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# LXR-α/ABCA1 PAIR ENHANCES AND DEREGULATES VEGF MODULATED BY DIOSGENIN IN LIVER CANCER CELL LINE (HUH-7)

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## ABSTRACT

*The nuclear liver x receptor (LXR) is an important regulator for the cholesterol, fatty acids and glucose homeostasis level in the human body. LXRs also play an essential role in the enlargement and progression of liver cancer. We study the effect of diosgenin on angiogenesis in vivo by chorioallantoic membrane (CAM) assay. Diosgenin stimulates the expression of LXR-α and its target ATP-binding cassette transporter (ABCA1) gene in Huh-7 cells. Diosgenin upregulates LXR-α and ABCA1 protein regulates angiogenesis and downregulates HIF1-α and VEGF in Huh-7 cells. Our study suggested that activation LXR interferes with angiogenesis through the stimulation of LXR-αand its target gene ABCA1, which in turn suppresses HIF1-α and VEGF signaling, an essential pathway regulating angiogenesis. An unrecognized role of the LXR was pointed out by the observation of LXR-α as an ABCA1 target gene in vascular biology. In conclusion, LXR-α may be a potential curative target for tumor angiogenesis.*

## KEY WORDS

*Angiogenesis, Diosgenin, Liver cancer, Liver X receptor-α,*

## INTRODUCTION

Liver cancer is a very common type of cancer related mortality in the world. The prognosis of HCC remains poorly understood despite various diagnosis and treatment, mainly due to its late diagnosis and advancements in the tumor [1]. Major cancer to the patients is the side effects caused by the chemotherapy and drug resistance [2]. The newer chemotherapeutic focuses on reducing the side effects to control the growth of the tumor cell invasion, metastasis and angiogenesis. Conventional chemotherapy is unsuccessful in treating HCC due to its high hepatoxicity. Dietary phytochemicals found naturally could be helpful in reducing the side effects of cancer [3].

New blood vessels are produced from preexisting vessels in a development called angiogenesis. It is an essential process that promotes growth, development, and wound healing, along with an important role in pathological conditions. Endothelial cells are concerned in hemostasis, thrombosis, and blood vessel permeability. Invasion and metastasis of cancer cells, which contributes to 90% of mortality rates in cancer, are known to be mediated by angiogenic processes [4].

The expression of various proteins which play a role in development, physiology, and disease are controlled by ligand-activated transcription factors known as Nuclear Receptors (NRs) [5,6]. In the function as receptors for various small lipophilic ligands such as metabolites, hormones, drugs, and environmental compounds, which in turn modulate the transcription activities of the receptors such as Liver X receptors α (LXRα) and β (LXRβ) are Nuclear Receptors which are involved in regulation of homeostasis and metabolism of lipids, cholesterol, and carbohydrates. Retinoid X Receptor (RXR) exhibit their transcriptional progress by enhancing the expression of genes encoding proteins concerned in lipid metabolism, mainly cholesterol efflux and fatty acid synthesis. LXRs may fasten cholesterol homeostasis to proliferation. Synthetic and natural LXR ligands have

been shown to restrain cell proliferation in cancer cell lines such as that of prostate, colon, ovarian, breast and leukemia. [5-8]

Vascular endothelial growth factor (VEGF) or vascular permeability factor, is a cytokine which has important role in angiogenesis and lymph angiogenesis [4]. VEGF binds to its receptor VEGF receptor 2 (VEGFR-2) and induces downstream signaling proteins which mediate VEGF's role in the progression of cancer, tumor growth, metastasis and formation of blood vessels [9]. VEGF is observed to stimulate endothelial cell mitogenesis, cell mitogenesis, sprouting and microvascular permeability. Human umbilical vein endothelial cells are isolated from the endothelium of the umbilical cord. It is used for the study its function and pathology. Previously it has been observed treatment with LXR agonist T0901317 and GW3965 suppresses the expression of VEGF receptor2 in lipid rafts via affecting endothelial cholesterol homeostasis and thus inhibited migration, tubulogenic and proliferation of HUVEC. However, it is not clear whether LXR target genes are involved in the regulation of angiogenesis by LXRs [10].

## MATERIALS AND METHODS

### *Chemicals and reagent*

Diosgenin ($C_{27}H_{42}O_3$; MW 414.62 KD), DCFH-DA (20, 70 - dichlorodihydro fluoresceine diacetate), AO/EB (Acridine orange /Ethidium bromide), Nile Red (9-diethylamino5H-benzo[α]phenoxazine-5-one) were purchased from Sigma–Aldrich Co. Diosgenin stock solution was prepared in DMSO. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), MTT [3-(4, 5-dimethylthiazol- 2yl)-2, 5-diphenyltetrazolium bromide], were purchased from Invitrogen. Antibodies against LXR-α, ABCA1, VEGF, HIF1- α and GAPDH were obtained from Sigma–Aldrich Co, USA.

