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der Universität zu Lübeck
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Contribution of Bruton's Tyrosine Kinase in Progression, Migration and Toll-Like Receptor induced Inflammation in Head and Neck Squamous Cell Carcinoma Cell lines

Dissertation

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DECLARATION

I hereby declare that this dissertation was completely written single-handed and no other sources have been used than those referred to in the dissertation itself. This dissertation in same or similar form has not been submitted in support of an application for any degree from the University of Lübeck or any other University.

Lübeck, 04 June 2015

ABSTRACT

Head and neck squamous cell carcinoma (HNSCC) is the sixth most frequent and aggressive malignancy worldwide. It uses diverse immuno suppressive strategies to activate high incidence of locoregional recurrence or distant metastasis leading to poor prognosis and has limited the overall survival rate of the patients. Toll-like receptors (TLRs) are crucial immune function regulators, whose aberrant activation was suggested to associate with chronic inflammation and tumor progression. The underlying mechanism driving this dual function of TLRs is still obscure. Recently, Bruton's tyrosine kinase (BTK) has emerged as a significant molecule involved in TLR signaling. Strategies targeting BTK with a clinically potent inhibitor, Ibrutinib (IBT) have successfully been implemented in a variety of B-cell malignancies. So far, the role of BTK in TLR signaling is unclear in HNSCC. Hence, it is important to understand its precise mechanism and its contribution to inflammation and HNSCC recurrence or resistance. The present study was focused to evaluate the molecular mechanisms of BTK and its contribution in TLR3- and TLR4-induced inflammation and the inhibitory influence of IBT in malignant HNSCC cell progression. *In vivo* analysis of different primary and their corresponding metastasis HNSCC cells revealed that inhibition of BTK by IBT modulates the expression of several genes related to cancer pathway and its function was appeared to be critical for the HNSCC cell survival, proliferation, migration and apoptosis. Furthermore, absence of BTK activity significantly impairs the production of TLR3- and TLR4-induced pro-inflammatory cytokines IL-1β, TNF-α and IL-8. Moreover, TLR3- and TLR4-induced activation of ERK1/2 and JNK MAP kinases was found to be dependent on BTK function. Inhibitory effect of combined treatment with IBT alone or in combination with TLR agonist Poly (I:C) led to increased apoptosis and inhibited tumor cell viability and cell migration. Therefore, in summary, the present study provides novel insights into the complex role of BTK in regulating TLR3- and TLR4-induced inflammation and indicates a possible involvement of BTK in regulating TLR-induced anti-apoptotic and migration strategies which could be either associated with distant metastasis or high locoregional recurrence. Hence, the present data suggests, targeting BTK would provide a promising and highly efficacious combined therapeutic approach for malignant HNSCC patients.

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LIST OF ABBREVIATIONS

(ds)RNA	(double stranded) Ribonucleic Acid
°C	Degree Celsius
µ	Micro (10^{-6})
µm	micrometre
BCL2L11	Bcl-2-like protein 11
BCR	B-Cell Receptor
bp	Base pairs
BSA	Bovine Serum Albumin
BTK	Bruton's Tyrosine Kinase
CO_2	Carbon dioxide
DAMP	Damage-Associated Molecular Patterns
DAPI	4',6-diamidino-2-phenylindole
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
dNTP	Deoxynucleotide Triphosphates
ECL	Enhanced chemiluminescence
ELISA	Enzyme-Linked Immunosorbent Assay
ERK	Extracellular signal-regulated kinases
et al	et alii (and others)
FACS	Fluorescence Activated Cell Sorting
FASLG	Fas Ligand
FBS	Fetal Bovine Serum
FL	Full Length
g	Earth's gravitational force
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

gm	Grams
h / hr	Hours
HMOX1	Heme oxygenase (decycling) 1
HNSCC	Head and Neck Squamous Cell Carcinoma
IBT	Ibrutinib
IF	Immuno Fluorescence
IFN	Interferon
IL	Interleukin
JNK	c-Jun N-terminal kinases
kb	Kilo base
kDa	Kilo Dalton
l	Litre
LPS	Lipopolysaccharides
M	Molar (mole/litre)
MAP2K3	Dual specificity mitogen-activated protein kinase kinase 3
MAPK	Mitogen-Activated Protein Kinases
mins	Minutes
ml	Millilitres (10^{-3} l)
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)
MYD88	Myeloid differentiation primary response gene 88
n.s	Non-significant
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
P	Phosphorylated protein
P38	p38 mitogen-activated protein kinases
PAMP	Pathogen-associated molecular pattern molecule
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction

PGF	Placental growth factor
PI	Propidium Iodide
Poly (I:C)	Polyinosinic: polycytidylic acid
PRR	pattern recognition receptors
qRT	Quantitative real time
rpm	Revolutions per minute
RTCA	Real-Time Cell Analyzer
S	Seconds
SDS	Sodium dodecyl sulphate
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRIF	TIR-domain-containing adapter-inducing interferon-β
U	Unit
UT	University of Turku

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1. INTRODUCTION

1.1. Head and neck squamous cell carcinoma (HNSCC)

1.1.1. Epidemiology and risk factors

According to National Cancer Institute at the National Institute of Health, head and neck squamous cell carcinoma (HNSCC) is defined as "cancer that arises from epithelial cells that line the mucosal surfaces of upper aero digestive track, including oral cavity, nasal cavity, paranasal sinuses, pharynx, larynx, and local lymph nodes". It is the sixth most frequent and aggressive neoplasm worldwide (Jemal*, et al.*, 2008) with approximately 644, 000 new cases diagnosed every year with two-thirds of these occurring in developing countries (Marur*, et al.*, 2008) The incidence of HNSCC is twice as high in men when compared with the cases as in women (Alibek*, et al.*, 2013). The epidemic rate of HNSCC cases in southern Asia accounts for 50% of all cases diagnosed per year. Whereas in central, southern Europe and United states 5% recorded cancer cases were associated with head and neck (Boyle*, et al.*, 2008)

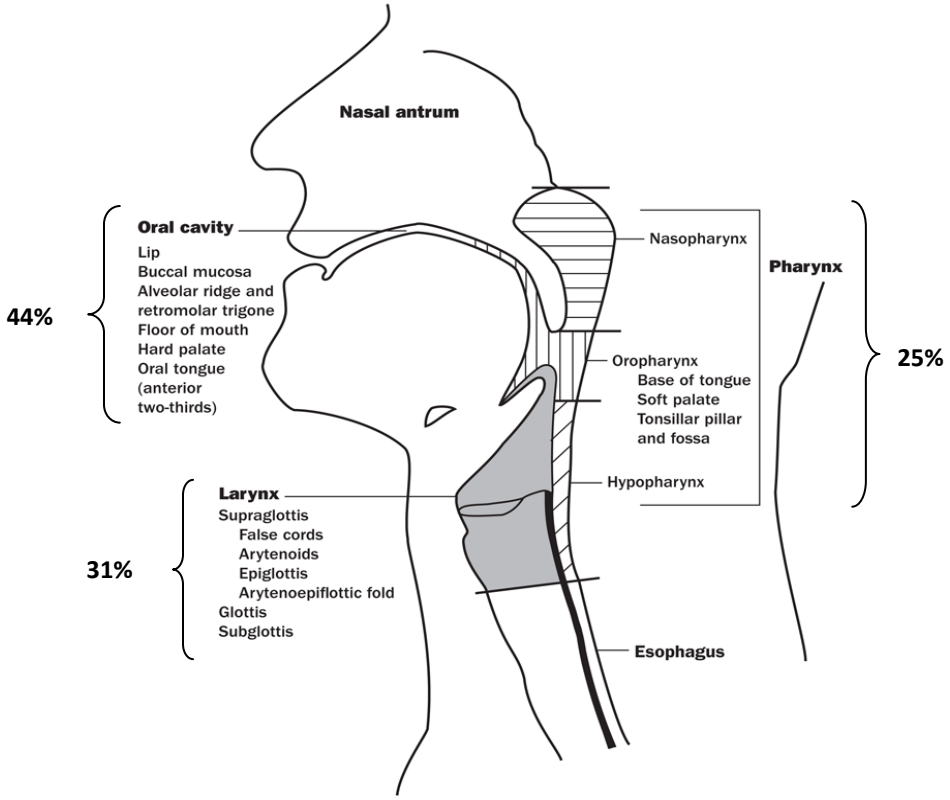


Figure 1. Anatomic illustration of head and neck squamous cell carcinoma (HNSCC) originates and its anatomical distribution of cancer according to Ridge JA. (Ridge*, et al.*, 2014).

HNSCC arises sporadically and the major incidence of cancer is due to number of life style related risk factors. Conventionally, 80%-90% of HNSCC have been attributed to chronic tobacco and alcohol consumption (Sturgis*, et al.*, 2007). The combined effect of consuming both tobacco and alcohol accounts for up to 70%, whereas the risk is reduced to fifteen fold in those who do not smoke or drink alone of all head and neck cancers that occur globally (Hashibe*, et al.*, 2009). Additionally in 15% patients, the probable cause of cancer has been linked to the presence of the oncogenic variants of viruses like Epstein-Barr virus (EBV, nasopharyngeal cancers) (Raab-Traub, 2002) and human papillomavirus (HPV, Oropharyngeal cancers), in particular type HPV-16 and 18 (Hennessey*, et al.*, 2009). Further, few other listed risk factors particularly associated with sinonasal carcinomas that includes occupational exposures to chromium, nickel, and radium (Marur*, et al.*, 2008). Albeit the listed factors were studied extensively, still other factors like genetic factors which might play a role in the development of the cancers still have to be studied comprehensively.

