© IJHG 2020 Int J Hum Genet, 20(1): 19-24 (2020)
PRINT: ISSN 0972-3757 ONLINE: ISSN 2456-6330 DOI: 10.31901/24566330.2020/20.01.746

Thymoquinone Down-regulates VEGFA and Up-regulates FLT1 Transcriptional Levels in Human Breast Cancer Cells

Z. Yegin[1*], T. Duran[2] and I.H. Yildirim[3]

[1]Medical Laboratory Techniques Program, Vocational School of Health Services, Sinop University, Sinop, Turkey
[2]Department of Medical Genetics, Faculty of Medicine, KTO Karatay University, Konya, Turkey; Department of Medical Genetics and Molecular Biology, Institute of Health Sciences, Faculty of Medicine, Kocaeli University, Kocaeli, Turkey
[3]Department of Genetics, Faculty of Veterinary, Dicle University, Diyarbakir, Turkey

KEYWORDS Angiogenesis. Breast Tumor. MCF-7 Cells. Thymoquinone. Transcription

ABSTRACT Angiogenesis is important for cancer progression index and angiogenesis factors related to tumorogenesis deserve to be investigated in detail. The use of minimally toxic phytochemical compounds as the new generation anticancer agents is an appreciated approach to manage angiogenesis factors. The purpose of this study was to investigate the potential effects of thymoquinone (TQ), the major constituent of the black seed, on the expression levels of VEGFA and its receptor FLT1 in human estrogen receptor-positive breast adenocarcinoma (MCF-7) cells. The researchers provide evidence that TQ down-regulated VEGFA and up-regulated FLT1 transcriptional levels in human breast cancer cells compared to HEK293 cells. To the best of the researchers' knowledge, this is the first study determining the effect of TQ in VEGFA and its receptor FLT1 in MCF-cells and more comprehensive investigations are highly recommended.

INTRODUCTION

Globally, breast cancer (BC) is the most common cancer among women, representing nearly 25 percent of all cancers. Incidence rates change greatly across the world, from 25 per 100,000 in Middle Africa and Eastern Asia to 92 per 100,000 in Western Europe and according to the estimates of World Health Organization (WHO), BC rates in the Middle East which is now 45.3 per 100,000 females are expected to double between 2012 and 2030 (Naja et al. 2019). Based on GLOBOCAN 2018, the widespread cancers in the Eastern Mediterranean Region are breast, colorectal, lung, liver, and bladder cancer and among women BC occupies 34.7 percent (Pourghazian et al. 2019). Though BC incidence increases with age, nearly 7-10 percent of women diagnosed are younger than age 40 (Rossi et al. 2019). Thus, developing new molecular strategies targeting breast tumors is very significant.

Angiogenesis is an important process in cancer growth and metastasis. It is a complicated mechanism including extracellular matrix remodelling, endothelial cell migration and proliferation, capillary differentiation and anastomosis. Vascular endothelial growth factor (VEGF) is one of the most important angiogenesis factors and the binding of VEGF to its receptors (VEGFRs) on endothelial cells and stromal fibroblasts plays a key role in angiogenesis (Seto et al. 2006; Hlobilkova et al. 2009). Tumor cells overexpress the ligand VEGFA (also known as VEGF) which has been identified as a predominant regulator of tumor angiogenesis. Afterwards, VEGF can bind to its receptors such as FMS-related tyrosine kinase 1 (FLT1, VEGFR1) and kinase insert domain receptor (KDR, VEGFR2) to initiate downstream signaling and angiogenesis. Although FLT1 has a high affinity for VEGFA (10-fold higher affinity than KDR), its angiogenic effects are weaker (Seto et al. 2006; Zhang et al. 2015a).

