relation between expression levels of selected biomolecules: HIF-1α, MMP-1, MMP-9 and cancer progression. Breast biopsies were collected from patients with four different grades of severity (Grade 0 - Grade 3). cDNA's was synthetized and quantified using Q-RT-PCR. Correlation between the expression level of these biomolecules and cancer progression / staging was done using statistical tests. Our results showed that expression levels of HIF-1α were significantly higher in G1 compared to G0; MMP-1 and MMP-9 were highly expressed at more advanced stages. As sensitivity/specificity statistical test confirmed, a sequential test is recommended in which HIF-1α is used as a marker for early stages of breast cancer, MMP-1 for determining G2 and G3 stages, and MMP-9 to confirm G3 staging.

Keywords: HIF-1α, MMP1, MMP9, Breast Cancer

*Corresponding Author Email: elias.chalhoub@balamand.edu.lb
Received 2 June 2016, Accepted 26 June 2016

Please cite this article as: Chalhoub E *et al.*, HIF-1α, MMP-1 & MMP-9: A prognostic tool for early breast cancer detection. British Journal of Medical and Health Research 2016.

INTRODUCTION

Cancer, a leading cause of death in the industrialized world, results from the progressive growth of progeny from a single transformed cell. In Lebanon, 69,5/100,000 women are diagnosed with breast cancer yearly [1] In the US, an estimated 40,030 breast cancer deaths (39,620 women, 410 men) were expected in 2013 [2].

A decrease of breast cancer death rates of 3% per year in women younger than 50 and 2% in women older than 50 years was noticed between 2005 and 2009, due to earlier detection and improved treatment. Despite progress made in the last 30 years in breast cancer screening and treatment, approximately half of diagnosed patients will eventually develop metastases. With early detection, the chances of cure increase since at early stages long term survival is over 95% [2].

The progression and survivorship of a formed solid tumor depend on several mechanisms such as the ability of the tumor to get nutrition and oxygen and its ability to eliminate cell wastes [3]. Solid tumors do not grow and develop in isolation; for their viability, tumors rely on the help of angiogenic factors secreted by non-tumoral surrounding cells including fibroblasts, endothelial cells and inflammatory cells such as monocytes-derived cells. The interactions between these cells are crucial to each step of tumorigenesis. Chemokines secreted by inflammatory cells are over expressed in tumor epithelial and fibroblast cells respectively; they enhance tumor cell proliferation, migration, and invasion and promote angiogenesis and metastatic spread [4; 5].

This study aims at describing the expression level of three biomolecules HIF-1α, MMP-1 and MMP-9 among a sample of breast cancer patients and according to breast cancer grades.

MATERIALS AND METHOD

97 paraffin embedded breast tissues gathered during 5 years (2004-2009) were collected from the Pathology laboratory at Saint George Hospital-UMC in Beirut Lebanon. Patients whose breast cancer is not primary and patients who suffer from other types of cancer, any chronic inflammation or disease were excluded from the study. Patients were subdivided into 4 groups according to clinical stages of invasive breast cancer: 12 samples at Grade 0-no malignancies detected, 19 at Grade 1-well differentiated tumor, 33 at Grade 2-moderately differentiated and 33 at Grade 3-poorly differentiated.

Clinical data:

Members of the research team filled a questionnaire and looked at pathology clinical data for each patient. Risk factors were calculated using the Gail model. An Informed consent was

obtained and all personal information were kept secure. This study was approved by the Institutional Review Board of Saint-George Hospital-UMC.

Paraffin Embedded Biopsies:

RNA extraction:

Breast biopsies were cut into four sections of 10µm each. Paraffin was removed by 1ml of xylene and washed with 1ml of ethanol 100%. Total RNA was extracted using RNeasy FFPE kit from Qiagen according to manufacturer protocol. Total RNA was eluted with 25µl of RNAse/Dnase free water. Presence, purity and yield of each sample were detected using NanoDrop ND1000 spectrophotometer and ND 1000 v3.5.2 software.

Two steps RT-PCR:

To perform cDNA synthesis, 1µl of oligo($dT_{)18}$ (0.5µg) were added to 12µl of extracted RNA. The reaction was carried on as described with the RevertAid™ M-MuLV Reverse Transcriptase kit of Sigma. cDNA were quantified by NanoDrop ND1000.

The quantification of the level of expression was performed using the QuantiFast™ SYBR® Green PCR kit-Qiagen and the MxPro- Mx3000P software, as listed in table1. Each sample was run in triplicate. The expression level of the housekeeping gene, *GAPDH* was used to calculate the relative amount of the target cDNA.