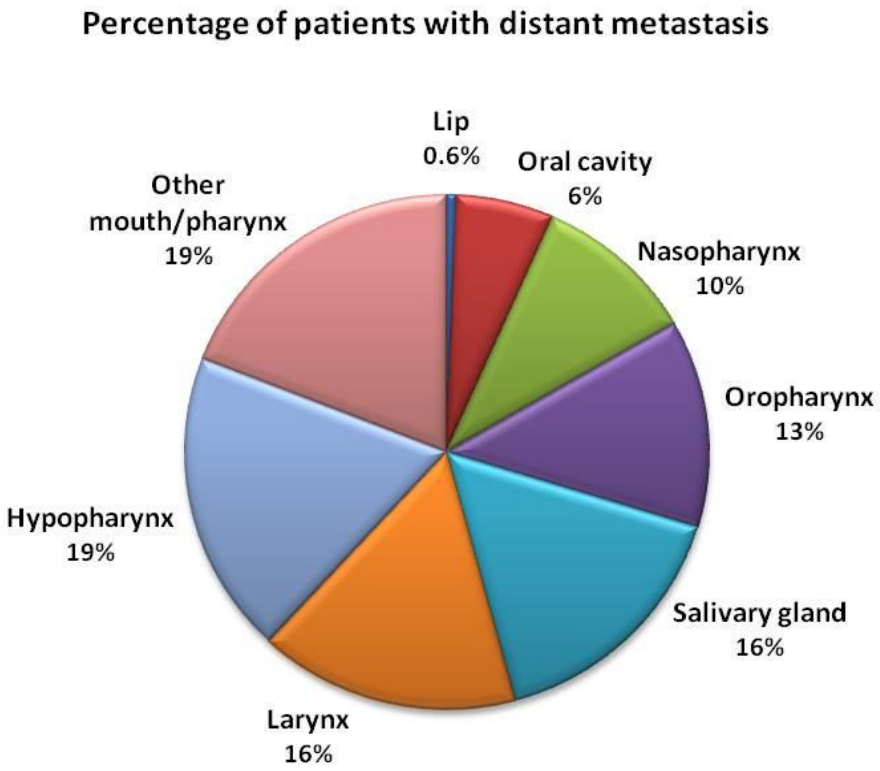


Figure 2. Metastasis of HNSCC to distant organs diagnosed in patients (1974-1999) (Carvalho*, et al.*, 2005).

1.1.2. Limitations of treatment

HNSCC is associated with high rates tumor recurrence leading to the increased mortality. The current treatment strategies for patients with HNSCC cover surgical resection, chemotherapy, radiation therapy, photodynamic therapy and targeted therapy for specific properties of cancerous cells. Many of these therapies increased the better quality of life, but the mortality rate of less than 50% has remained unchanged for decades (Haddad*, et al.*, 2008). The cure rate of 70-90% was achieved in approximately one-third of HNSCC patients diagnosed and treated in the early-stage of the disease (Argiris*, et al.*, 2008). Conversely within 5years, disease recurrence was experienced in majority of the patients with distant metastasis (Figure 2) resulting in death due to loco-regionally advanced disease (stage III or stage IV) (Chin*, et al.*, 2005). The other prognostically most important factor of HNSCC is the ability to metastasize to lymph nodes and distant organs from primary site, (Carvalho*, et al.*, 2005) by influencing the host immune system early (Duray*, et al.*, 2010). And the primary challenge to progress in search of better cure would require more understanding of HNSCC cellular mechanisms.

1.1.3. Molecular mechanisms involved

In most of the cancer cells the immune responses are misdirected through several mechanisms, which result in failure of recognizing the transformed cells and subsequent immune attack. The main alterations of cancerous cells were characterized by six hall marks according to the molecular, biochemical and cellular features, (Hanahan*, et al.*, 2011), such as:

1.1.3.1. Self-sufficiency / Inhibition of growth signals

Cancer cells are self-sufficient in the growth signals and grow independently. They can generate growth factors themselves or influence cells through their microenvironment to produce over-expressing receptors for growth factors and evade normal growth suppressors to undergo extensive cell proliferation, enhancing tumor development (Hanahan*, et al.*, 2011). Epidermal growth factor receptor (EGFR) is a cell surface receptor belonging to epidermal growth factor (EGF) family. It is one of the growth

factors that is persistently produced by cancer cells (Walker*, et al.*, 2009). There are several studies demonstrating over-expression of EGFR in HNSCC cells (Santini*, et al.*, 1991; Saranath*, et al.*, 1992), which correlates with poor prognosis. Due to its critical role in cell survival and proliferation, the EGFR has been a target of anticancer treatment (Burtness, 2005).

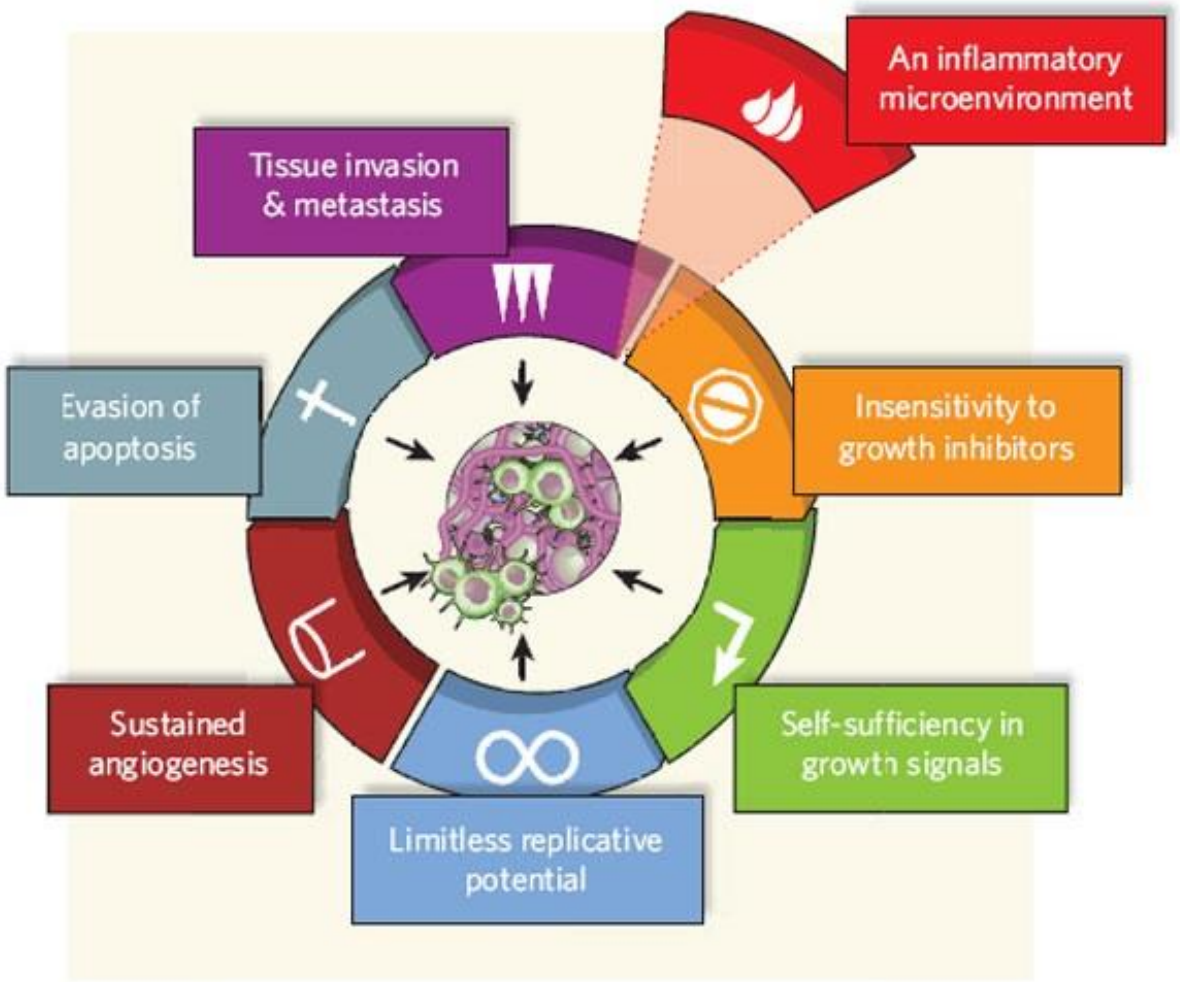


Figure 3. Illustration of six hallmarks of cancer proposed by Hanahan and Weinberg (Hanahan*, et al.*, 2011) and the inflammatory microenvironment as an emerging seventh hallmark (Laird*, et al.*, 2011).

1.1.3.2. Limitless growth potential

The proteins involved in regulation of cell division, mutate and lack function in most cases, eventually driving inappropriate cell stages and reproducing uncontrolled cell

growth of cancer cells. In HNSCC tumors, over expression of e.g. Cyclin D1 and its correlation with progression of the disease is widely observed (Jares*, et al.*, 1994). And likewise, in contrast to the condition in normal cells, the activity of telomerase is found to be high in HNSCC cell lines (100%), invasive tumors (90%), dysplastic lesions (100%) and hyperplasic lesions (100%). The activation might occur early in the tumorigenesis process and the active state of the enzyme is found to be consistent (Mao*, et al.*, 1996).