The present study aimed to analyze the varied expression levels of angiogenesis factors in MCF-7 cells with the use of TQ. The use of phytochemicals with minimal side-effects is a quite novel approach in preventing and controlling cancer. TQ is the major biologically active component of *Nigella sativa* and is commonly used in the Middle East both as a spice and in traditional medicine. TQ displays antimicrobial, antiparasitic, anti-inflammatory, antioxidant and immunomodulatory properties. It also has signifi-

*Address for correspondence:
E-mail: zyegin@sinop.edu.tr

cant antineoplastic activity and protects non-tumor tissues from chemotherapy-induced damage (ElKhoely et al. 2015). The role of TQ in inhibiting major oncogenic transcription factors such as NF-κB, STATs, Nrf2/ARE and Wnt/β-catenin was reported in diverse cancer models (Shanmugam et al. 2018). A strong synergism inducing apoptosis, necrosis and autophagy against both MCF-7 and breast ductal carcinoma (T47D) cells was shown between gemcitabine and TQ (Bashmail et al. 2018). TQ augmented the effect of cyclophosphamide in Her2 + and Her2- breast cancer cell lines (Khan et al. 2019). TQ-mediated molecular mechanisms in triple-negative breast cancer (TNBC) which is the most aggressive and chemoresistant subtype of breast cancer were investigated and decreased TNBC cell proliferation and migration/invasion regulated partially by NF-κB/miR-603/eEF-2K signaling were shown (Kabil et al. 2018). The in vivo therapeutic potential of TQ-Pac combination in managing multiple cascades including extrinsic apoptosis, tumor suppressor genes, and p53 signaling was previously shown in TNBC (Sakalar et al. 2016).

Objectives

The researchers aimed to investigate the effects of thymoquinone at the molecular level by assessing the transcriptional levels of angiogenesis factor VEGFA and its receptor FLT1 in MCF-7 and HEK-293 cell lines.

MATERIAL AND METHODS

Cell Lines and Culture Conditions

Human estrogen receptor-positive breast adenocarcinoma cell line (MCF-7) and as control human embryonic kidney cell line (HEK293) were used in this study. MCF7 (ATCC® HTB-22™) and 293T (ATCC® CRL-3216™) cell lines were purchased from the American Type Culture Collection (Manassas, VA). MCF-7 cells were cultured in Dulbecco's modified Eagle's medium with 10 percent fetal bovine serum and 1percent PBS antibiotics in a 5 percent CO_2 humidified incubator at 37°C. HEK293 cells were cultured in the same supplements and conditions as in MCF-7 cells. Cell lines were serially passaged following trypsinization by 0.05% trypsin/0.02% EDTA.

MTT Proliferation Assay

The viability and proliferation of MCF-7 and HEK293 cells were quantified by standard Methyl-thiazolyl-tetrazolium (MTT) proliferation assay. MCF-7 and HEK293 cells were seeded in 96-well tissue culture plates at a density of about $5x10^3$ cells per well with 100 µl of culture medium and cells were cultured for 48 hr. Then, thymoquinone was applied to wells at 15 µM, 25 µM, and 35 µM concentration for 24, 48 and 72 hours. Thymoquinone treated and untreated cells were subjected to 10 µl of 12 mM MTT solution and incubated at 37°C for 4 hours. Afterwards, the media were removed and replaced with 50 µl of DMSO and incubated for 20 minutes with gentle shaking to dissolve the crystal blue melts. The absorbance at 540 nm was recorded by using microplate reader and IC_{50} dosage (represents the concentration of thymoquinone that exhibited 50 percent cell viability for MCF-7 and HEK293 cells in vitro) was recorded.

Analyses of VEGFA and FLT1 mRNA Expression Levels

MCF-7 and HEK293 cells were seeded and treated with 25 µM TQ for 48 hours. Total RNAs were isolated with Trizol reagent and treated with RNase-free DNase I to remove all potential genomic DNA molecules. RNA concentrations were quantified with UV-VIS spectrophotometer and the integrities of the molecules were evaluated with the gel image of 28S/18S rRNA bands. First-strand cDNA was synthesized from 100 ng total RNA with oligo (dT)18 primer using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Lithuania) in a total volume of 20 µl. cDNAs encoding target genes (VEGFA, FLT1) and endogenous control (GAPDH) were amplified with Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Lithuania) using Light Cycler 480 analyzer. To eliminate possible genomic DNA and reagent contaminations, RT- (reverse transcriptase minus) and NTC (no template control) were included in all the reactions performed. Real-time PCR experiments for both target genes and internal control were performed in triplicate.