### *Cell Culture*

Human liver cancer cell line, Huh-7, was purchased from the National Centre for Cell Science (NCCS), Pune, India. Cells were consequently grown in DMEM and supplemented with 10 % (v/v) FBS and 1 % (w/v) penicillin/streptomycin at 37° C in a humidified atmosphere 95 % air and 5 % $CO_2$.

### *MTT Assay*

Cell viability was tested by the MTT assay. The cells were implanted in 96-well plates at a mass of 5 × $10^3$ cells/well and treated with diosgenin at 10–100 µM for 24 hr. Then the medium was changed along with the cells and were incubated with MTT (50 µl) for 3 h. The amount of dead cell per dish is directly proportional to the mixture of formazan, which was solubilized in isopropanol, and deliberate spectrophotometrically at 570 nm.

### *Acridine orange/ethidium bromide (AO/EtBr) staining*

In a six well plate, cells were incubated with 40 µM ($IC_{50}$) of diosgenin for 24 hr. Then the medium was removed and with the use of PBS the cells were swabbed and stained with 10 µM AO/EB (1 mg/ml) for 5 min. Morphological changes were observed with a fluorescent microscopy (Floid cell imaging station).

### *Chorioallantoic membrane assay (CAM)*

Fertilized chicken eggs were incubated at 37°C in an 80% humidified air. On day 6, as the yolk was placed in the center of the chorioallantoic membrane (CAM), Diosgenin diluted in ethanol solution was placed onto the nylon membrane. Angiogenesis responses were examined 5 days after implantation. The number of blood vessels is a key of angiogenesis and was assessed by counting the branches of blood vessels.

### *Semi-Quantitative RT- PCR*

Huh-7 cells were treated with 40µM diosgenin for 24 hr. Trizol was used to isolate total RNA after incubation. cDNA was synthesized using a cDNA kit by taking equal quantities of RNA from each sample. 5ul PCR master mix was used to carry out semi-quantitative RT-PCR. For RNA template normalization GAPDH gene was used.

### *Western blotting*

After diosgenin treatment cells were isolated and swabbed with ice-cold PBS two times, then the cells were lysed in RIPA lysis buffer : 20 mM Tris HCl, pH 8, 150 mMNaCl, 0.5 % sodium deoxycholate, 5 mM EDTA, 1 % Nonidet P-40, 0.1 % SDS and centrifuged at 12,000 g for 10 min at 4°C. Cell lysate was analyzed and equal quantity of total protein was separated by SDS PAGE electrophoresis and transferred onto 0.2 um nitrocellulose membrane. The membrane was blocked using blocking buffer for an hour at room temperature and then suitable antibodies were added overnight at 4°C. The primary antibody was used at 1:1000 dilutions.After washing, the respective secondary antibodies built-in alkaline phosphatase-conjugated goat anti-mouse IgG and anti-rabbit IgG were added and the membranes were keep warm for 2 h at 4°C. Signals were visualized by using a chromogenic substrate BCIP/NBT (Amresco, USA).

***Statistics***

One-way ANOVA using GraphPad Prism software (version 6.0) was used for all the statistical analysis. A difference with P of <0.05 is considered statistically significant.

## RESULT

***Cytotoxic effect of Diosgenin on Huh-7 cells***

MTT assay was carried out to assess the cytotoxicity effect of diosgenin on Huh-7 cells. Treatment with diosgenin (10-100 µM) inhibited cell viability in a dose-dependent manner. The $IC_{50}$ value of diosgenin for Huh-7 cell was 40 µM, (Fig.1). This result suggests that diosgenin inhibit cell proliferation and cause cell death in a dose dependent manner.