1.1.3.3. Ability to sustain angiogenesis

Basically a tumor cannot grow beyond 1-2 mm in size due to a limited supply with nutrients and oxygen. Therefore, angiogenesis plays a very critical role. One of the factors to switch on angiogenesis is regulated by tumor secreted growth factors such as vascular endothelial growth factors (VEGF) (Kyzas*, et al.*, 2005) promoting formation of blood vessels and directional growth. Hence, targeting angiogenic mechanisms has been considered as one of the important anti-cancer approaches.

1.1.3.4. Ability to evade apoptosis

One of the primary characteristics of malignant cell is to acquire the ability to resist apoptotic stimuli and abnormal regulation of apoptosis. Events like mutations in tumor suppressor genes such as, p53, polymorphisms of cell surface receptor FAS and its interacting ligand FASLG (Zhang*, et al.*, 2006) and or uncontrolled expression of anti-apoptotic genes like, Bcl-2 and Bcl-XL, have been associated with increased susceptibility to a variety of cancers including HNSCC. The expression of anti-apoptotic genes inhibits the apoptosis via preventing the release of crucial pro-apoptotic proteins like Bax and cytochrome C (Reed, 2000; Vousden*, et al.*, 2002)

1.1.3.5. Tissue invasion and metastasis

Cancer cells can migrate to distant organs through blood vessels, seed there and grow. This property of cancer cells is due to their ability, to e.g. destroy the basement membrane of the surface epithelium, invade, metastasize (Scanlon*, et al.*, 2013),

activate various extracellular proteases like 'Matrix Metalloproteinase' (MMPs), and to inhibit the 'Tissue Inhibitors of Metalloproteinase' (TIMPs). They play a major role in Epithelial-mesenchymal transition (EMT) and tissue remodeling. EMT facilitates invasion of cancer cells by developing motile, mesenchymal-like cells from non-motile parent epithelial cells. Consistently growing evidences state that EMT plays a significant role in HNSCC invasion and metastasis. Several protein biomarkers of EMT have been identified in HNSCC such as E-Cadherin (Biddle*, et al.*, 2011), N-Cadherin, (Nguyen*, et al.*, 2011), Vimentin (Chen*, et al.*, 2011), β-catenin (Goto*, et al.*, 2010), SNAIL1(Mendelsohn*, et al.*, 2012) and many more. Also over expression of MMPs is associated with degradation of tissues in many chronic inflammatory diseases like cancer and thus increase in the activity of MMPs influence the process of metastasis and tumor (Tang*, et al.*, 2005). In case of HNSCC MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-10, MMP-11, and MMP-13, levels were over expressed. In particular MMP-9 might be useful for evaluating the malignant potential in individual HNSCC (Pornchai*, et al.*, 2001).

1.2. Inflammation and cancer

Inflammation is a complex and strictly regulated immunological response against pathogen invasion, external stimuli such as chemical or physical stress, environmental pollutions and tissue injury controlled by the cells of innate and adaptive immune system. Even though dynamic inflammatory response is critical for host defensive mechanism resulting in healing process, the prolonged inflammatory responses can induce chronic inflammation resulting in tissue destruction and development of cancer. A german physician Rudolf Virchow in 1863 proposed association of chronic inflammation in tumor progression and this was perceived until today and supported by epidemiological studies revealing the relationship of chronic inflammation in developing cancer, in about 15-20% of all global cancers (Balkwill*, et al.*, 2001; Mantovani, 2009).

One of the key players of inflammation are macrophages as they have a central function in mediating innate immune inflammatory responses by recognizing microbial pathogens and host tissue injury through different pathogen recognition receptor (PRR) families (Takeuchi*, et al.*, 2010). Following the recognition of pathogen or host injury, a cascade of the events is initiated: i) production of soluble inflammatory

mediators such as inflammatory cytokines, chemokines and complement components; ii) concurrent recruitment and migration of leukocyte to inflammatory tissue region, orchestrated by enzymes, in turn to activate immune cells to clear infection and tissue repair (Araki*, et al.*, 2005). In accord, the key orchestrators that initiate the inflammatory responses include transcription factors and pro-inflammatory cytokines (Balkwill, 2009; Rius*, et al.*, 2008). The transcription factors including nuclear factor-kappa B (NF-κB) are activated downstream of TLR-signaling and induce the expression of the inflammatory cytokine cascade such as Interleukin (IL)-1β, IL-6, IL-8 and Tumor necrosis factor-alpha (TNF-α) (Colotta*, et al.*, 2009). The recent studies on chronic inflammatory responses in gastrointestinal tract and the liver provide evidence of their involvement in tumor initiation and progression in tissues (Colotta*, et al.*, 2009). As NF-κB is a downstream target of toll-like receptors, we emphasize to study the TLR induced inflammation process in HNSCC cells in particular.

1.3. Pro-inflammatory cytokines

Cytokines are a variety of soluble factors that regulates host responses towards infection, immune response and inflammation. Some cytokines clearly promote inflammation and act to worse the disease and are called as proinflammatory cytokines. Whereas, some serve to suppress the activity of proinflammatory cytokines, reduce inflammation and promote healing, which are known as anti-inflammatory cytokines e.g. interleukin (IL)-4, IL-10 and IL-13 are potent anti-inflammatory agents to suppress genes for pro-inflammatory cytokines such as IL-1, Tumor necrosis factor (TNF) and chemokine IL-8 (Dinarello, 2000). Proinflammatory cytokines, IL-6 and TNF-α have been suggested to play certain role in variety of squamous cell carcinomas (SCCs) including HNSCCs (Druzgal*, et al.*, 2005; Hoffmann*, et al.*, 2007; Mojtahedi*, et al.*, 2011; Skrinjar*, et al.*, 2015; St John*, et al.*, 2004). Hence, the present study was focused to investigate the influence of the following proinflammatory cytokines in contributing to acute inflammation which could also be involved in promoting tumor recurrence and anti-tumor resistance in HNSCC cell lines.

1.3.1. Interleukin (IL)-1β

Interleukin (IL)-1β is the well characterized molecular form of IL-1 and is one of the potent pro-inflammatory cytokine that exert pleiotropic effect on variety of cells. IL-1β is crucial for the host-defense in response to infection and is one of the important soluble mediators of acute and chronic inflammation (Dinarello, 1996). IL-1β is produced and secreted by variety of cell types and signals through IL-1 type 1 receptor (IL-1R1) which recruits IL1 receptor accessory protein (IL-1RAcP) at the cell membrane leading to the activation of intracellular signaling (Ren*, et al.*, 2009). Activation of IL-1β requires processing from an inactive precursor by cysteine protease caspase-1 via the inflammasome, an intracellular multi-protein complex and regulator of inflammation (Martinon*, et al.*, 2007).

Constitutive and upregulated production of IL-1β was documented in solid tumors including breast, colon, head and neck cancers and was generally associated with a bad prognosis. The IL-1 expression can exhibit autocrine behavior by enhancing the tumor cell to invade and proliferate by itself, or exhibit paracrine effect on stromal cells in tumor microenvironment. It is also known to induce expression of metastatic genes like matrix metalloproteinase's (MMP) that are involved in the production of angiogenic proteins and growth factors such as VEGF, IL-8, IL-6, TNF-α, and tumor growth factor beta (TGFβ) (Lewis*, et al.*, 2006). The importance and necessity of IL-1 in the tumor growth and invasion has resulted in the investigations to target IL-1 receptors as a novel therapeutic agent.

1.3.2. Interleukin (IL)-6

Interleukin (IL)-6, is a multi-functional cytokine produced by T-cells and macrophages. It was well characterized as a critical regulator of immune and inflammatory responses during infection. It transduces the signals upon binding to ligands through a heterodimeric receptor that contains the ligand binding IL-6 alpha receptor (αIL-6R) and associates with gp130, thus involves in the activation of JAK/STAT, ERK and PI3K signaling pathways. There are recent reports stating the following: i) elevated expression of IL-6 in multiple epithelial tumors; ii) ability of IL-6 to induce B-cell differentiation; iii) role of IL-6 in induction of IL-2 and IL-2 receptor; iv) proliferation and differentiation in T-cells that are involved in the tumor proliferation. In addition, IL-6 was

implicated in tumorigenesis and it was also shown to promote malignancy in certain carcinomas (Hirano*, et al.*, 1990; Schafer*, et al.*, 2007).

1.3.3. Interleukin (IL)-8

Interleukin (IL)-8, is a pro-inflammatory cytokine alternatively known as CXCL8, belongs to the CXC chemokines family. IL-8 is responsible for induction of chemotaxis, facilitating directed migration of cells to the site of inflammation. It is known that IL-8 expression is regulated by different stimuli including inflammatory signals such as IL-1β, TNF-α, environmental stress and many others. The biological effects are mediated through binding of IL-8 to two cell-surface G protein-coupled receptors called CXCR1 and CXCR2 that promote activation of Akt, PKC or MAPK signaling cascades. Tumor derived IL-8 has a profound effect on enhancing tumor cell proliferation and survival. In addition, IL-8 activates endothelial cells to promote angiogenesis and induce a chemotactic infiltration of neutrophils into tumor microenvironment. It can also promote tumor cell invasion and migration by inducing secretion of tumor-associated growth factors. Therefore, due to its multiple effects, targeting CXC-chemokines signaling might have important implications in therapeutic treatment (Waugh*, et al.*, 2008).