Table 1: Primer sequences and cycling conditions for real-time quantification analysis

Primers	Sequences	Annealing/extension	temperature(°C)
HIF-1α (F)	5'-TGCTCATCAGTTGCCACTTCC-3'		62
HIF-1α (R)	5'-CCAAATCACCAGCATCCAGAAGT-3'	(13)	
MMP-1 (F)	5' 5'-AAGGCCAGTATGCACAGCTT-3'		58
MMP-1 (R)	5'-TGCTTGACCCTCAGAGACCT-3'	(11)	
MMP-9 (F)	5'-CCTGCCAGTTTCCATTCATC-3'		57
MMP-9 (R)	5'-GCCATTCACGTCGTCCTTAT-3'	(11)	
GAPDH (F)	5′- AACCTGCCAAATATGATGAC -3′		62
GAPDH (R)	5′- TTGAAGTCAGAGGAGACCAC -3′	(14)	

General cycling conditions : Denaturation step at 95°C for 5 min. two steps cycling for 35 cycles: Denaturation at 95°C /10 seconds. Combined annealing/extension / 30 seconds

Statistical analysis:

We used classical univariate descriptive analysis using means and standard deviations to summarize our quantitative data. We used non-parametric tests (Mann Whitney and Kruskal Wallis) to compare levels of expression of our studied markers according to Grades. p-value below 0.05 was considered significant. All analysis was performed using SPSS V20.

RESULTS AND DISCUSSION

Real Time PCR:

HIF-1α

Hypoxia Inducible Factor-1α was amplified in all samples. The expression variations of HIF-1α were illustrated in figure 1a, and showed a gradual increase of this biomarker according to different tumor grades from $9.53E^{-03}$ at G0 to $1.93E^{+00}$ at G3. An increase 140 folds is detected between grade G0 and grade G1 (p =0.007), while its expression remained almost stable during the remaining grades ($1.34E^{+00}$ at G1, to $1.93E^{+00}$ at G3).

MMP-1

The means of the relative concentrations of MMP-1 cDNA was transferred into the bar charts figure 1b. It varies between $2.49E^{-03}$ and $8.26E^{-01}$. The expression of MMP-1 increased ≈18.5 folds between G0 and G1 and ≈6 folds between G1 and G2 (p = 0.558); and it raised ≈3 folds between G2 and G3.

MMP-9

The means of the relative concentrations of MMP-9 cDNA was transferred into the bar charts figure 1c. The relative expression of MMP-9 during G3 is eliminated from the chart in figure 1d to shed light on the expression dissimilarity between G0, G1 and G2. The results below showed an important expression of MMP-9 at G3 of $6.10E^{+03}$ ng/µl compared to all other grades; with p value of MMP-9 expression between G2 and G3 was estimated to be 0.030. It increases ≈$3x10^3$ folds between G0 and G1 and decreases ≈30 folds between G1 and G2.

Specificity and sensitivity test:

To determine the rate of false positive/negative results that could arise from the data above, a characteristic test of sensitivity and specificity was performed. Sensitivity was defined as the true positive rate and specificity as the negative one. In the aim of limiting potential diagnostic error, false negative samples should be reduced to the max, such low levels can be achieved using an error cut of 3-5% generating a sensitivity of 98.6%, 95.7% and 95 % respectively for our three markers mixed with a specificity of >95%.

Using this approach on our data base, it showed no false negative results for HIF-1α; meaning that all biopsies classified as pathologically negative did not show any expression of this biomarker at a level equal or above $3E^{-02}$ (mean of expression level of HIF-1α at G1 is $1.34E^{+00}$). These negative samples for HIF-1α seemed also to be negative at the molecular level for the two MMPs. It is important to note that Patients age ranged between 22 and 84 at diagnosis (average of 50.62).