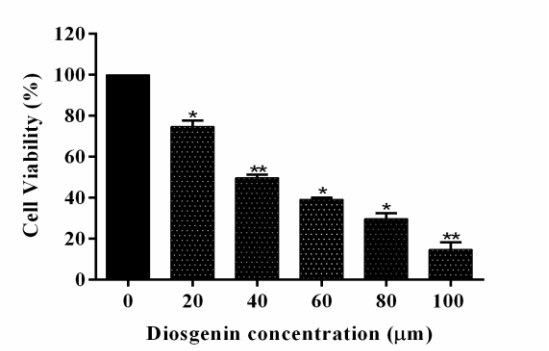


**Figure 1. MTT assay: Cells were treated with diosgenin (10-100 µM) for 24 h to determine the cytotoxicity of diosgenin using MTT assay. Cell viability was significantly reduced at 40 µM diosgenin concentration compared with the control and treated cells (*P< 0.05).**

***Diosgenin induces apoptosis and morphological changes in Huh-7 cells***

Cell death persuade by diosgenin was examined in Huh-7 cells by using the Acridine Orange/Ethidium Bromide (AO/EtBr) dual staining. AO/EtBr was used to assess the nuclear morphology of apoptotic cells. AO stains both live and dead cells, but EtBr stains only individual cells that have misplaced their membrane integrity. Cells are stained green, yellow, and reddish/orange representing viable cells, early apoptotic cells, and late apoptotic cells respectively. In the control, consistently green live cells with normal and large nucleus were observed, though in diosgenin treated cells were stained orange (Fig.2). These outcomes affirmed that diosgenin significantly persuade the apoptosis in Huh-7 cells.

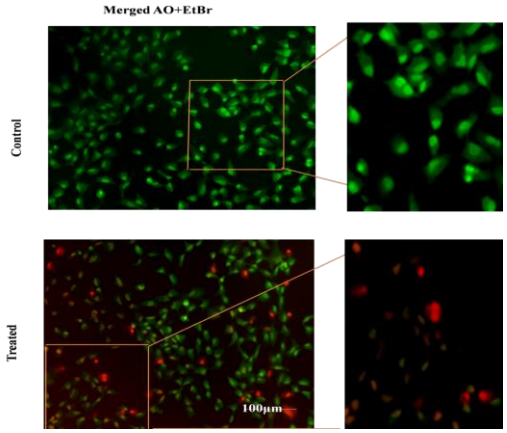


**Figure 2. Morphological changes in apoptotic Huh-7 cells. Cells were treated with 40 µM diosgenin to observe the morphological changes with a cell imaging station following staining with AO/EB. After staining and visualization, control cells appeared green and diosgenin-treated cells appeared orange indicating apoptosis.**

***Diosgenin inhibits angiogenesis in vivo in the CAM assay***

The CAM assay is used to learn proangiogenic and antiangiogenic substance in vivo. Effects is directly

observed by studying the configuration of the vascular plexus in the CAM following treatment with diosgenin. Doses of up to 40µM were also tested but was toxic to the embryo, but it caused growth retardation and death (Fig.3)


**Figure 3. Antiangiogenic effects of diosgenin in the CAM assay. (A) CAMs of 4-day-old chickens were incubated for 7days with control solution (–) diosgenin and 40µM (+) Treatment with diosgenin inhibited CAM vascularization.**

***Diosgenin upregulates the LXR-α and ABCA1in Huh-7 cell line***

The highlights of the effects of LXR stimulation on cancer angiogenesis. The cells control the protein level of cholesterol carrier ABCA1 upon treatment with diosgenin. To investigate the LXRα and ABCA1 expression in Huh-7 cell, we initially studied the expression using Semi-Quantitative RT- PCR and western blotting. Both LXRα and ABCA1 were upregulated in Huh-7, an expression of LXR target gene ABCA1 was increased in Huh-7 cells indicating that ABCA1 is the direct target of LXRα in liver cancer (Fig 4a-d).