1.3.4. Tumor necrosis factor (TNF)-α

Tumor necrosis factor (TNF)-α is a homotrimeric proinflammatory cytokine which is also characterized as ‘cachectin’ and belongs to TNF superfamily. TNF-α secretion can be induced by several pathogen associated molecular patterns (PAMPs) and it is known to exist in transmembrane and soluble forms. Its bioactivity is regulated by binding TNF-α with two distinct receptors TNFR1 (p60) and TNFR2 (p80) to facilitate the activation of several inflammatory cascades (Wu*, et al.*, 2010). Although TNF-α plays a crucial role in apoptosis, cell survival, inflammation and host immune responses, elevated production of TNF-α and persistent immune responses were identified to contribute to several pathological processes such as chronic inflammation and malignant disease. In certain tumor types, TNF-α is widely known to induce hemorrhagic necrosis and tumor progression (Balkwill, 2009). As TNF-α receptors are expressed on both epithelial and stromal cells, constitutive production of TNF-α in

tumor environment can not only directly facilitate cancer progression by regulating neoplastic cells but also it can act indirectly through endothelial and other inflammatory cells. TNF-α induces tumor initiation and promotion mediated through activation of NF-κB, PKCα dependent pathways. It induces expression of growth factors like EGFR and TGF-α, leading to increased tumor proliferation, it is also known to regulate tumor angiogenesis by IL-8 and VEGF via a JNK and AP-1 pathways. In a tumor environment TNF-α also confers tumor cell invasion by upregulating migration-inhibitory factor (MIF) in macrophages through enhanced production of MMPs (Hagemann, *et al.*, 2005). Therefore, these pleiotropic effects of TNFs in multiple tumor-promoting activities suggest that inhibition of TNF-α as an effective strategy for cancer therapy (Wu, *et al.*, 2010).

1.4. Pathogen recognition

The initial sensing of microbial infection is mediated by innate pattern recognition receptors (PRRs) which are expressed on both intracellular and extracellular matrix of macrophages, dendritic cells and also in nonprofessional immune cells (Janeway, *et al.*, 2002). They recognize structures conserved among microbial species called Pathogen-associated molecular patterns (PAMPs) and endogenous molecules released from damaged cells, termed danger-associated molecular patterns (DAMPs) (Matzinger, 2002). These receptors are classified into four PRR families based on their location, function and expression. The four recognized PRR families including transmembrane proteins such as the Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), as well as cytoplasmic proteins such as the Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs) (Martinon, *et al.*, 2005; Takeuchi, *et al.*, 2010). Upon PAMP or DAMP recognition, PRRs signal the presence of infection to the host and trigger the transcription of genes by that are involved in inflammatory responses like induction of proinflammatory cytokines, type-I interferons (IFNs), chemokines, cell adhesion molecules, and immunoreceptors (Akira, *et al.*, 2006).

1.4.1. Viral (ds) RNA recognition by TLR3

Viruses possess several structurally diverse PAMPs, including surface glycoproteins, DNA, and RNA species (Mogensen*, et al.*, 2005). Toll like receptor 3 (TLR3) specifically detects viral double stranded (ds) RNA (Alexopoulou*, et al.*, 2001). Structural analysis of the receptor revealed that the leucine rich receptors (LRRs) form a solenoid horseshoe shape of which one-side of it is masked by carbohydrate while the other side is glycosylation-free (Choe*, et al.*, 2005). From the analysis of crystal structure of TLR3 bound to dsRNA, it was established that dsRNA binds to the N-terminal and C-terminal portions of LRRs (TLR3) and tends to form the dimer of TLR3 molecules upon binding to the ligand was established (Liu*, et al.*, 2008).

TLR3 is mostly thought of as an intracellular receptor, resident on the plasma membranes of endosomal vesicles. Flow cytometry analysis with human TLR3 antibodies showed that human fibroblasts and epithelial cells express TLR3 both on the cell surface and in the endosome. However, immature human DCs only express endosomal bound (Matsumoto*, et al.*, 2003). TLR3 transduce signaling *via* the intracellular Toll/IL-1 receptor (TIR) domain by recruitment of adaptor protein TIR-domain containing adaptor inducing interferon-β (TRIF) (also called TICAM-1). It contains alanine in position 795 in the protruding BB loop of the TIR domain rather than the proline amino acid moiety, which is conserved amongst other TLRs (Oshiumi*, et al.*, 2003a).

Polyinosinic polycytidylic acid (Poly (I:C), a synthetic analog for dsRNA was found to be the most effective TLR3 agonist (Sha*, et al.*, 2004). The cellular uptake, internalization and trafficking of Poly (I:C) to the endosome where TLR3 is localized is either facilitated directly by the binding of CD14 on the cell surface to the ligand, Poly (I:C) or cooperates with TLR3 on the cell surface of human fibroblasts to internalize dsRNA (Lee*, et al.*, 2006). TLR3 upon activation by extracellular dsRNA will typically cause or stabilize receptor dimerization by cross linking (Takada*, et al.*, 2007) and activates several intracellular signaling cascades leading to the activation and nuclear translocation of the transcription factors (IRF3, NF-κB) and upregulation of cytokine expression (interferon-β and proinflammatory cytokines). These signaling cascades results in turn activation of interferon stimulated genes (ISGs) and production of anti-viral proteins, thus amplifying the anti-viral immune response (Dunlevy*, et al.*, 2010).

1.4.2. Lipopolysaccharide (LPS) recognition by TLR4

Toll like receptor 4 (TLR4) sense the infection by recognizing lipopolysaccharides (LPS), a compound derived from the outer membrane of gram-negative bacteria which is known to be a cause of septic shock. LPS, particularly the lipid A portion, is a prominent feature of gram-negative bacteria, being one of the most potent PAMPs known and responsible for the inflammatory response observed during endotoxic shock (Akira*, et al.*, 2006; Trent*, et al.*, 2006). LPS that is liberated from gram-negative bacteria associates with the extracellular acute-phase protein called as LPS-binding protein (LBP) (Schumann*, et al.*, 1990). This complex binds to the co-receptor CD14 expressed at the cell surface which allows transfer of LPS to the accessory molecule MD2, which is associated with the extracellular domain of TLR4 (Akira*, et al.*, 2006; Hailman*, et al.*, 1994; Tobias*, et al.*, 1995). Two complexes of TLR4-MD2-LPS interact symmetrically to form a TLR4 homodimer (Park*, et al.*, 2008) and the dimerized TLR4 subsequently activates the early innate immune responses through MYD88 dependent pathway (Wesche*, et al.*, 1997) and later responses through adaptor TRIF (Yamamoto*, et al.*, 2003a).

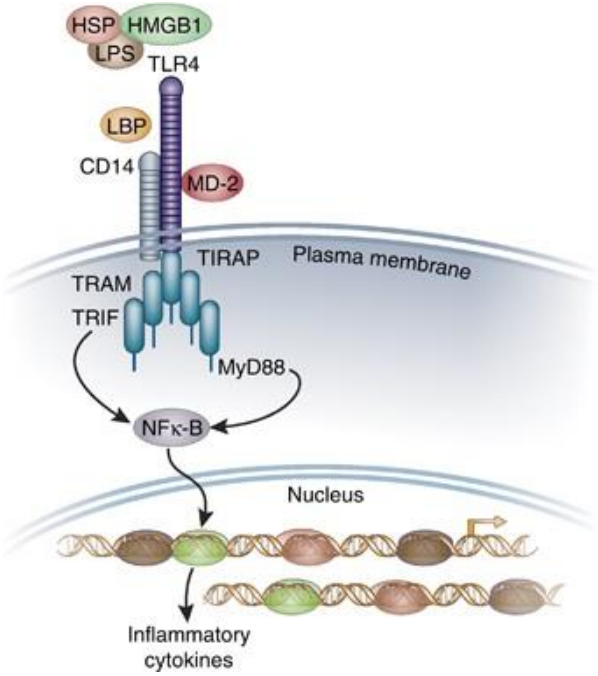


Figure 4. Illustration of lipopolysaccharide (LPS) recognition by TLR4 and the involvement of molecular complex during the process. Data adapted from (Leventhal, et al., 2012).

1.5. Toll-Like Receptor (TLR) Signaling

Toll receptors are evolutionarily conserved between insects and humans (Anderson, 2000). Toll-like receptor (TLR) signaling, regardless of the stimulated receptor, activates NF-κB and MAP kinases to induce regulatory responses. Activation of TLR is initiated by recognition of the pathogenic ligands which in turn lead to TLR oligomerization (Saitoh*, et al.*, 2004). A conformational change in TLR triggers its cytoplasmic TIR domain to recruit different TIR domain-containing adaptor molecules such as myeloid differentiation primary response protein (MYD88), MYD88 adaptor-like (Mal)/Toll/IL-1R domain-containing adaptor Protein (TIRAP), TIR-containing adaptor inducing interferon-β (TRIF) (TRIF; also known as TICAM-1), TRIF-related adaptor molecule (TRAM). Activation of different TLRs recruits different TIR domain-containing adaptor molecules and leads to different pattern of gene expression profiles that are involved in innate immune responses (Doyle*, et al.*, 2002; Hoshino*, et al.*, 2002; Toshchakov*, et al.*, 2002).

