9.1. Effect of [6]-shogaol on JAK-1/STAT-3 and PI3K/AKT signaling pathway

Introduction

Several signaling pathways involved in development of oral squamous cell carcinoma. STAT (Signal Transducers and Activators of Transcription) are a family of cytoplasmic proteins which acts as signal messengers and transcription factors. These transcription factors are activated via the tyrosine phosphorylation cascade after ligand binding and stimulation of the cytokine and growth factor. They migrate from the cytosol to the cell nucleus for the purpose of activating transcription of target gene. Constitutive activation of STAT-3 is involved in many cellular processes including proliferation, survival, inflammation, invasion, metastasis and angiogenesis.

Another signaling molecule of PI3K/AKT is important intracellular signaling pathway. This pathway is one of the most frequently activated proliferation and survival pathways in cancer. The PI3K/AKT pathway is able to control major cellular functions such as proliferation, cell growth, apoptosis and malignant transformation. Aberrant activation of JAK-1/STAT-3/PI3K/AKT molecules has been documented in a wide range of human pathogenesis, including hematopoietic malignancies and solid tumors such as oral cancer, prostate, and breast cancers (Wang et al., 2012; Samsonov et al., 2013; Zhang et al., 2015). However, the inhibition of JAK-1/STAT-3/PI3K/AKT transcriptional factor plays a crucial role in suppression of angiogenesis and metastasis of tumor cells (Denley et al., 2013; Gurbuz et al., 2014). Extensive research paying attention in natural agents interferes with multiple cell-signaling pathways that can potentially preventive of cancer as well as for treatment. In the present study, we investigate that [6]-shogaol inhibits angiogenesis and metastasis through inhibition of JAK-1/STAT-3/PI3K/AKT on DMBA induced HBP carcinogenesis.
9.2. Results

9.2.1. Effect of [6]-shogaol on IL-1, IL-6 and EGFR expression

The mRNA expression of IL-1, IL-6 and EGFR levels in control and experimental groups were depicted in Fig. 9.1. In DMBA induced animals shows increased expression of IL-1, IL-6 and EGFR as compared to control group. Whereas, dietary oral administration of [6]-shogaol significantly decreased expression of IL-1, IL-6 and EGFR in DMBA induced hamsters. Control and [6]-shogaol alone treated hamsters were no significant changes in IL-1, IL-6 and EGFR expression.

![Graph 1](image1)

![Graph 2](image2)

Fig. 9.1. (a) Effect of [6]-shogaol on DMBA induced mRNA expressions of IL-1, IL-6 and EGFR were analyzed by RT-PCR. (b) The densitometry data represent means ± SD from three independent experiments and are shown as relative density of mRNA normalized to respective GAPDH. Values are not sharing a common marking (* and **) differ significantly at $P < 0.05$ (One way ANOVA followed by DMRT).
9.2.2. Effect of [6]-shogaol on cell signaling molecules analysed by Western blotting

The protein expression of PI3K, pAKT, AKT, JAK-1 and STAT-3 in control and experimental group were illustrated in Fig. 9.2. Our result shows increased expression of PI3K, AKT, JAK-1, STAT-3 and decreased pAKT expression in DMBA induced hamsters. While, oral administration of [6]-shogaol significantly decreased expression of PI3K, AKT, JAK-1, STAT-3 and significant inhibition of pAKT expression in DMBA induced hamsters. However, no significant changes were noted in [6]-shogaol alone and the control group.

![Western Blot Image]

**Fig. 9.2.** (a) Effect of [6]-shogaol on PI3K, pAKT, AKT, JAK-1 and STAT-3 expression levels in control and experimental groups were analysed by Western blot. The quantification was performed by densitometric analysis using Image-studio software (LI COR, USA). (b) The densitometry data represent means ± SD from three independent experiments. Values are not sharing a common marking (* and **) differ significantly at $P < 0.05$ (One way ANOVA followed by DMRT).
9.2.3. Effect of [6]-shogaol on cell signaling molecules analysed by RT-PCR

Furthermore, the mRNA expression of PI3K, AKT, JAK-1 and STAT-3 were analysed by RT-PCR in control and experimental groups were shows in Fig. 9.3. In our results increased mRNA expression of PI3K, AKT, JAK-1 and STAT-3 in DMBA induced hamsters. However, oral administration of [6]-shogaol to DMBA treated significantly decreased the expression of PI3K, AKT, JAK-1 and STAT-3 levels. In control and [6]-shogaol alone treated hamsters has no changes in the mRNA expression.