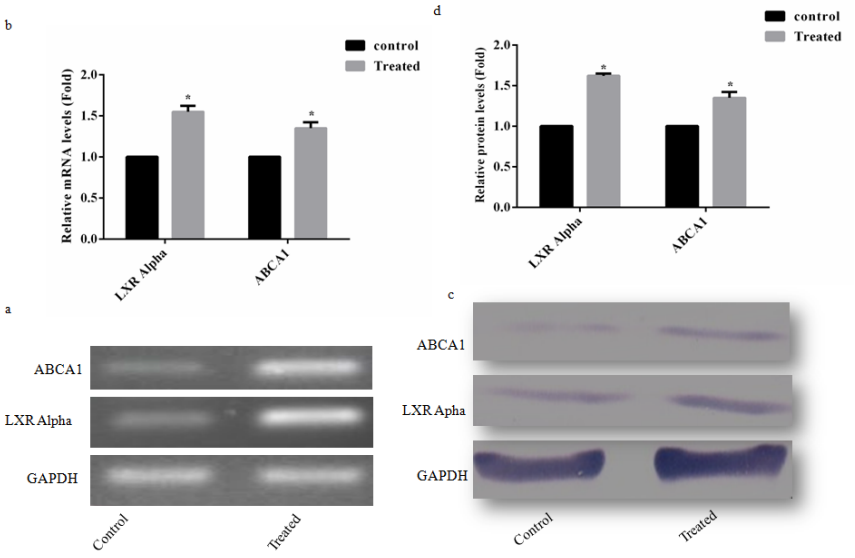


**Figure 4. Diosgenin upregulates LXR-α and ABCA1. The expressions of LXR-α, ABCA1mRNA and protein expressions Cells were treated with 40 µM diosgenin for 24 hr. mRNA and protein were determined by Semi-quantitative RT-PCR and Western blot. b, d) mRNA and protein quantification data (*p< 0.05). GAPDH was used as internal controls GAPDH was used to normalize the gene expression.**

***LXR Activation Reduces Signaling of VEGF***

Adverse effects of diosgenin suggested a concern of LXR angiogenic signaling in the endothelial cells. Though, treatment ofHuh-7with diosgenin functional activation of LXR-α and ABCA1 as established by reduced phosphorylation of VEGF in abridged angiogenesis.

Finally, we addressed the potential mechanisms by which how diosgenin activates LXRα and ABCA1 expression. The VEGF signaling pathway is an important pathway for regulating angiogenesis. The VEGF, HIF1-α gene expressions were analyzed. In the diosgenin-treated cells, expression of VEGF, HIF1-α mRNAs and protein were downregulated compared to control cells (Fig. 5a-d). These results suggested that diosgenin reduce the VEGF signaling pathway and thus reduce angiogenesis in Huh-7 cells.

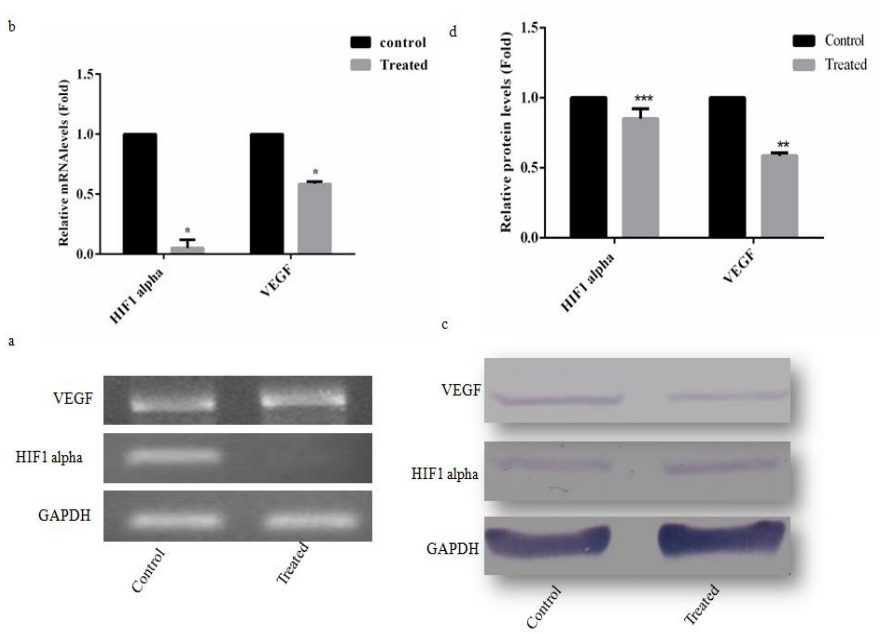


**Figure 5. Diosgenin down regulate HIF1-α and VEGF. The expression HIF1-α and VEGF mRNA and protein expressions cells were treated with 40 µM diosgenin for 24 hr. mRNA and protein were determined by Semi-quantitative RT-PCR and Western blot. b,d) mRNA and protein quantification data (*p< 0.05). GAPDH was used as internal controls GAPDH was used to standardize the gene expression.**

## DISCUSSION

The antiangiogenic possessions of LXR are due to the decrease by impairing Lipid Raft Localization and signaling of VEGF[11].Stimulating the liver X receptor restrain angiogenesis via stimulation of ApoD [10].Further study must be in a manner to understand the function of LXR-dependent VEGF regulation.T0901317, a synthetic liver X receptor agonist, which persuades high density lipoprotein cholesterol (HDL-C) in mice. T0901317 healing of stroke, excited angiogenesis, vascular maturation and development of functional outcome following stroke by rising endothelial nitric oxide synthase (eNOS) phosphorylation factors [12]