Most of the TLRs (except for TLR3) signal through MYD88, whereas, TLR3 signals through the TRIF adaptor molecule. TLR coupled adaptor proteins recruit and activate IL-1R-associated kinase (IRAK) family members (Kobayashi*, et al.*, 2002; Li*, et al.*, 2002). Phosphorylated IRAK activates TRAF6, a member of tumor necrosis factor receptor (TNFR)-associated factor (TRAF), which interacts with and activates TGF-activated kinase 1(TAK1), TAB1 and TAB2, ubiquitylating factors, ubiquitin conjugation enzyme E2 variant 1 (UEV1A) and ubiquitin-conjugating enzyme 13 (UBC13) (Deng*, et al.*, 2000).

Activated TAK1 triggers the phosphorylation of the IκB kinases (IKKs) and catalyzes the phosphorylation and degradation of IκBα, which leads to the activation of nuclear factor κB (NF-κB), interferon regulatory factors (IRFs) and mitogen activated protein kinase (MAPK) signaling pathways. TAK1 is a MAP3K that activates downstream MAPK Kinase-3, (MKK3) MKK6 and MKK7 and subsequently p38 and JNK MAPK (Wang*, et al.*, 2001) subsequently resulting in the upregulation of nuclear AP-1 transcription factor dependent cytokine production (Sato*, et al.*, 2005).

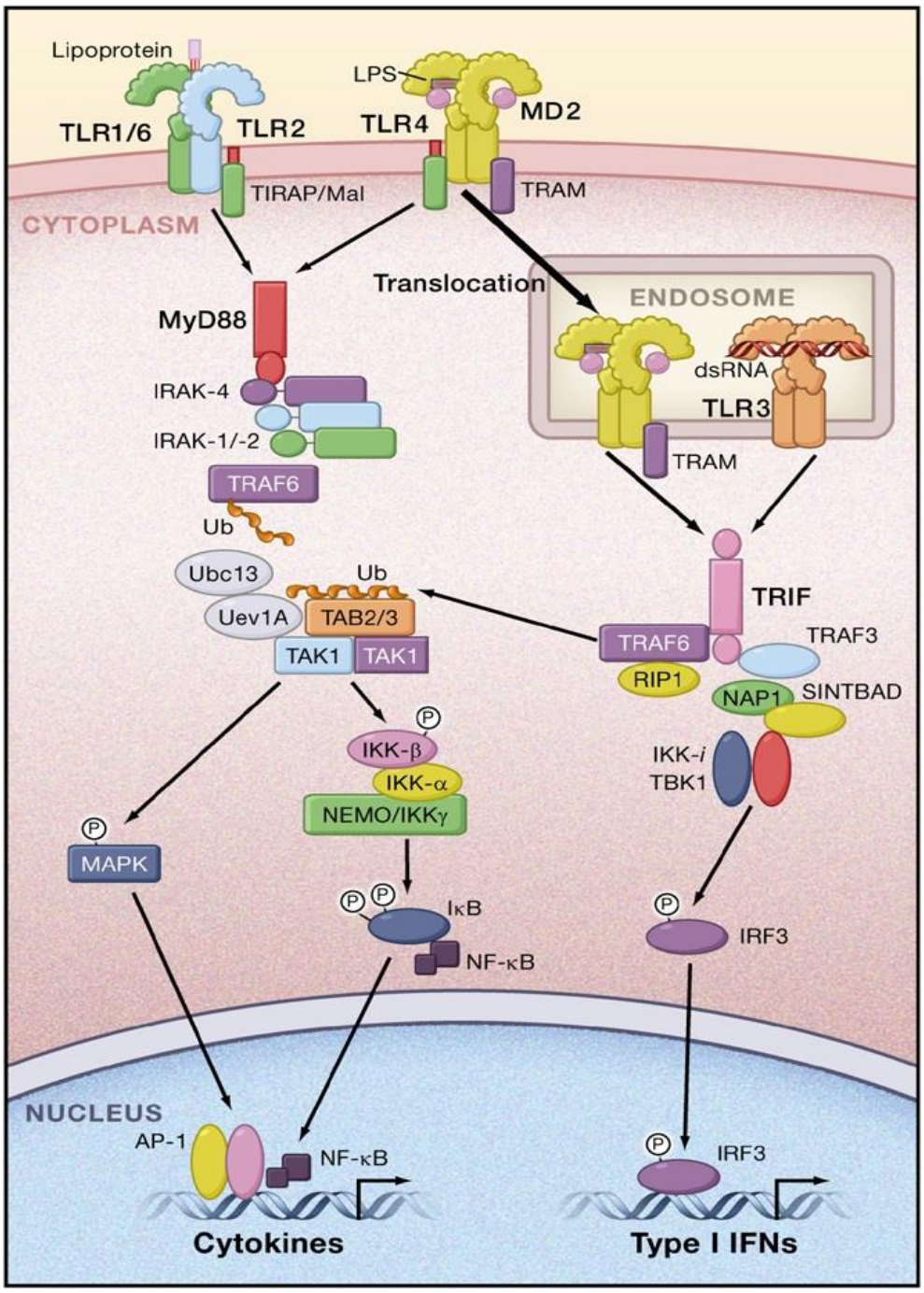


Figure 5. Illustration of TLR signaling cascade: MYD88 dependent signaling and TRIF dependent signaling (Adapted from (Takeuchi*, et al.*, 2010).

1.5.1. MYD88 dependent signaling

MYD88 has a key role in numerous immune modulated processes including host defense, infection, inflammation, and disease (O'Neill, 2008a). It was first shown as an

essential TIR domain containing adaptor molecule to trigger the TLR activated inflammatory cytokine TNF-α and IL-12 expression (Hayashi*, et al.*, 2001; Hemmi*, et al.*, 2002; Schnare*, et al.*, 2000; Takeuchi*, et al.*, 2000). MYD88 is composed of C-terminal TIR domain required for homodimeric interaction with TLR, an intermediary domain (ID), and an N-terminal death domain (DD) essential for protein interaction and downstream signaling (Burns*, et al.*, 1998). Studies using MYD88 deficient mice revealed that most TLRs like TLR2, TLR4, TLR5, TLR7 and TLR9 transduce signals via MYD88 adaptor protein except TLR3. This shows the critical role of MYD88 in TLR induced innate immunity (Muraille*, et al.*, 2003).

TLR activation of the MYD88 dependent pathway result in rapid NF-κB activation and production of proinflammatory cytokines such as: tumor necrosis factor alpha (TNF-α), interleukin (IL-) 1β, IL-6 and chemokines like macrophage inflammatory protein 3α (MIP-3α), monocyte chemo attractant protein-1 (MCP-1), and IL-8 (Zughaier*, et al.*, 2005). Upon TLR activation, MYD88 associates with Type I IL-1R (IL-1R1), which was also observed in TLR4 signaling (Wesche*, et al.*, 1997) in studies using MYD88 deficient mice. This revealed that TLR4 follows both MYD88-dependent (Feng*, et al.*, 2003; Miyake, 2004) and MYD88-independent pathways (Hoebe*, et al.*, 2003a; Oshiumi*, et al.*, 2003b). The activation of TLR4 signaling cascade via MYD88 dependent pathway is important for dendritic cell maturation and provides and link between the innate and adaptive immune responses (Hoebe*, et al.*, 2003b).

1.5.2. TRIF dependent signaling

In response to stimulation with dsRNA, TLR3 recruits TRIF (also called as TICAM-I*),* which is another TIR domain containing adaptor molecule, identified by database screening (Yamamoto*, et al.*, 2002) and also by yeast-two-hybrid screening with TLR3 (Oshiumi*, et al.*, 2003b). TRIF is a large protein consisting of 712 amino acids in humans. It is comprised a C-terminal receptor-interacting protein (RIP) homotypic interaction motif (RHIM), a TIR domain in center, and a consensus TRAF6 binding motifs (T6BM) in the N-terminal region (Oshiumi*, et al.*, 2003b; Takeuchi*, et al.*, 2010). It was demonstrated that, TRIF involves binding of TANK Binding Kinase-1 (TBK1) to its N-terminal (Sato*, et al.*, 2000; Tabeta*, et al.*, 2004) and RIP1 to its C-terminal to mediate downstream signaling (Meylan*, et al.*, 2004).

TRIF dependent pathway is peculiar to the TLR3 and TLR4 signaling cascade (Akira*, et al.*, 2001). Pathogenic activation of TRIF-dependent pathway results in rapid activation of interferon regulatory factor 3 (IRF3) (Kawai*, et al.*, 2001; Oshiumi*, et al.*, 2003b) leading to release of interferon-β (IFN-β) but delayed kinetics of NF-κB (Hoebe*, et al.*, 2003b; Kawai*, et al.*, 1999). Studies from TRIF deficient mice have shown poor direct interaction between TLR4 and TRIF (Yamamoto*, et al.*, 2003a) and later, identified TRAM as an important bridging adaptor protein to transduce TLR4 induced signals by TRIF dependent pathway (Fitzgerald*, et al.*, 2003; Oshiumi*, et al.*, 2003b; Yamamoto*, et al.*, 2003b).