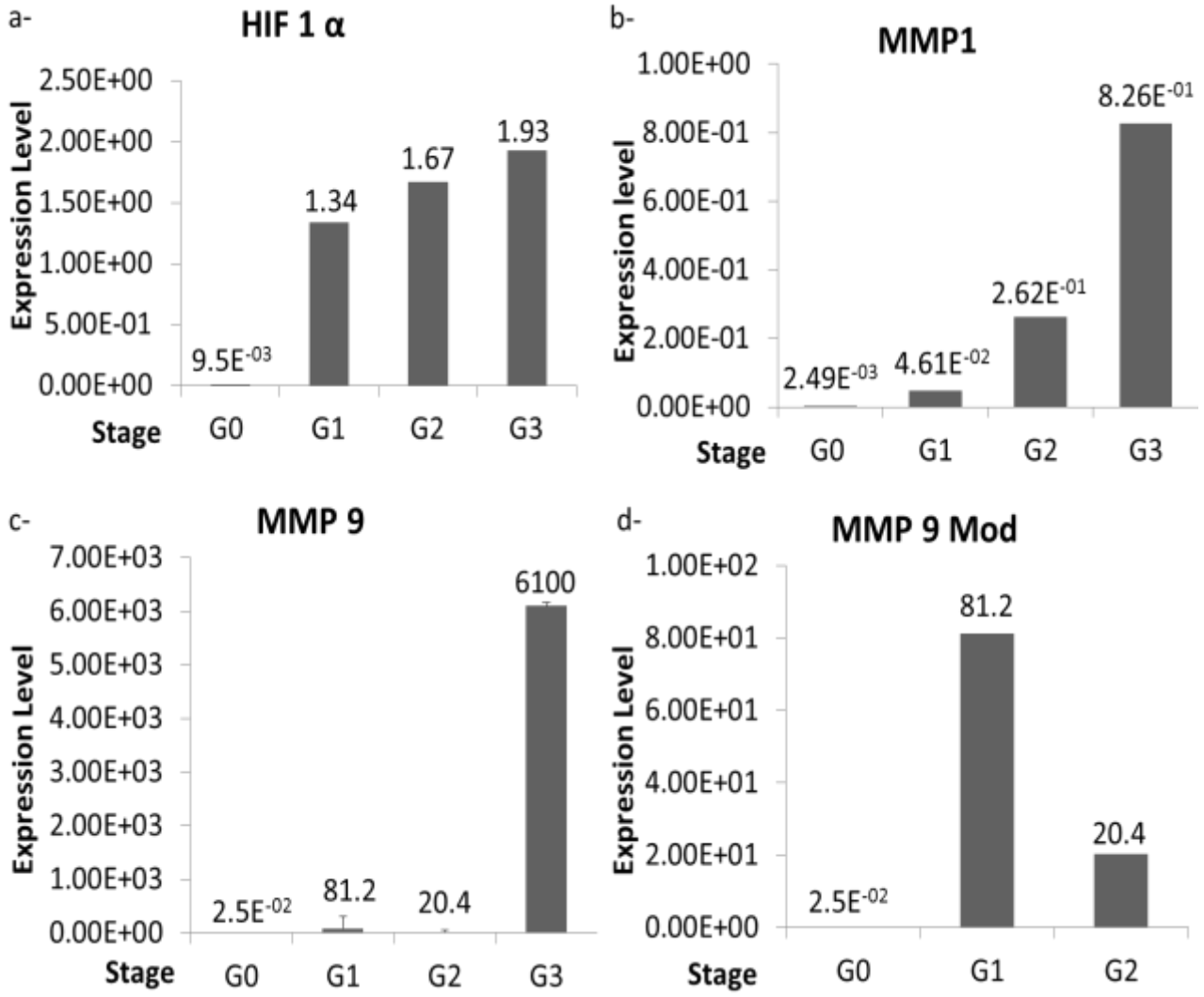

Figure 1: a. Variations of the level of expression of HIF-1*α*. b. Mean variations of the level of expression of MMP-1. c. Mean variations of the level of expression of MMP-9 between G0, G1, G2 and G3. d. Mean variations of the level of expression of MMP-9 between G0, G1 and G2.

DISCUSSION:

MMP-1 and MMP-9 as staging markers:

MMPs, secreted mainly by immune cells, may promote angiogenesis by two different mechanisms: either by degrading the ECM barriers and allowing cell invasion or by secreting factors that promote and maintain angiogenesis. Two types of matrix metalloproteinases are important in ECM degradation and analyzed in this study: MMP-1, a collagenase that has a preference to degrade collagen type III, an ECM substrate; and MMP-9, a gelatinase B that cleave type IV collagen of ECM basement membrane. They are correlated with poor outcome of breast cancer patients.[16; 17].