Diosgenin activates the LXR-Alpha and ABCA1 to reduce the angiogenesis through the VEGF signaling pathway in Huh-7 cell. There has been solid proof linking cancer and cholesterol. In the early ages as it predicted that cancer was due to the crystallization of cholesterol from living cells, in the due course, cholesterol was seen to be deposited in cancer cells, which led to the theory that there is a link between carcinogenesis and inflammation [13]. It becomes applicable enough to know, that the molecular link which might exist connecting LXR-α with inflammation and cholesterol homeostasis in esteem to cancer is common and to obtain a mechanism procedure in which a cancerous cell might espouse to be reprogrammed into apoptotic condition by selectively activating LXR-α in the cancerous cells [14].

LXR activation in breast cancer cells will decrease the level of cholesterol needed for its growth by altering its efflux, thus leading to the inhibition of cell proliferation and induction of apoptosis [16]. LXR activation has stopped proliferation, and initiated apoptosis of melanoma cell [17]. Dendrogen was seen to exhibit lethal autophagy via a different mechanism by inducing the expression of pro-autophagic factor Nur77, NOR1, and LC3-II under the control of LXR [18].

In A549 cancer cell line hTERT gene expression is down regulated because of diosgenin activity on telomerase. Diosgenin stops the movement and capturing of PC-3 cells restrains the MMP action. It also inhibits JNK, ERK

and PI3K/AKT singling pathway as well as NF-kB activity [19]. Diosgenin exhibits anti proliferative effect in liver cancer cells by inducing apoptosis and G2/M cell cycle arrest. Additionally, up regulation of p27 and p21 expression and activation of the cascade has been observed with diosgenin though it is independent of p53 [20]. Diosgenin induces apoptosis in IGF-1 simulated primary human thyrocytes via capsize dependent pathways and also prohibits the FLICE inhibitory protein (FLIP)and activates cascade 8 through PI3K singling. Diosgenin produces ROS, brings a neutral stability between Bcl-2 and Bax and activates caspase-9[21]. It causes autophagy via the mTOR singling pathway. Diosgenin also sharply increases ROS level in cml cells [20]. Diosgenin inhibits actin polymerization, Vav2 phosphorylation and reduces Cdc42 activation in human breast cancer (MDA-MB-231) cells [22]. Diosgenin reduces the HGF-induced Mdm2 and vimentin by down-regulating phosphorylated Akt and mTOR in prostate cancer [23].

LXR Agonist T901317 and 22(R)- hydroxylcholestrol treatment bypasses the proliferation of progression stage of LnCaP human prostate cancer cell and other frequent human cancer cell line [24] Diosgenin enhances ABCA1- dependent cholesterol efflux and suppresses aortic atherosclerosis progression by silencing macrophage miR-19b expression [25]. T0901317 can reverse EGFRTKI resistance of lung cancer cell lines A549 and H1650. Gas 6 stimulated the activity of LXR and was enhanced through an interaction between LXRα and STAT-1 on the DNA promoter of ARG 2. Gas 6 suppresses lipopolysaccharide that stimulates nitrite production in a STAT-1 and LXR pathway dependent manner in bone marrow derived macrophages. And it also reduced the Mer- neutralizing antibody in LXR and Arg-2 expression in lung tissue and acute lung injury [26]. LXR activation can reduce the angiogenesis and LXR activation can reduce angiogenesis and has a direct impact on the endothelial cholesterol homeostasis as coincubation of HUVEC or arotic rings with exogenous cholesterol inhibit the LXR on endothelial tubulogenesis, proliferation and mounting. [27]. Protein levels of cholesterol transport ABCA1 and ABCG1 were amplified by LXR activation, which endorse cholesterol output towards apolipoprotein and also trans endothelial high-density lipoprotein traffic [28,29]. In the ubiquitin mediated degradation, LDLR is targeted by the low-density lipoprotein receptor which is an inducible degrader due to the expression of mRNA in T0901317 [30]. The present study proves that diosgenin reduce the angiogenesis by the induction of LXR α and ABCA1 in Huh-7 cells.

## CONCLUSION

Activation of endothelial LXRs decreases angiogenesis by restraining cholesterol dependent VEGF signaling. Our study indicated that activation of LXR by Diosgenin diminishes angiogenesis in Huh-7 via induction of LXR-α and its target ABCA1. LXR interacts with ABCA1 and decrease the VEGF and HIF1-α expression. Hence, manipulation of LXR signaling maybe an alternative treatment for cancer angiogenesis.

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### Declaration of Conflicting Interests

The authors have no conflict of interest to declare

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