1.6. Protein kinases in TLR signaling

Modification mechanism of proteins by phosphorylation is a prominent mechanism which regulates the activity of several signaling molecules involved in multiple cellular processes. Usually in eukaryotes, the amino acid repertoire of a protein, which can act as a phosphorylation sites are serine, threonine, tyrosine and histidine (Ciesla*, et al.*, 2011). Protein kinases are key enzymes that direct the function and activity of other proteins by addition of phosphates. In TLR signaling, protein serine/threonine kinases (PSTKs) and protein tyrosine kinases (PTKs) play an important role in inducing the innate and adaptive immune responses.

1.6.1. Protein tyrosine kinases

Protein tyrosine kinases (PTKs) are key mediators of trans-membrane signaling. They are non-receptor tyrosine kinases (RTKs) found in the cytoplasm with no transmembrane segment and function downstream in constitutive or inducible association with receptor tyrosine kinase (RTK) (Blume-Jensen*, et al.*, 2001; Ghoreschi*, et al.*, 2009). The protein tyrosine kinase (PTK) activity coordinates a broad spectrum of cellular processes, including proliferation, differentiation, survival, adhesion and motility (Hunter, 2009). Recent understandings of tyrosine kinases have highlighted their imperative role in oncogenic activation and molecular pathogenesis of cancer (Vlahovic*, et al.*, 2003). Perusal of the recent studies conducted on these kinases suggests their major role in inflammation and immune responses. The three

main families of tyrosine kinases listed, sarcoma (Src), tyrosine kinase in hepatocellular carcinoma (Tec) and spleen tyrosine kinase (Syk) are known to be involved through TLR signaling (Page*, et al.*, 2009). The present study was performed on Bruton's tyrosine kinase (BTK), a member of Tec family and the results outcome was discussed in detail in the subsequent sections.

1.6.1.1. Bruton's Tyrosine Kinase (BTK)

Bruton's tyrosine kinase (BTK) is a non-receptor tyrosine kinase present in the cytoplasm of B-cells and all cell lineages of hematopoietic system with exception to plasma cells and T-lymphocytes (Brunner*, et al.*, 2005). It is an essential activator downstream molecule of several receptors thereby involved in diverse signaling cascades and cellular processes such as regulation of B-cell proliferation, apoptosis, differentiation and inflammation (Bolen, 1993; Khan*, et al.*, 1995). BTK is involved in signaling via a variety of receptors including the BCR, FcRs (Kawakami*, et al.*, 1994) TLRs (Jefferies*, et al.*, 2003; Liljeroos*, et al.*, 2007), G protein-linked receptors (Langhans-Rajasekaran*, et al.*, 1995; Ma*, et al.*, 1998), the death receptors and cytokines receptors (Deng*, et al.*, 1998; Matsuda*, et al.*, 1995; Sato*, et al.*, 1994).

Auto-regulatory N-terminal pleckstrin homology (PH) domain of BTK specifically binds to membrane phospholipids and multiple proteins, allowing the BTK recruitment on the cell membrane (Tsukada*, et al.*, 1994). The PH domain is followed by a Tec-homology domain (TH), which is composed of BTK Homology (BH) region and by one or two proline rich regions (PR). Apart from PH, TH domains, the BTK is characterized to harbor SH1 (Catalytic domain), SH2 and SH3 domain (Src homology). The Src-homology (SH)-3 domains recognize the proline rich sites and the SH2 domain aids in binding to activated tyrosine-kinase receptors by recognizing specific phosphorylated tyrosine. Whereas the catalytic domain (SH1) localized at the C-terminal, is characterized by tyrosine-kinase activity (Miller*, et al.*, 2002; Mohamed*, et al.*, 2009). BTK possess two regulatory phosphorylation sites, Tyr-223 and Tyr-551 in this domain (Figure 6) that participate in kinase activation (Rawlings*, et al.*, 1996).

Activation of BTK is a multi-step process initiated upon interaction of cell surface receptors with corresponding ligands which recruits phosphatidylinositol 3-kinase

(PI3K) that acts on phosphatidylinositol 4, 5-bisphosphate (PIP2) and generates phosphatidylinositol 3, 4, 5 trisphosphates (PIP3). This binds to the PH domain and translocates BTK to the plasma membrane and gets activated and functional by phosphorylating at tyrosine residue 551 (Tyr-551). Active BTK forms a complex with adapter protein with its SH2 domain and activates phospholipase-Cλ (PLC-λ) and protein kinase C (PKC), resulting in activation of multiple transcriptional signaling molecules such as Nuclear factor-κB (NF-κB), MAP kinases (ERK, p38 and JNK) etc., (Bajpai*, et al.*, 2000; Kurosaki, 2000; Petro*, et al.*, 2001; Qiu*, et al.*, 2000).

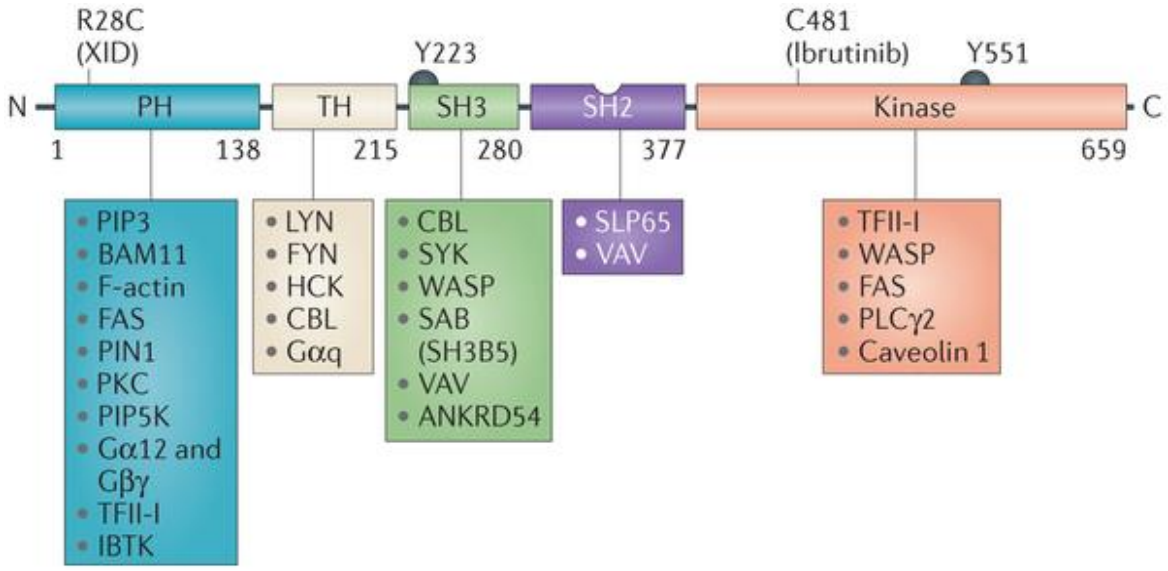


Figure 6. The domain structure of the BTK and its phosphorylation sites at the tyrosine residue 223 and 551. Below is the list of proteins known to interact with the individual BTK domain. R28C represents the mutation that is present in X-linked immunodeficiency (XID) mice, and C481 is the binding site of clinically potent BTK inhibitor, Ibrutinib Adapted from (Hendriks*, et al.*, 2014).

Human BTK protein sequence shared 98.3% homology with that of mouse (Lindvall*, et al.*, 2005) and the mutations in the BTK gene lead to severe inherited immunodeficiency disease, X-linked agammaglobulinemia (XLA) in humans and X-linked immunodeficiency (Xid) in mice (Satterthwaite*, et al.*, 2000). These diseases were characterized by reduced B cell maturation and defective humoral immune responses (Conley, 1985; Desiderio, 1997). Although it isa result from a variety of point mutations in BtK, the severity of B cell depletion in humans XLA is more than

compared to mice Xid (Hardy*, et al.*, 1984; Scher, 1982) and basis for the distinct severity among the two species is still unclear.

Most XLA patients were observed to be more prone to recurrent bacterial and viral infections (Lindvall*, et al.*, 2005) which suggest a possible role of BTK in immune responses. Nevertheless, several studies have demonstrated the expression of BTK in innate immune cells such as macrophages (Kaukonen*, et al.*, 1996; Weil*, et al.*, 1997) and dendritic cells (DCs) (Gagliardi*, et al.*, 2003). In Macrophages it was found to be an essential kinase involved in triggering the TLR induced inflammatory responses (Mukhopadhyay*, et al.*, 2002). In addition BTK was identified to interact with TLR receptors via the intracellular TIR-domain (Jefferies*, et al.*, 2003) and to associate with TLR downstream signaling molecules like MYD88, Mal, IRAK and with TRIF by adding phosphates (Gray*, et al.*, 2006; Lee*, et al.*, 2012). BTK activity has been shown to be essential in elevating the cytokine production such as IL-10, IL-6 and TNF upon TLR stimulation intimating its role in immune regulation (Levy, 2007; Schmidt*, et al.*, 2006). Given its predominant role in mediating large array of receptor signaling and its expression by immunocompetent cells, BTK was considered as a potent target in many cancer types (Akinleye*, et al.*, 2013).