In this study, MMP-9 increased 3235 folds between G0 and G1, 75 times between G2 and G3 (p = 0.030) and 243 017 folds between G0 and G3. It was expected that the level of

expression of MMP-9 will be at its highest point at G3 where it plays an important role in facilitating cancer invasion. It correlates with VEGF by stimulating the degradation of ECM and releasing the sequestered VEGF. MMP-3, MMP-7, MMP-9 and MMP-12, can degrade plasminogen, generating the angiogenesis inhibitor angiostatin[6,7,10]. Thus, MMP-9 cooperates with Integrin modulating ligand recognition, maximizing tumor cell motility, migration and invasion. Integrin activation caused enhanced breast cancer cell migration toward fibronectin, vitronectin, and fibrinogen. MMP-9 initiates the degradation and the release of this latter substrate attracting more tumor cells as a potent chemotaxins for neutrophils[15]. These variations in the level of expression illustrated the profile of expression of MMP-9 during cancer progression and were compatible with previous data where a higher amount of mRNA was detected in G3 when compared to G2 [11].

On the other hand, MMP-1 was shown to cleave and activate the protease activated receptor 1 (PAR-1) identified as an oncogene, a G-protein-coupled receptor, which induce invasiveness and breast cancer tumorigenesis. MMP-1 and MMP-3 release FGF, a potent endothelial mitogen that bound to ECM [8,10]. In this work, MMP-1 expression level seemed to be correlated with tumor grades and increased 331 folds between G0 and G3 and 18 folds between G1 and G3. There are no significant differences in its expression between G2 and G3, confirming already published data[11]. This could be explained by an increase in the invasion rate and aggressiveness of breast cancer at stage G2 and G3. The profile of expression of MMP-1 is associated proportionally with tumor grades; however the expression of MMP-9 rose at early stage of cancer (G1), subsequently declined at G2 then increased abruptly at G3. These two metalloproteinases had a higher expression at G3 higher rate of invasion and were involved in metastasis and could be implicated in the degree of aggressiveness, proving that MMPs may have a role in detecting patients with high risk of recurrence [16]. Both MMP-1 and MMP-9 showed no post-transcription regulation (data not shown); a proportional elevation was confirmed between mRNA and protein by ELISA and Western Blot in previous studies [11]. We could estimate a gradual role of MMP-1 in cancer development while the main function of MMP-9 arose at G3, a crucial step of invasion were the level of expression of MMP-9 is highly considerable compared to MMP-1.

HIF-1α **a potential marker for early onset of breast cancer:**

One of the major causes of cancer development is hypoxia through over-expression of two Hypoxia Inducible Factors HIF-1α and HIF-2α that induce VEGF formation and release. However, a balance between the stimulating and tumor inhibiting level of HIF protein is essential for optimal tumor growth. Published studies showed that the most aggressive cancer lines has the lowest expression of HIF protein, the smallest increase in VEGF mRNA and the

lowest induction of VEGF protein[12]. Both HIF-1 subunits belong to helix-loop-helix periodic acid Schiff proteins [12]. In low oxygen tension media, HIF-1α is stabilized, translocate to the nucleus where it form a dimer with HIF-1β and activated transcription of essential genes for tumor progression and development [18].

