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Assessment Of The Anti-angiogenic Effect Of Imatinib On Laryngeal Carcinoma Cell Line (Hep2)"

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Introduction

Head and neck squamous cell carcinoma (HNSCC) is the most common epithelial malignancy arising in the upper aerodigestive tract (Bran et al., 2009). Over 500,000 new cases of HNSCC are reported annually worldwide with about 50% five year survival rate (Marur and Forastiere, 2016).

Angiogenesis is a physiological process of formation of new capillaries from pre-existing vessels (Gacche, 2015). It plays a crucial role in survival of cancer cells, tumor growth and the development of distant metastasis (Bran et al., 2009). The intensity of angiogenesis was shown to increase in various human tumors, including HNSCC (Vassilakopoulou et al., 2015).

In 1971, Judah Folkman, a pioneer researcher in tumor angiogenesis, explained the importance of vasculature for the growth and proliferation of solid tumors. He demonstrated that "If a tumor is deprived from generating its own blood supply, it would not grow more than 1–2 mm in size or it may wither and die" (Folkman, 1971).

Vascular endothelial growth factor is considered the key player that regulates the angiogenesis process predominantly. The VEGF family consists of seven different factors, these are VEGF-A , which is commonly referred as VEGF, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, and PLGF (Fagiani et al, 2016).

VEGF-A is the most important endothelial cell-specific mitogen and angiogenesis inducer. It is engaged at almost every stage of physiologic and pathologic vascular development (Zhao and Adjei , 2015). The function of paracrine VEGF, released

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by tumor, myeloid or other stromal cells, is to increase vessel branching and render tumor vessels abnormal. On the other hand, autocrine VEGF, released by endothelial cells (ECs), has a crucial role in vascular homeostasis (Lee et al., 2007).

Imatinib is a tyrosine kinase inhibitor which is also known as Imatinib mesylate, STI571 or Gleevec. It was manufactured by Novartis, Basel, Switzerland and was first used for the treatment of chronic myeloid leukemia (Bran et al, 2009). In addition, Imatinib appeared to be highly effective in treatment of unresectable or metastatic gastrointestinal stromal tumors (Blanke et al, 2008; Heinrich et al, 2017), malignant melanoma (Hodi et al, 2013) and AIDS-related Kaposi's sarcoma (Koon et al, 2014). It mainly acts by inhibiting the tyrosine kinase activity of C-Kit protein the receptor of stem cell factor (SCF) and PDGFRs (Iqbal and Iqbal, 2014).

The use of Imatinib as an anti- angiogenic drug was widely supported by many studies on a variety of cancer cell lines and xenograft models like non-small-cell lung cancer, ovarian cancer and HNSCC cell lines (Matei et al, 2007;Vlahovic et al, 2007; Bran et al, 2009).

Our study was conducted to evaluate the antiangiogenic effect of Imatinib on VEGF expression in Hep2 cell line.

Materials and Methods

Materials

Imatinib , 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) and all cytotoxicity assay chemicals were purchased from Sigma Aldrich CO., St. Louis, Mo, USA. RNA extraction kit was purchased from Thermo Fisher Scientific Inc. Germany (Gene JET , Kit,#K0732). qRT-PCR kit was provided by Bioline, a median life science company, UK (SensiFAST™ SYBR® Hi-ROX One-Step Kit, catalog no.PI-50217 V). (RIPA) lysis buffer PL005 of western blot technique was provided by Bio BASIC INC. (Marhham Ontario L3R 8T4 Canada). TGX Stain-Free™ FastCast™ Acrylamide Kit (SDS-PAGE) was provided by Bio-Rad Laboratories, INC, USA Catalog. NO. 161-0181. The primary antibody for VEGF and Horse Raddish peroxidase (HRP)-conjugated secondary antibody (Goat antirabbit IgG- HRP-1mg) were obtained from (Novus Biologicals USA, AF-293-NA).

2-2 Cell Culture and Cell Viability Assay

Human laryngeal squamous cell carcinoma, Hep2, cell line was purchased from Cell Culture Unit - VACSERA, Egypt. Hep2 cells were imported from the "American Type Culture Collection (ATCC)" in the form of frozen vials. Hep2 cells were cultured in Dulbeco`s Modified Eagle's Medium (DMEM) supplemented in 10% fetal bovine serum (FBS) (Cambrex BioScience, Copenhagen, Denmark) and 1% streptomycin-penicillin. The cells were grown at 37°c in humidified atmosphere of 5% CO2 in air. Imatinib (Sigma, USA) was dissolved in distilled water.