Statistical Analysis

SPSS version 21 was used for statistical analysis. According to the results of student's t-test, comparisons for the analyzed genes were performed. The levels of VEGFA and FLT1 expressions were analyzed by the 2–ΔΔCt method developed by Livak and Schmittgen (2001).

RESULTS

Comparisons between cell lines in terms of the analyzed genes were made according to the results of Student's t- test. The results for FLT1 were as follows: $p<0.000$ for MCF-7 and HEK293 cell comparison, $p=0.014$ for MCF-7 untreated and TQ-treated cells, $p=0.399$ for HEK293 untreated and TQ-treated cells. These results clearly show the effects of TQ on MCF-7 cells but not on HEK293 cells for FLT1 gene. The results for VEGFA were as follows: $p=0.011$ for MCF-7 and HEK293 cell comparison, $p<0.000$ for MCF-7 untreated and TQ-treated cells, and $p<0.000$ for HEK293 untreated and TQ-treated cells. Thus, it can be said that TQ alters the transcriptional levels of VEGFA both on MCF-7 and HEK-293 cell lines.

Effect of Thymoquinone on Cell Viability

As shown in Table 1, TQ was applied to wells at ranging concentrations of 15 μM, 25 μM, and 35 μM at different time intervals. The IC_{50} concentration (producing half-maximal inhibition) at 24 h was found as 25 μM.

Thymoquinone Treatment Down-regulates VEGFA Expression in MCF-7 Cells

Inhibition of VEFGA has been targeted as a promising strategy for cancer therapy. As shown in Table 2, TQ enormously down-regulated the transcriptional level of VEGFA compared with the HEK-293 cells.

Thymoquinone Treatment Up-regulates FLT1 Expression in MCF-7 Cells

Though the effects of TQ in terms of expression was not as striking as in the example of VEGFA, compared with the control HEK-293 cells, a substantial increase in the expression of FLT1 in MCF7 cells was recorded (Table 2).

Table 1: Results of the MTT Assay

		Thymoquinone administration durations		
		24 hr	*48 hr*	*72 hr*
Concentration	0 μM	0.323±0.024	0.463±0.0628	0.293±0.082
	15 μM	0.320±0.073	0.233±0.0273	0.245±0.028
	25 μM	0.368±0.090	0.267±0.0237	0.293±0.077
	35 μM	0.290±0.072	0.293±0.0311	0.231±0.044

*MTT assays were performed six times for each sample. The mean values and standard deviations were given in the Table.

Table 2: Results of gene expression studies

	MCF-7 not exposed		*MCF-7 exposed*				*HEK293 not exposed*		*HEK293 exposed*			
	Mean	*Standard deviation*	*Mean*	*Standard deviation*	*Difference of the means*	*p*	*Mean*	*Standard deviation*	*Mean*	*Standard deviation*	*Difference of the means*	*p*
FLT1	17.79	0.70	15.23	0.34	-2.56	0.014	23.7	0.25	18.05	0.10	-5.65	0.399
VEGFA	19.25	0.30	31.52	0.61	12.27	<0.000	24.62	0.60	23.28	0.32	-1.34	<0.000

*The mean values and standard deviations of the Ct values after normalization with the housekeeping gene were given in the Table. Experiments were performed in triplicate.