Fig. 9.3. Effect of [6]-shogaol on PI3K, AKT, JAK-1 and STAT-3 were analyzed by RT-PCR. (b) The densitometric analysis performed by using Kodak GL 100 imaging system. The densitometry data represent means ± SD from three independent experiments and are shown as relative density of mRNA normalized to respective GAPDH. Values are not sharing a common marking (* and **) differ significantly at \( P < 0.05 \) (One way ANOVA followed by DMRT).
9.2.4. Effect of [6]-shogaol on apoptotic markers

We analyzed apoptotic markers (wild-type p53, mutant p53, Bcl-2 and Bax) expression in control and experimental hamsters in each group were shown in Fig. 9.4. Over expression of mutant p53, Bcl-2 and decreased expression of wild-type p53, Bax were noticed in DMBA induced hamsters. Oral administration of [6]-shogaol (20 mg/kg b.wt) to DMBA induced hamsters, showed elevated expression of wild-type p53 and Bax thus inhibit mutant p53, Bcl-2 expression levels. Control and [6]-shogaol alone treated hamsters no significant changes in p53, Bcl-2 and Bax expression.

![Image](image_url)

Fig. 9.4. Effect of [6]-shogaol on apoptotic markers of wild p53, mutant p53, Bcl-2 and Bax in control and experimental groups were analysed by Western blot. (a) The band intensities were quantified by densitometric analysis using Image-studio software (LI COR, USA) and normalized to respective β-actin loading control. (b) The representative graph shows the relative protein expression of fold changes. Values are expressed as mean ± SD for three independent experiments. Values that do not share a common superscript letter (a, b, c) between groups differ significantly at $p < 0.05$ (One way ANOVA followed by DMRT).
9.2.5. [6]-shogaol induce apoptosis by TUNEL assay

To evaluate the [6]-shogaol induced apoptosis in DMBA induced HBP was performed by TUNEL assay (Fig. 9.5). DMBA induced HBP shows few or lesser apoptotic cells. In contrast, oral administration of [6]-shogaol significantly induces apoptosis was confirmed by dark brown stained cells in DMBA induced HBP. Meanwhile, unstained cells were observed in untreated control and [6]-shogaol alone treated animals.

Fig. 9.5. Apoptotic cells were revealed by TUNEL assay in buccal tissue of control and experimental group (A-D). Representative microphotographs of TUNEL staining of (A) control, (B) DMBA induced buccal tissue, (C) DMBA + [6]-shogaol (20 mg/kg b.w) arrows marks indicate positive cells, (D) [6]-shogaol alone.
9.2.6. Effects of [6]-shogaol on MMP-2, -9 / TIMP-2 and VEGF expression

The effect of [6]-shogaol on MMP-2, MMP-9, TIMP-2 and VEGF expression were noticed in control and experimental animal in each groups shows in Fig. 9.6. In DMBA treated hamsters shows significantly increased the expression of MMP-9, MMP-2, VEGF and decreased expression of TIMP-2 as compared to control (Group I). Conversely, oral supplementation of [6]-shogaol to DMBA induced hamsters shows significantly enhanced the expression of TIMP-2 which could inhibit the MMP-2, MMP-9 and VEGF expression as compared with DMBA treated hamsters (Group II). Control and [6]-shogaol alone treated hamsters has no significant changes in the angiogenesis markers.