Cells were incubated with or without Imatinib into 96-well plates ($5x10^3$ cells / well) for 24 h, cell viability was evaluated using MTT assay as described previously by Hsu et al, 2004. The relative cell viability and the IC50 were obtained. The groups of this study were the IC50 dose for 24 h coded (I1) and one higher dose coded (I2) in addition to untreated control group coded (CON).**2-3 RT-PCR**

RNA was extracted using extraction kit purchased from Thermo Fisher Scientific Inc. Germany. Primer sequence for the studied target genes (VEGF) and reference housekeeping gene (B-actin) were obtained from gene bank. RT-PCR master mix was prepared and applied to the samples. The prepared reaction mix samples were applied in real time PCR machine (Step One Applied Biosystem, Foster city, USA).

After the RT-PCR run, the data were expressed in Cycle threshold (Ct). The PCR data sheet includes Ct values of assessed gene (VEGF) and the house keeping (reference) gene (B-actin). The relative quantitation (RQ) of each target gene is quantified according to the calculation of delta-delta Ct (ΔΔCt).

We calculated the RQ of each gene by taking $2^{-\Delta\Delta Ct}$ as following:

$\Delta\Delta Ct = [(Ct\ target, Sample)-(Ct\ ref, Sample)]-[(Ct\ target, Control)-(Ct\ ref, Control)]$

Where: Ct target, Control = Ct value of gene of interest in control DNA

Ct ref, Control = Ct value of reference gene in control DNA

Ct target, Sample = Ct value of gene of interest in tested sample

Ct ref, Sample = Ct value of reference gene in tested sample

2-4 Western Blot

Cells were harvested by trypsinization, suspended in cold protein lysis buffer (RIPA) PL005, and incubated for 30 minutes in ice. Cell debris was removed by centrifugation at ~16,000×g for 30 minutes at 4°C. 20 µg protein concentration of each sample was loaded with an equal volume of 2x Laemmli sample buffer. The pH was checked and brought to 6.8. The mixture was then boiled at 95°C for 5 minutes to ensure denaturation of protein before loading on polyacrylamide gel electrophoresis.

The prepared resolving and stacking gels were submerged in electrophoresis chamber, the prepared running buffer was poured in the chamber. 20 µg of total protein was loaded per mini-gel well. The gel was run for 20 mins at 50 V to allow samples migration in stacking layer. The voltage was increased to 100–150 V to allow protein migration and separation in resolving layer to finish the run in about 1 hr. The separated proteins were blotted to polyvinylidene difluoride (PVDF) membrane using BioRad Trans-Blot Turbo Instrument.

The membrane was blocked in tris-buffered saline with Tween 20 (TBST) buffer and 3% bovine serum albumin (BSA) at room temperature for 1 hour. The primary antibody for VEGF was diluted in TBST. Incubation was done overnight in each primary antibody solution, against the blotted target protein, at 4°C. The blot was rinsed 3–5 times for 5 mins with TBST. Incubation was done in the Horse Raddish peroxidase (HRP)-conjugated secondary antibody (Goat antirabbit IgG-HRP-1mg Goat mab) solution against the blotted target protein for 1 hr at room temperature. The blot was rinsed 3–5 times for 5 mins with TBST. The chemiluminescent substrate (ClarityTM Western ECL substrate - BIO-RAD, USA cat#170-5060) was applied to the blot according to the manufacturer's recommendation. Briefly, equal volumes were added from Clarity western luminal/enhancer solution and speroxidase solution. The chemiluminescent signals were captured using a Charged Coupled Device (CCD) camera-based imager.

Image analysis software was used to read the band intensity of the target proteins against control sample by total protein normalization on the Chemi Doc MP imager.

Data was entered and coded using the statistical package SPSS version 25. Data was summarized using mean and standard deviation and standard error of the mean for quantitative variables. Comparisons between groups were done using unpaired t test when comparing 2 groups and analysis of variance (ANOVA) with multiple comparisons post hoc test when comparing more than 2 groups

Results

The calculated IC50 for Imatinib was 7.75µg/ml. The other used dose was 15.5 µg/ml.

Western blot bands of VEGF after treatment of cells with Imatinib appeared to be thinner in relation to the control group. I2 revealed a thinner band than I1 (Fig.1)

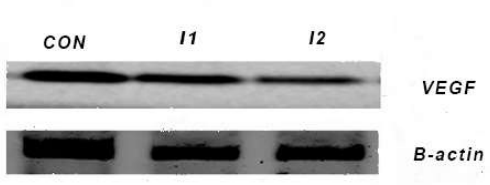

Fig.1 Western blot bands of VEGFin control group and in Imatinib groups after 24 h in comparison to B- actin.