DISCUSSION

Angiogenesis is an important process for the creation of new blood and lymphatic vessels sustaining the growth of the tumor and both VEGFA and its receptors are responsible for stimulatory signals for the angiogenesis (Skirnisdottir et al. 2016). The prognostic roles of VEGF and its receptors have been investigated in various cancer studies. Chen et al. (2005) showed the increase of VEGF and FLT1 mRNA in human laryngeal carcinoma cells. In a stage I non-small-cell lung cancer study, tumors expressing VEGF or KDR displayed poorer outcomes (Seto et al. 2006). High mRNA levels of both VEGF and FLT1 were associated with the development of multiple myeloma (Liu et al. 2007). In astrocytoma pathogenesis, though expressions of FLT1 and KDR showed no significant difference between low and high-grade tumor groups, expressions of VEGF and MMP-9 showed an increase in the high-grade group (Hlobilkova et al. 2009). A significant correlation between HIF-1 alpha (involved in the transcriptional regulation of VEGF) nuclear staining and VEGF staining was found in small cell lung carcinoma and HIF-1 alpha+/VEGF+ cases were shown to be associated with poor survival (Ioannou et al. 2009). VEGF and its two receptors (FLT1 and KDR) were detected with immunocytochemistry in tumor cells of patients with oesophagogastric cancer and in comparison to the normal oesophageal epithelium, VEGF, FLT1, and KDR were upregulated (Gray et al. 2013). VEGFR1 (FLT1) was shown to be related to the gastric cancer recurrence since 9 out of 10 cases with the highest FLT1 expression belonged to the recurrence group (Suspitsin et al. 2013). Glioma patients whose tumors co-expressed VEGFA and FLT1 mRNA at the high level had shorter survival (Zhang et al. 2015a). In lung carcinoma, the high expression of VEGFA and one of the two receptors (FLT1 or KDR) were associated with worse survival outcome (Zhang et al. 2015b). Angiogenesis regulators VEGFR2 and VEGFA showed an association with p53 status in terms of disease recurrence and survival in epithelial ovarian carcinoma (Skirnisdottir et al. 2016). High VEGF expression level was proposed as an important aggressiveness factor in breast tumors (Rydén et al. 2003; Balasubramanian et al. 2007; Srabovic et al. 2013; Bhat et al. 2019). In the light of studies conducted in different tumor types, it is an apparent fact that the overexpression of VEGF is related to tumor development and progression. Thus, the results of this study demonstrate the downregulation of VEGFA by TQ is a desirable circumstance in the control of breast tumors. The above mentioned studies conducted in different tumors also usually reflected the increased levels of VEGFA's receptor FLT1. In this study conducted in MCF-7 cells, TQ increased the levels of FLT1 and this highlights to a different point of view in contrast to the expected situation. The studies evaluating the expression level of FLT1 in breast carcinomas are quite limited and more studies are needed. Though Srabovic et al. (2013) showed the overexpression of VEGFR-1 and VEGF at the immunohistochemical level in breast tumors, this is conflictive with some previous studies. Schmidt et al. (2008) suggested VEGFR-1 expression as an indicator of better prognosis and Wülfing et al. (2005) reported rare Flt-1 expression in ductal breast carcinoma in situ. The improved survival rate stemming from the overexpression of Flt-1 was proposed by Zhukova et al. (2003). Based on these three studies correlating upregulation of VEGFA's receptor FLT1 with better prognosis in breast tumors, results of this study are also promising since upregulated FLT1 levels by TQ in MCF-7 cells were shown. More studies conducted on expression levels of FLT1 both in breast cancer cells and breast tumors could help to draw a precise conclusion.

TQ has been shown to possess promising anti-tumor activities through different molecular mechanisms. In the breast cancer study of Arafa et al. (2011), TQ transcriptionally upregulated PTEN, which then led to phosphorylation of Akt and induction of p53 protein and its transcriptional target p21. This resulted with the induction of G2/M phase arrest and apoptosis in doxorubicin-resistant MCF-7/DOX cells. TQ treatment also caused an increase in the Bax/Bcl2 ratio via upregulating Bax and down-regulating Bcl2 proteins. The molecular mechanisms of TQ have also been investigated in liver cancer and induction of G2/M cell cycle arrest and increase in the ratio of Bax/Bcl2 were emphasized (ElKhoely et al. 2015). In addition to these benefits TQ offers as a phytochemical-based anticancer agent, there are also a few handicaps such as poor water solubility, and bioavailability. To overcome these handicaps,

nanostructurally combined materials can be developed. Complex with β-cyclodextrin (CD) nanoparticles was shown to improve TQ solubility with increased antiproliferative activity on MCF7 cells and little toxicity to normal human periodontal fibroblasts (Abu-Dahab et al. 2013). The encapsulation of TQ in chitosan myristic acid nanogel was proved to be more efficient than sole TQ solution in MCF7 cells (Dehghani et al. 2015). The development of TQ-nanostructured lipid carrier as a drug for the treatment of breast cancer has also been offered by Ng et al. (2015). The recently suggested F2 gel (nanofibers of poly-N-acetyl glucosamine) loaded with doxorubicin and TQ enhanced antitumor activity with minimal toxicity in MCF-7 and HEPG2 cells (Zidan et al. 2018). Therefore, as long as TQ exerts the expected molecular effects to be used as an anticancer drug, nanotechnology methods could help to overcome the poor solubility effects.