1.6.1.2. BTK inhibitor-Ibrutinib (IBT)

Ibrutinib (formerly PCI-32765 or Imbruvica^{TM}) is an orally bioavailable, specific and highly potent BTK inhibitor with phenomenal clinical activity. It covalently binds to the cysteine residue (Cys-481) at the active site of BTK (TK/SH1 domain), thereby resulting in irreversible inhibition of kinase activity (Honigberg*, et al.*, 2010; Pan*, et al.*, 2007). Using Ibrutinib, several studies have been carried out for B-cell malignancies including chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL), multiple myeloma (MM), diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL). Thus far, it has been approved for CLL and MLL treatment by FDA (Cameron*, et al.*, 2014).

Numerous studies on CLL cell lines has demonstrated that Ibrutinib potently inhibits cell proliferation by suppressing TLR induced AKT, ERK and NF-κB signaling and induces dose- and time-dependent cytotoxicity *via* activation of caspase-3 dependent

apoptosis (Herman*, et al.*, 2011). It is also known to block pro-survival pathways and inhibits DNA replication in CLL by down regulating expression of CCL-3 and CCl-4 (Ponader*, et al.*, 2012) and antagonizes BTK-dependent chemotaxis to CXCL12 and CXCL13 (de Rooij*, et al.*, 2012).

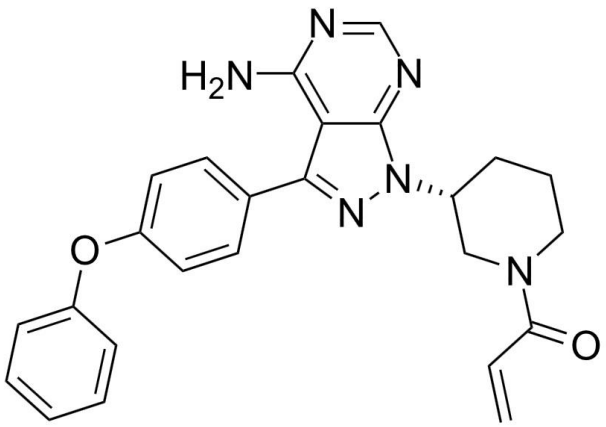


Figure 7. The chemical structure of BTK inhibitor, Ibrutinib (also known as PCI-32765 and available in market as Imbruvica^{TM}). Adapted from encyclopedia (encyclopedia, 2015).

The phenomenal activity of single agent Ibrutinib in clinical trials has raised several investigators to explore its synergic efficacy in combined treatment with chemo immunotherapy regimens to achieve the possibility of enhanced response and disease cure (Brown, 2013). Ibrutinib with ACY1215 and a selective histone deacetylase 6 (HDAC6) inhibitor, showed 3-fold increase in induction of apoptosis indicating direct synergistic anti-tumor effect on MCL tumor cell lines (Vij*, et al.*, 2012). Also studies addressing Ibrutinib plus bendamustine and rituximab (BR) appear to produce profound clinical response (ORR=93%) in relapsed/refractory CLL patients (Brown, 2012). Given its predominant effect, Ibrutinib appears to be one of the most active inhibitor to target BTK activity thereby study its effect in different cancer types.

1.7. Mitogen-Activated Protein Kinase (MAPK)

Mitogen-activated protein kinases (MAPKs) are signaling components, comprise a family of highly conserved serine/threonine kinases. MAPK including ERK, JNK and P38 are important in converting extracellular stimuli (Pearson*, et al.*, 2001), into wide range of cellular responses such as regulation of cell proliferation, cell survival, migration, inflammation and apoptosis (Johnson*, et al.*, 2002). All MAPKs include central three-tiered "core signaling modules" which is evolutionarily conserved Thr-X-Tyr motif in the activation loop of the kinase sub domain VIII. The concomitant phosphorylation of Tyr and Thr within the conserved region results to the activation of MAPKs (Kyriakis*, et al.*, 2012).

In mammals, the extracellular signal-regulated kinases 1 and 2 (ERK1/2) MAPKs are generally activated by mitogen and were found to be upregulated in tumors. Inappropriate activation of TLRs, over expression of EGFR, activating mutations of RAS and RAF results in aberrant activation of ERK and is considered as a key contributing factor in many human cancer types (Kohno*, et al.*, 2011). Sustained ERK signaling promotes phosphorylation and stabilization of genes such as Fos, Jun and Myc (Murphy*, et al.*, 2004) thereby, promoting cell cycle entry by accumulating cyclin D1 and suppress the expression of genes which inhibit proliferation (Yamamoto*, et al.*, 2006). Thus, inhibition of the ERK pathway represents a mechanism-based to cancer treatment.

Two other major MAPKs, the stress activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK), and p38 MAPK are activated by environmental and genotoxic stresses and play a key role in inflammation and tissue homeostasis thereby regulating cell survival, differentiation, proliferation and migration of specific cell types (Wagner*, et al.*, 2009). The JNK MAPK can exert pro-and anti-oncogenic function in different cell types and cancer development. Several studies have demonstrated that inhibition of JNK impairs the liver cell proliferation and tumor formation (Hui*, et al.*, 2008). In several human cancer cell lines, the loss of the tumor suppressor PTEN protein leads to AKT activation and increased JNK activity (Vivanco*, et al.*, 2007). Whereas, several studies on mice elaborated the tumor suppressor function of P38α, and many negative regulators of P38 signaling have been found to be over-expressed in human tumors and cancer cell lines (Bulavin*, et al.*, 2002; Yu*, et al.*, 2007). Also, increased p38

MAPK activation induces apoptosis in hepatoma cell lines (Iyoda*, et al.*, 2003). However, increased levels of phosphorylated p38α was found to be correlated with malignancy in various cancers, including breast carcinomas, follicular lymphoma, thyroid, lung cancers and head and neck squamous cell carcinomas (Elenitoba-Johnson*, et al.*, 2003; Esteva*, et al.*, 2004; Junttila*, et al.*, 2007).

2. AIMS OF THE STUDY

A chronic inflammatory response at the tumor microenvironment is apparently an important mechanism to evade effective antitumor immune responses. Since chronic inflammation is known to associate with tumor development and progression, extensive research has been carried out on many cancer types in this respect. However to evade from effective immune responses, malignant HNSCCs develop complex immunosuppressive strategies. Beside the known mechanisms of immune receptors (TLRs) in active innate immune responses, increasing evidences were also found that their aberrant activation at the tumor microenvironment leads to prolonged inflammation that in turn activates host immune escape mechanisms, anti-apoptotic activity and cancer progression. Recently, BTK was reported as a critical molecule that is involved in active TLR signaling including (TLR 2-4, 7-9) (Doyle*, et al.*, 2007; Horwood*, et al.*, 2006; Jefferies*, et al.*, 2003; Lee*, et al.*, 2012). BTK has emerged as an attractive target for therapeutic interventions due to its function in diverse range of cellular processes. Extensive studies in B-cell malignancies using clinically potent BTK inhibitor, Ibrutinib (Honigberg*, et al.*, 2010; Pan*, et al.*, 2007) has revealed BTK to involve in tumor progression. So far in malignant HNSCC cells, the role of BTK is unclear and hence it is important to understand its precise molecular mechanism and its contribution to inflammation and tumor recurrence or resistance.

Hence the present study was aimed to evaluate the molecular mechanisms of BTK and to understand its contribution in TLR3 and TLR4 induced inflammation in malignant HNSCC cells. And to study the inhibitory influence of Ibrutinib (IBT) in malignant HNSCC cell survival, progression, migration and its ability to induce apoptosis. In order to address these aims, attempts were made to characterize the TLR3 and TLR4 signaling in an *in vitro* established permanent HNSCC cell lines and auxiliary analysis on the role of BTK in TLR induced inflammation was studied by exploiting the clinically potent BTK inhibitor, Ibrutinib. This analysis was in turn performed in conjunction to reckon the anti-tumor potential of treatment with Ibrutinib and TLR agonists in combination.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1. Laboratory equipments

Description of the apparatus	Details of the manufacturer
Basic Power Supply, PowerPac™	Bio-Rad Laboratories Inc., USA
BD FACS Canto™ Flow cytometer	BD Biosciences, San Jose, USA
Cell Analyzer, Cedex XS	F. Hoffmann-La Roche AG, Switzerland
Centrifuge, Allegra 25R/X-12R	Beckman Coulter GmbH, Germany
CO_2 Incubator, CB 53	BINDER GmbH, Tuttlingen, Germany
CO_2 Incubator, INC 153	Memmert GmbH + Co.KG, Germany
Electrophoresis cell, Sub-Cell® GT	Bio-Rad Laboratories Inc., USA
Electrophoresis mini-PROTEAN® Tetra cell	Bio-Rad Laboratories Inc., USA
Electrophoresis power supply, EPS 601	GE Healthcare GmbH, Germany
Electrophoresis transfer Cell, Mini Trans-Blot®	Bio-Rad Laboratories Inc., USA
Fluorescence microscope, Axiovert 200M	Carl Zeiss Jena GmbH, Germany
Gel Doc™ XR-Molecular Imager	Bio-Rad Laboratories Inc., USA
Gel documentation System Fusion FX7 (Fluorescence & Chemiluminescence)	Vilber Lourmat Deutschland, GmbH, Germany
Inverted microscope, Wilovert	Helmut Hund GmbH, Germany
Laminar air flow, HERAsafe KSP12	Thermo Electron LED GmbH, Germany
Magnetic Stirrer, IKA RH basic	IKA-Werke GmbH & Co.KG, Germany
Micro centrifuge, Heraeus Biofuge fresco	Kendro Laboratory Products-Service
Microcentrifuge-Microfuge 18	Beckman Coulter GmbH, Germany