The PCR and western blot statistical results revealed a statistically significant difference in the mean VEGF gene and protein expressions between CONT and (I1 and I2) (P value<0.001). A statistically significant difference could also be detected between I1 and I2 at the gene level (P value<0.001) and at protein level (P value= 0.014) (Fig 2,3).

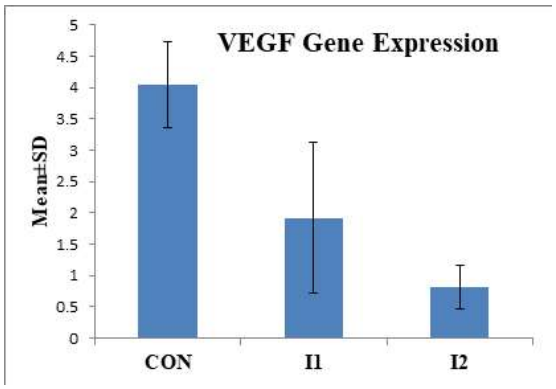

Fig.2: Bar chart showing the mean of VEGF gene expression in different groups

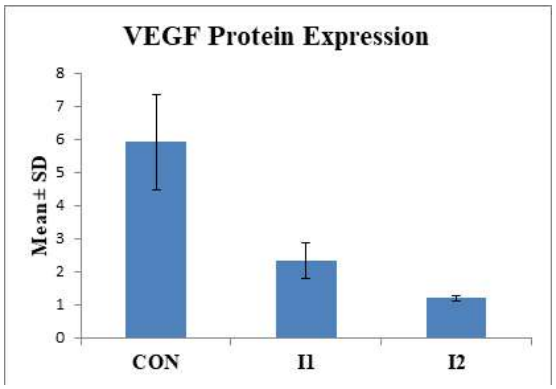

Fig.2: Bar chart showing the mean of VEGF protein expression in different groups

Discussion

Imatinib is, a tyrosine kinase inhibitor, used widely in treatment of chronic myeloid leukemia (Bran et al, 2009). It is also considered a powerful anti-angiogenic agent (Vlahovic et al, 2007). Our results revealed a statistically significant decrease in the mean ofVEGF gene and protein expressions from CON to (I1 and I2). In addition, the mean VEGF gene and protein expressions decreased significantly in I2 than I1.

These aforementioned results are similar to those of Beppu et al, 2004 who previously detected the inhibitory effect of Imatinib on VEGF protein expression in neuroblastoma cells by using Western blot and in culture media using ELISA. The researchers found that the effect was concentration dependent. They also observed that the concentration of Imatinib associated with a 50% reduction in VEGF was similar to the biologic IC50 of Imatinib in those cells.

In addition, they detected that the inhibition of VEGF correlated with the reduction in tumor size in the in vivo xenograft model and with cell proliferation in vitro. They suggested that these results might be due to Imatinib blockage of PDGFR and c-Kit receptor, preventing their activation by their pro-angiogenic ligands PDGF-BB and SCF respectively. It acted by inhibiting phosphorylation of protein kinase B (AKT) and Extracellular Signal-Regulated kinase ERK1/2.

Another study, targeting ovarian cancer by Imatinib, a selective PDGFR inhibitor, claimed that the drug inhibited PDGF induced posphoinositol 3′-kinase protein kinase B (PI3K/Akt) pathway and partially reduced PDGF induced MAPK1, 2 phosphorylation and activation. The inhibition of those pathways blocked PDGF induced VEGF secretion and promoted HIF1 degradation with strong correlation between PDGF and VEGF (Matei et al, 2007).

Moreover, Bran et al, 2009, examined PDGF and VEGF protein and gene expressions using ELISA and PCR respectively. They conducted their study on a number of HNSCC cell lines using ascending doses of Imatinib of 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 μmol/l. A statistically significant reduction in protein expression was detected at 24 and 72 hours.

However, the PCR results revealed that the expression of PDGF-A was stable and not influenced by the Imatinib treatment while the expression of PDGF-B remained absent. On the other hand, VEGF 165 expression was reduced significantly after 24 as well as after

72 hours.

The ELISA results but not the PCR results of Bran et al, 2009 explained that Imatinib inhibited HNSCC cell growth, angiogenesis and survival. It acted by blocking PDGFR followed by inhibiting PDGF/PDGF-R autocrine pathway as well as inhibiting VEGF paracrine loop. Schultz et al, 2011 also detected similar results, when conducting their experiment on HNSCC cell lines using different doses.

In conclusion, our data suggested that Imatinib could be effective in inhibiting angiogenesis in HNSCC.

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