CONCLUSION

The researchers provide evidence that TQ down-regulated VEGFA and up-regulated FLT1 transcriptional levels in human breast cancer cells. The present data raise the possibility that VEGF and FLT1 play key roles in the growth of human breast carcinoma cells.

RECOMMENDATIONS

As recapitulated at the beginning of the discussion part in detail, the exact roles of VEGFA and its receptor FLT1 in breast tumors need to be investigated in larger patient cohorts both at mRNA and protein levels. Thus, the consensus implying the overexpression of VEGFA with a worse clinical outcome and overexpression of its receptor FLT1 with a better prognosis scheme may reflect the importance of our cell culture study in terms of opening new avenues in chemoprevention strategies by the use of phytochemical agent TQ. According to the best of the researchers' knowledge, this is the first study evaluating the effect of TQ on VEGFA/FLT1 molecular pathway in breast carcinoma cells. Therefore, the researchers also strongly recommend extra cell culture studies targeting angiogenesis factors both at mRNA and protein level.

Abbreviations

TQ: Thymoquinone; VEGFA: Vascular endothelial growth factor A; FLT1: Fms-related tyrosine kinase 1; MCF-7: Michigan Cancer Foundation-7; HEK293: Human embryonic kidney 293; BC: Breast cancer; WHO: World Health Organization; GLOBOCAN: Project of the International Agency for Research on Cancer (IARC); NF-κB: Nuclear factor κB; STATs: Signal Transducers and activators of transcription; Nrf2: Nuclear factor erythroid 2-related factor 2; ARE: Antioxidant-response element; T47D: Breast ductal carcinoma cells; FASN: Fatty acid synthase; TNBC: Triple-negative breast cancer; miR-603: microRNA-603; eEF-2K: Eukaryotic elongation factor-2 kinase; Pac: Paclitaxel; PBS: Phosphate-buffered saline; MTT: Methyl-thiazolyl-tetrazolium; DMSO: Dimethylsulfoxide; IC_{50}: The half maximal inhibitory concentration; KDR: Kinase insert domain-containing receptor; MMP-9: Matrix metallopeptidase 9; HIF-1: Hypoxia-inducible factor 1; PTEN: Phosphatase and tensin homolog; Akt: Serine/threonine kinase 1; PTEN: Phosphatase and tensin homolog; siRNA: Small interfering RNA; F2 gel: Nanofibers of poly-N-acetyl glucosamine; HEPG2: Human liver hepatoma; FGF: Fibroblast growth factor; WNT: Wingless-type MMTV integration site family; SPSS: Statistical package for social science

REFERENCES

Abu-Dahab R, Odeh F, Ismail SI, Azzam H, Al Bawab A 2013. Preparation, characterization and antiproliferative activity of thymoquinone-beta-cyclodextrin self assembling nanoparticles. *Pharmazie*, 68(12): 939-944.

Arafa el-SA, Zhu Q, Shah ZI, Wani G, Barakat BM, Racoma I, El-Mahdy MA, Wani AA 2011. Thymoquinone up-regulates PTEN expression and induces apoptosis in doxorubicin-resistant human breast cancer cells. *Mutat Res*, 706(1-2): 28-35.

Balasubramanian SP, Cox A, Cross SS, Higham SE, Brown NJ, Reed MW 2007. Influence of VEGF-A gene variation and protein levels in breast cancer susceptibility and severity. *Int J Cancer*, 121(5): 1009-1016.

Bashmail HA, Alamoudi AA1, Noorwali A1, Hegazy GA, AJabnoor G, Choudhry H, Al-Abd AM 2018. Thymoquinone synergizes gemcitabine anti-breast cancer activity via modulating its apoptotic and autophagic activities. *Sci Rep*, 8(1): 11674.