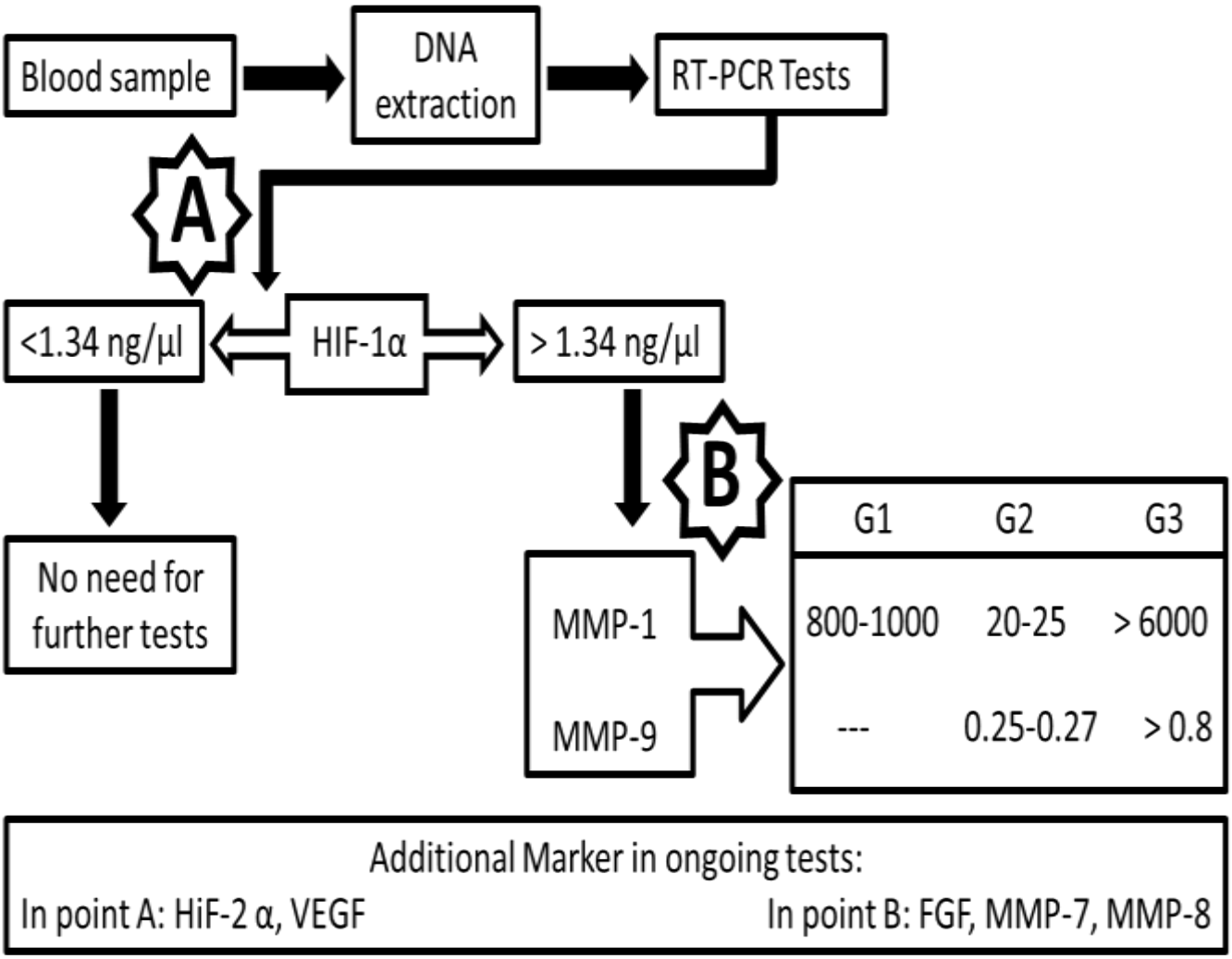

Figure 2: Proposed sequential blood test

This study showed that HIF-1α was highly expressed after breast cancer diagnosis at G1, and that its expression remains approximately at the same level throughout (*p=0.629)*. However, the raise of its expression 140 folds between G0 and G1 (p = 0.007) indicate an important role in early onset of breast cancer. Deniz et *al.* [19] showed that HIF-1α is associated with tumor grade and pattern of growth in urothelial carcinoma of the upper urinary tract. The regulation at the protein level (data not shown) has also been confirmed in previous work that an up-regulation of mRNA expression of HIF-1α correlates with higher protein expression [20]. Knowing that despite the fact that HIF proteins are necessary for optimal tumor growth and angiogenesis *in vivo*, over-expression of these molecules could also be detrimental to tumor growth [12]. A correlation between HIF-1 over-expression and patient mortality, poor prognosis, or treatment resistance has been noted in many studies [21]. Consequently, the

previous results may estimate a higher expression of HIF-1α mRNA at G1, G2 and G3 compared to G0 and could be used as a potential marker for early onset of breast cancer.

Based on all the data the above, and further to all Sensitivity/specificity test results; we suggest the following sequential blood test to determent breast cancer grading (figure 2).

CONCLUSION:

Breast tumor is one of the most frequent and aggressive type of cancers. Under stress and hypoxia conditions, it expresses HIF-1α that affects neo-angiogenesis and necessitates the secretion of clusters of growth factors and chemokines such as MMP-1/9, which facilitate invasion and metastasis [15; 16]. We come with this article proposing a sequential blood test as a prognostic tool for early breast cancer detection. We suppose that, further to clinical validation on a higher number of cases and ongoing additional marker tests, it could be proposed as an alternative and more accurate prognostic tool than the one currently in use.

ACKNOWLEDGEMENTS

We thank Dr. Nadim Karam, Dean of the Faculty of Health Sciences at the University Of Balamand for his continuous support. We are indebted to the Saint George Hospital University Medical Center Pathology laboratory for their help in selecting and cutting the Biopsies.