Fig. 9.6. (a) Effect of [6]-shogaol on DMBA induced angiogenesis and metastasis markers. The mRNA expressions of MMP-2, MMP-9, TIMP-2 and VEGF were analyzed by RT-PCR. (b) Densitometric analysis was performed by using Kodak GL 100 imaging system. The densitometry data represent means ± SD from three independent experiments and are shown as relative density of mRNA normalized to GAPDH mRNA. Values are not sharing a common marking (*) and **) differ significantly at P < 0.05 (One way ANOVA followed by DMRT).
9.3. Discussion

Understanding the signaling mechanisms are involved in the pathogenesis of oral cancer would help to improve its diagnosis, prognosis and development of new therapeutic approaches. Phytonutrients block specific signal stimulating enzymes and growth factor receptors (GFRs) involved in cancer cell growth. These nutrients are able to eventually inhibit transmission of signal molecule to malignant cell replication (Wang et al., 2012). In this study, we investigate the effect of [6]-shogaol inhibit angiogenesis, invasion and metastasis through suppression of JAK-1/STAT-3 and PI3K signaling activation on DMBA induced HBP experimental model.

The JAK/STAT is one of the major signaling pathway mediated by several cytokine, environmental carcinogen and growth factors contributes to various oncogenic process in cancer (Mali, 2015). Anomalous activation of JAKs phosphorylates tyrosine residues in the cytoplasmic region of the receptor creating binding sites that offer STAT-3 activation. The STAT-3 form dimers that translocate to the nucleus when phosphorylated on highly conserved tyrosine residues of pSTAT through active JAK-1. The STAT dimers bind specific promoter sequences and modulate transcription of genes confer cellular processes such as proliferation, metastasis, angiogenesis and resistance to apoptosis resulting in cancer (Kowshik et al., 2014; Mali, 2015).

In our study, we noticed over expression of JAK-1, STAT-3 and its upstream molecules IL-6, EGFR in DMBA induced hamsters. Whereas, oral administration of [6]-shogaol 20 mg/kg b.wt to DMBA induced hamsters shows significantly inhibit the activation of JAK-1, STAT-3, IL-6 and EGFR expression levels. Similarly, [6]-shogaol inhibits cancer cells proliferation through inhibition of STAT-3 and NF-κB signalling pathway (Saha et al., 2014). Similarly, several dietary agents particularly targets molecular signaling like green tea (Tedeschial, 2004), resveratrol (Wung et al., 2005), and curcumin (Bharti et al., 2003) have been shown to suppress STAT activation in tumor cells. Thus evidence suggested that [6]-shogaol mightly inhibit JAK-1/STAT-3 activation in DMBA induced HBP carcinogenesis.
Accumulating evidence suggest that aberrant activation PI3K/AKT signalling events which controls cell proliferation and survival, angiogenesis, invasion and metastasis has achieved major importance as a target for cancer therapy (Kavitha et al., 2013; Mali, 2015). The cytosolic located PI3K/AKT functionally loss or activation can leads to cause neoplastic transformation in different types of cancers (Vivanco and Sawyers, 2002; Mali, 2015). Abrogation of JAK phosphorylates to activation of PI3K/AKT resulting in enhance cell proliferation, angiogenesis, invasion and metastasis in DMBA induced oral cancer (Kavitha et al., 2013). Similar results were observed in our study. Conversely, oral administration of [6]-shogaol inhibit cytosolic active molecules of PI3K and AKT in DMBA induced hamsters. Thus, inhibition of PI3K and AKT could prevent subsequent activation of angiogenesis, invasion and metastasis in cancer. The phenolic compound of curcumin induces apoptosis via suppression of NF-κB/PI3K and AKT signaling pathway (Qiao and Jiang, 2013). Similarly, Kavitha et al. (2013) and Kowshik et al. (2014) reported that dietary agents suppressed abrogate cell proliferation, invasion and angiogenesis markers by block STAT-3 and PI3K/AKT signaling activation in DMBA induced HBP carcinogenesis.