Bhat S, Tania RP, Hussain S, Sahaf B, Ansari I 2019. Expression of VEGF in breast lesions: An immunohistochemical study. *Int J Adv Med*, 6(1): 12-17.

Chen G, Ding J, Luo L, Liu Y, Yan K, Chen P, Song P, Fu Y, Gong S 2005. [Expression of vascular endothelial

growth factor and its receptors in laryngeal carcinoma cell and its significance]. *Lin Chuang Er Bi Yan Hou Ke Za Zhi*, 19(18): 842-844.

Dehghani H, Hashemi M, Entezari M, Mohsenifar A 2015. The comparison of anticancer activity of thymoquinone and nanothymoquinone on human breast adenocarcinoma. *Iran J Pharm Res*, 14(2): 539-546.

ElKhoely A, Hafez HF, Ashmawy AM, Badary O, Abdelaziz A, Mostafa A, Shouman SA 2015. Chemopreventive and therapeutic potentials of thymoquinone in HepG2 cells: Mechanistic perspectives. *J Nat Med*, 69(3): 313-323.

Gray RT, O'Donnell ME, Maxwell P, McGuigan JA, Spence GM 2013. Long-term follow-up of immunocytochemical analysis of vascular endothelial growth factor (VEGF), and its two receptors, VEGF-R1 (Flt-1) and VEGF-R2 (Flk-1/KDR), in oesophagogastric cancer. *Int J Biol Markers*, 28(1): 63-70.

Hlobilkova A, Ehrmann J, Knizetova P, Krejci V, Kalita O, Kolar Z 2009. Analysis of VEGF, Flt-1, Flk-1, nestin and MMP-9 in relation to astrocytoma pathogenesis and progression. *Neoplasma*, 56(4): 284-290.

Ioannou M, Papamichali R, Kouvaras E, Mylonis I, Vageli D, Kerenidou T, Barbanis S, Daponte A, Simos G, Gourgoulianis K, Koukoulis GK 2009. Hypoxia inducible factor-1 alpha and vascular endothelial growth factor in biopsies of small cell lung carcinoma. *Lung*, 187(5): 321-329.

Kabil N, Bayraktar R, Kahraman N, Mokhlis HA, Calin GA, Lopez-Berestein G, Ozpolat B 2018. Thymoquinone inhibits cell proliferation, migration, and invasion by regulating the elongation factor 2 kinase (eEF-2K) signaling axis in triple-negative breast cancer. *Breast Cancer Res Treat*, 171(3): 593-605.

Khan A, Aldebasi YH, Alsuhaibani SA, Khan M1 2019. Thymoquinone Augments Cyclophosphamide-Mediated inhibition of cell proliferation in breast cancer cells. *Asian Pac J Cancer Prev*, 20(4): 1153-1160.

Liu JR, Luo SK, Li J, Su C 2007. Expression and clinical significance of vascular endothelial growth factor and its receptors in multiple myeloma. *Ai Zheng*, 26(6): 652-656.

Livak KJ, Schmittgen TD 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, 25(4): 402-408.

Naja F, Nasreddine L, Awada S, Ahmad RES, Hwalla N 2019. Nutrition in the prevention of breast cancer: A Middle Eastern perspective. *Front Public Health*, 7: 316.

Ng WK, Saiful Yazan L, Yap LH, Wan Nor Hafiza WA, How CW, Abdullah R 2015. Thymoquinone-loaded nanostructured lipid carrier exhibited cytotoxicity towards breast cancer cell lines (MDA-MB-231 and MCF-7) and cervical cancer cell lines (HeLa and SiHa). *Biomed Res Int*, 263131.

Pourghazian N, Sankaranarayanan R, Alhomoud S, Slama S 2019. Strengthening the early detection of common cancers in the Eastern Mediterranean Region. *East Mediterr Health J*, 25(11): 767-768.

Rossi L, Mazzara C, Pagani O 2019. Diagnosis and treatment of breast cancer in young women. *Curr Treat Options Oncol*, 20(12): 86.