SOURCES OF FUNDING

G.K and E.S were supported by successive grants from The Balamand Internal Research Grant BIRG fund

REFERENCES

1- Shamseddine, A & Musallam K (2010). Cancer Epidemiology in Lebanon. Middle East Journal of Cancer; 1(1): 41-44.
2- American Cancer Society (2013). *Cancer Facts and figures 2013* [Report].
3- Milliken, D. *et al.* (2002). Analysis of Chemokines and Chemokine Receptor Expression in Ovarian cancer ascites. *Clinical Cancer Research,* 8, 1108–1114.
4- Gasparini, G. (1995). Biological and clinical role of angiogenesis in breast cancer. *Breast Cancer Research and Treatment*. 36, 103-107.
5- Edythe C. Real breasts, real health: the only A-B-C guide book for surgical & non-surgical breast augmentation using the p-method. [n.p.]: Real Health Method e-Books; 2004.
6- Hagemann, T., *et al.,* (2004). Enhanced invasiveness of breast cancer cell lines upon co-cultivation with macrophages is due to TNF-a dependent up-regulation of matrix metalloproteases. *Carcinogenesis.* 25, 1543-1549.

7- Boudreau, N. & Myers, C. (2003). Breast cancer-induced angiogenesis: multiple mechanisms and the role of the microenvironment. *Breast Cancer Research*, 5, 140-146.

8- Nguyen, N. *et al.* (2006). Tumor-derived Cyr61 (ccn1) promotes stromal matrix metalloproteinase-1 production and protease-activated receptor 1–dependent migration of breast cancer cells. *American Association for Cancer Research*, 66(5), 2658-2666.

9- Ke, Q., & Costa, M. (2006). Hypoxia-inducible factor-1 (hif-1). *Molecular pharmacologist*, 70, 1469–1480.

10- Duffy, MJ. e*t al.* (2000). Metalloproteinases: role in breast carcinogenesis, invasion and metastasis. *Breast cancer Research*, 2, 252–257.

11- Köhrmann, A. *et al.* (2009). Expression of matrix metalloproteinases (MMPs) in primary human breast cancer and breast cancer cell lines: new findings and review and breast cancer cell lines: new findings and review. *BMC journal*, 9, 188-208.

12- Blancher, C. *et al.* (2000). Relationship of hypoxia-inducible factor (hif)-1a and hif-2a expression to vascular endothelial growth factor induction and hypoxia survival in human breast cancer cell lines. *Cancer Research*, 60, 7106–7113.

13- Catrina, SB. *et al.,* (2006). Hypoxia-inducible factor-1aand hypoxia-inducible factor-2a are expressed in kaposi sarcoma and modulated by insulin-like growth factor-i. *American Association for Cancer Research*, 12 (15), 4506-4514.

14- Rizzi F, *et al.* (2008). A Novel Gene Signature for Molecular Diagnosis of Human Prostate Cancer by RTqPCR. *PLoS ONE* 3 (10), 3617-3625.

15- Rolli, M. *et al.,* (2003). Activated integrin v3 cooperates with metalloproteinase mmp-9 in regulating migration of metastatic breast cancer cells. *PNAS*, 100 (16), 9482–9487.

16- Kerkelä, E., & Saarialho-Kere, U. (2003). Matrix metalloproteinases in tumor progression: focus on basal and squamous cell skin cancer. *Experimental Dermatology*, 12, 109–125.

17- Eiseler, T. *et al.* (2009). Protein kinase D1 regulates matrix metalloproteinase expression and inhibits breast cancer cell invasion. *Breast Cancer Research*, 11, 1-12.

18- Pugh, CW., Gleadle, J., & Maxwell, P. (2001). Hypoxia and oxidative stress in breast cancer hypoxia signaling pathways. *Breast Cancer Research*, 3, 313–317.

19- Deniz, H. (2010). Evaluation of relationship between HIF-1α immune-reactivity and stage, grade, angiogenic profile and proliferative index in bladder urothelial

carcinomas. *Interior Urology and nephrology*, 42, 103-107.

20- Jiang BH, *et al.* (1997). V-SRC induces expression of hypoxia-inducible factor 1 (HIF-1) and transcription of genes encoding vascular endothelial growth factor and enolase 1: involvement of HIF-1 in tumor progression. *Cancer Research*, 57, 5328–5335.

21- Semenza GL (1998) Hypoxia-inducible factor 1: master regulator of O2 homeostasis. *Current Opinion of Genetic Development, 8***,** 588–594.

BJMHR is

- **Peer reviewed**
- **Monthly**
- **Rapid publication**
- **Submit your next manuscript at**

editor@bjmhr.com