Alteration of oncogenes and tumor suppressor genes expression are associated with apoptosis and/or tumor development (Wong, 2011). Loss of the p53 function is probably one of the most consistent abnormalities found in human tumours. In many different types of cancer including oral cancer show a high incidence of p53 mutation (Gimenez-Conti et al., 1996; Muller and Vousden, 2013). This mutation leads to a loss or diminution of the wild type activity of p53 and function as a dominant negative inhibitor. Numerous in vitro and xenograft models have confirmed that the ability of mutant p53 enhances invasion and motility, with evidence mutant p53. There is evidence that the presence of mutant p53 may dampen the response to restoration of wild type p53 (Wang et al., 2011), which reflects a dominant negative activity of mutant p53. Recently, studies indicated that the retention of wild-type p53 can be detrimental to the therapeutic response in cancer.
In our study, we noticed the same in DMBA induced cancer bearing animals. This clearly indicated that the inhibition/suppression of mutant p53 may restore the wild type p53 function, leading to therapeutic response and improved clinical outcome, likely [6]-shogaol treatment improved therapeutic response may be associated with restoration of the wild type p53 function. Previous studies demonstrated that functional mutation of p53 and altered expression of Bcl-2/Bax ratio may shift in favour of cell proliferation by dysregulating the apoptotic pathway (De Sousa et al., 2009). Similarly, in our study increase in p53 and Bcl-2 expression which are related to redox imbalance and oxidative insult during DMBA metabolism. [6]-shogaol treatment significantly inhibits expression of mutant p53, Bcl-2 and increased expression of Bax. Activation of Bax, inhibits Bcl-2 which leads to release cytochrome c from mitochondria, its activate apoptotic stimuli. These mitochondrial apoptotic stimuli trigger caspase dependant intrinsic apoptosis (Fig.9.5). Similar class of phenolic acid curcumin induces apoptosis through activation of Bax, caspase in colorectal carcinoma cancer cells (Guo et al., 2013; Guo et al., 2015).

Several phytochemicals have been documented to inhibit hypoxia induced angiogenesis by modulating the expression of pro-angiogenic and invasive molecules can be used as early targets for preventing cancer metastasis (Chiodoni et al., 2010; Chien et al., 2012). In DMBA induced tumor bearing animal shows increased mRNA expression of MMP-9 and MMP-2 were depicted in Fig. 9.6. Oral administration of [6]-shogaol significantly, decreased MMP-9 and MMP-2 expression in DMBA treated animals. Enhanced expression of TIMP-2, a key inhibitor specifically inhibits the expression of MMP-9/MMP-2 in turn block tumor invasion. Similarly, Ling et al. (2010) reported that ginger and its derivatives inhibits breast cancer cell invasion by decrease MMP-9 expression via blockade of NF-kB activation. Dietary agent could inhibit malignant cell invasion and migration through abrogate MMP-2, MMP-9 and its inhibitors through sustain or inhibition of JAK/STAT pathway in hamster experimental model (Kowshik et al., 2014). Thus, inhibition of neo-angiogenesis by suppressing JAK-1/STAT-3 may represent an attractive strategy in preventing or delaying new tumour formation.
Angiogenesis is a very essential process for a metastasized neoplastic cell to survive and develop at a secondary neighbour site. Human squamous cell carcinoma (HSCC) is typically hypervascular, in which a high density of tumor vasculature surrounds the cells, resulting in poor prognosis (Folkman, 2002). VEGF is a 45 kDa of homodimeric glycoprotein which is the key mediator of angiogenesis. Over expression of VEGF has well documented in oral cancer (Carmeliet and Jain, 2000; Seki et al., 2011). Therefore, inhibiting VEGF expression is a promising strategy for the anti angiogenesis and cancer therapy (Ferrara and Kerbel, 2005). Here, we noticed over expression of VEGF in DMBA induced hamster. However, oral administration of [6]-shogaol significantly decreased the VEGF expression (Fig.9.6). Previous study reported that inhibition of cytokine and growth factor resulting in reduces the formation of vessels arrangement and inhibits the primary cell proliferation and metastatic tumors progression (Ferrara, 2010). Similarly, [6]-shogaol and [6]-gingerol inhibit invasion and angeogenesis in hepatocarcinoma cells. Another study also documented that dietary agent of ginger inhibits NF-κB activation and subsequent inhibition of angiogenic factors VEGF and IL-8 expression ovarian cancer cells (Rhode et al., 2007). Weng et al., (2012) suggested that [6]-shogaol has double bond on the carbon side chain and α, β-unsaturated carbonyls that might inhibit invasion angiogenesis of cancer cell. Therefore, these findings suggest that [6]-shogaol exerts anti tumor angiogenesis effect through inhibition of STAT-3/PI3K/AKT independent signaling pathways.