Rydén L, Linderholm B, Nielsen NH, Emdin S, Jönsson PE, Landberg G 2003. Tumor specific VEGF-A and VEGFR2/KDR protein are co-expressed in breast cancer. *Breast Cancer Research and Treatment*, 82(3): 147–154.

Sakalar Ç, Izgi K, Iskender B, Sezen S, Aksu H, Çakir M, Kurt B, Turan A, Canatan H 2016. The combination of thymoquinone and paclitaxel shows anti-tumor activity through the interplay with apoptosis network in triple-negative breast cancer. *Tumour Biol*, 37(4): 4467-4477.

Schmidt M, Voelker HU, Kapp M, Dietl J, Kammerer U 2008. Expression of VEGFR-1 (Flt-1) in Breast Cancer is Associated with VEGF Expression and with Node-negative Tumour Stage. *Anticancer Research*, 28: 1719-1724.

Seto T, Higashiyama M, Funai H, Imamura F, Uematsu K, Seki N, Eguchi K, Yamanaka T, Ichinose Y 2006. Prognostic value of expression of vascular endothelial growth factor and its flt-1 and KDR receptors in stage I non-small-cell lung cancer. *Lung Cancer*, 53(1): 91-96.

Shanmugam MK, Arfuso F, Kumar AP, Wang L, Goh BC, Ahn KS, Bishayee A, Sethi G 2018. Modulation of diverse oncogenic transcription factors by thymoquinone, an essential oil compound isolated from the seeds of Nigella sativa Linn. *Pharmacol Res*, 129: 357-364.

Skirnisdottir I, Seidal T, Åkerud H 2016. The relationship of the angiogenesis regulators VEGF-A, VEGF-R1 and VEGF-R2 to p53 status and prognostic factors in epithelial ovarian carcinoma in FIGO-stages I-II. *Int J Oncol*, 48(3): 998-1006.

Srabovic N, Mujagic Z, Mujanovic-Mustedanagic J, Softic A, Muminovic Z, Rifatbegovic A, Begic L 2013. Vascular endothelial growth factor Receptor-1 expression in breast cancer and its correlation to vascular endothelial growth factor A. *International Journal of Breast Cancer*, Article ID 746749.

Suspitsin EN, Kashyap A, Shelekhova KV, Sokolenko AP, Kuligina ESh, Iyevleva AG, Kornilov AV, Ehemann V, Yanus GA, Aleksakhina SN, Preobrazhenskaya EV, Zaitseva OA, Yatsuk OS, Klimashevsky VF, Togo AV, Imyanitov EN 2013 Evidence for angiogenesis-independent contribution of VEGFR1 (FLT1) in gastric cancer recurrence. *Med Oncol*, 30:644.

Wülfing P, Kersting C, Buerger H, Mattsson B, Mesters R, Gustmann C, Hinrichs B, Tio J, Böcker W, Kiesel L 2005. Expression patterns of angiogenic and lymphangiogenic factors in ductal breast carcinoma in situ. *Br J Cancer*, 92(9): 1720-1728.

Zhang SD, Leung KL, McCrudden CM, Kwok HF 2015. The prognostic significance of combining VEGFA, FLT1 and KDR Mrna expressions in brain tumors. *J Cancer*, 6(9): 812-8.

Zhang SD, McCrudden CM, Kwok HF 2015. Prognostic significance of combining VEGFA, FLT1 and KDR mRNA expression in lung cancer. *Oncol Lett*, 10(3): 1893-1901.

Zhukova LG, Zhukov NV, Lichinitser MR 2003. Expression of Flt-1 and Flk-1 receptors for vascular endothelial growth factor on tumor cells as a new prognostic criterion for locally advanced breast cancer. *Bulletin of Experimental Biology and Medicine*, 135(5): 478–481.

Zidan AA, El-Ashmawy NE, Khedr EG, Ebeid EM, Salem ML, Mosalam EM 2018. Loading of doxorubicin and thymoquinone with F2 gel nanofibers improves the antitumor activity and ameliorates doxorubicin-associated nephrotoxicity. *Life Sci*, 207: 461-470.

Paper received for publicaiton in September, 2019
Paper accepted for publication in January, 2020