Microoven Microwelle Privileg 8020	IRE Beteiligungs GmbH, Germany
Micropipettes, eppendorf Research Plus	Eppendorf AG, Germany
Microplate spectrophotometer	Bio-Rad Laboratories Inc., USA
PCR, Mastercycler EP Gradient S	Eppendorf AG, Hamburg, Germany
Pipette controller, Accu-jet® pro	BrandTech Scientific Inc, USA
Precision balance, EW620-3NM	Kern & Sohn GmbH, Germany
Real-Time PCR System, LightCycler® 96	F. Hoffmann-La Roche AG, Switzerland
RTCA Analyzer, xCELLigence	F. Hoffmann-La Roche AG, Switzerland
Scanner, CanoScan 8000F	Cannon Deutschland GmbH, Germany
Shaking waterbath 1083	GFL, Gesellschaft für Labortechnik GmbH, Germanyl
Thermoshaker TS1	Biometra GmbH, Germany
Tilt rocker, ST 5	CAT Ingenieurbüro M. Zipperer GmbH, Germany
UV Transilluminator FirstLight	UVP Inc., USA
UV-VIS Bio Photometer	Eppendorf AG, Germany
UV-VIS Spectrophotometer, NanoDrop 2000	Thermo Fisher Scientific Inc, USA
Vortexer, MS1 IKA Minishaker	IKA-Werke GmbH & Co.KG, Germany

Table 1. List of laboratory equipments used in the present study.

3.1.2. Laboratory Consumables

Description of the article	Details of the manufacturer
Aluminium Foil	Carl Roth GmbH & Co. KG, Germany
Antistatic weighing dishes	Th. Geyer GmbH & Co. KG, Germany
BD Falcon culture slides	BD Biosciences, USA
Cedex smart slide	F. Hoffmann-La Roche AG, Switzerland
Cell culture flask (T25, T75, T175)	Sarstedt AG & Co., Germany
Cover slips	Gerhard Menzel GmbH, Thermo Fisher Scientific, Germany
Cryo Storage box	Greiner Bio-One International AG, Austria
Cryogenic vials	Greiner Bio-One International AG, Austria
Culture-Insert, µ-Dish^{35mm,high}	ibidi GmbH, Germany
Cuvettes, 8.5mm	Sarstedt AG & Co., Germany
Disposable bags	Sarstedt AG & Co., Germany
Facial tissue	Werner Hassa GmbH, Germany
Falcon tubes, sterile(15ml, 50ml)	Sarstedt AG & Co., Germany
Flitopur S 0.2 syringe filter	Sarstedt AG & Co., Germany
Gel-Loading pipet tips (10-200 µl)	Greiner Bio-One International AG, Austria
LightCycler® 480 Multiwell Plates 96	F. Hoffmann-La Roche AG, Switzerland
LightCycler® 480 Sealing Foils	F. Hoffmann-La Roche AG, Switzerland
Medical gloves (Nitrile rubber, Vinyl)	Paul Hartmann AG, Germany
Microfuge tubes (0.5ml, 1ml, 2ml, 5ml)	Sarstedt AG & Co., Germany
Microscope slides set	NeoLab, Germany
Mini-PROTEAN® TGX™ Precast Gels	Bio-Rad Laboratories Inc., USA
Nitrocellulose Membran (0.2, 0.45 µm)	Bio-Rad Laboratories Inc., USA
Parafilm	Pechiney Plastic Packaging, USA

PCR 4-tube RNase free strip	Sarstedt AG & Co., Germany
Pipette tips (1000µl, 100µl, 10µl)	Sarstedt AG & Co., Germany
Proliferation E-Plate 16	ACEA BioSciences, Inc., USA
SafeGuard™ Filter tips (0.1-20µl, 1-100µl, 100-1000µl)	PEQLAB Biotechnologie GmbH, Germany
Scalpel	Feather Safety Razor Co., Japan
Serological pipettes (1, 5, 10, 25, 50ml)	Sarstedt AG & Co., Germany
Super PAP Pen Liquid Blocker	Science services, Germany
Tissue culture plates (12, 24, 96 wells)	Greiner Bio-One International AG, Austria
Tissue culture plates (6 wells)	Sarstedt AG & Co., Germany
Tweezers	Carl Roth GmbH & Co. KG, Germany
Whatman filter paper	GE Healthcare, UK
WypAll paper towels	Kimberly-Clark Co., USA

Table 2. List of consumables used in the present study.

3.1.3. Chemical substrates

Description of material	Details of the manufacturer
2x RNA loading dye	Thermo Fisher Scientific Inc., USA
6x DNA loading dye	Thermo Fisher Scientific Inc., USA
Accutase	PAA Laboratories GmbH, Germany
Acetic acid 100%	Merck KGaA, Germany
Acetone	Avantor Performance Materials, USA
Agarose	Biozym Scientific GmbH, Germany
Antibody dilution buffer	DCS Innovative Diagnostik-Systeme, GmbH & Co., Germany
Aprotinin	Sigma-Aldrich Co., USA
APS (Ammonium per sulphate)	Carl Roth GmbH & Co. KG, Germany
Bacillol® AF	Bode Chemie GmbH, Germany
Bromophenol blue	Carl Roth GmbH & Co. KG, Germany
BSA (Bovine Serum Albumin)	Sigma-Aldrich Co., USA
Cell lysis buffer (10X)	Cell Signaling Technology Inc., USA
Coomassie Brilliant Blue G-250	Thermo Fisher Scientific Inc., USA
DAPI (4′, 6-Diamidin-2-phenylindol)	F. Hoffmann-La Roche AG, Switzerland
DEPC-Water	Thermo Fisher Scientific Inc., USA
Disodium phosphate	Merck KGaA, Germany
DMEM- Dulbecco's Modified Eagle Medium; GIBCO	Life Technologies, USA
DMSO (Dimethyl sulfoxide)	Sigma-Aldrich Co., USA
DNase I (1U/µl), RNase-free	Thermo Fisher Scientific Inc., USA
dNTP Mix (10 mM)	Thermo Fisher Scientific Inc., USA
DPBS-Dulbecco's Phosphate-Buffered Saline	PAA Laboratories GmbH, Germany

EDTA (Ethylene diamine tetra acetic acid)	Carl Roth GmbH & Co. KG, Germany
Fluoromount-G®, Slide mounting medium	Southern Biotech, USA
Ethanol, Absolute ≥ 99.8	Avantor Performance Materials, USA
Ethanol, denatured (70%)	Apotheke Lübeck, Germany
Ethidiumbromide (EtBr) (10mg/ml)	Life Technologies, USA
Ethidiumbromide Destroyer Sprayer	Favorgen Biotech Co., Taiwan
Fetal Bovine Serum (FBS Gold)	PAA Laboratories GmbH, Austria
Formaldehyde 37%	Sigma-Aldrich Co., USA
GeneRuler DNA Ladder (100bp, 1kb)	Thermo Fisher Scientific Inc., USA
Glycerine	Carl Roth GmbH & Co. KG, Germany
Glycerol	Carl Roth GmbH & Co. KG, Germany
Glycin	Carl Roth GmbH & Co. KG, Germany
Ibrutinib (PCI-32765)	Selleckchem.com, USA
Isopropanol	Fischar Otto GmbH & Co. KG, Germany
Leupeptin	Sigma-Aldrich Co., USA
Lipofectamine®2000 reagent	Life Technologies, USA
Lipopolysaccharide, E.Coli 026:B6	Sigma-Aldrich Co., USA
Loading dye 6x, DNA samples	Thermo Fisher Scientific Inc., USA
Magnesium chloride MgCl2 (25mM)	Ampliqon III, Denmark
Methanol	Avantor Performance Materials, USA
Methylene blue	Sigma-Aldrich Co., USA
Milk powder	Carl Roth GmbH & Co. KG, Germany
Monopotassium phosphate	Merck KGaA, Germany
MOPS (3-(*N*-morpholino)propanesulfonic acid)	Sigma-Aldrich Co., USA
MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-	Sigma-Aldrich Co., USA

diphenyltetrazolium bromide)	
Mycoplasma-Off, Spray	Minerva Biolabs GmbH, Germany
Opti-MEM® Medium	Life Technologies, USA
PageBlue Protein Staining Solution	Thermo Fisher Scientific Inc., USA
PCR Standard buffer (with 15mM $MgCl_2$)	Thermo Fisher Scientific Inc., USA
Pepstatin-A, 2mg/ml	Sigma-Aldrich Co., USA
PFA (Paraformaldehyde) (16 %)	Science services, Germany
PIC (Phosphatase inhibitor cocktail)	Sigma-Aldrich Co., USA
PMSF (Phenyl methane sulfonyl fluoride)	Sigma-Aldrich Co., USA
Poly I:C (Polyinosinic-Polycytidylic acid)	InvivioGen, USA
Ponceau S	Sigma-Aldrich Co., USA
Precision Plus Protein-Standard marker	Bio-Rad Laboratories Inc., USA
Quick Start™ Bradford (1 x) dye	Bio-Rad Laboratories Inc., USA
Restore™ Western blot stripping buffer	Thermo Fisher Scientific Inc., USA
RiboLock RNase Inhibitor	Thermo Fisher Scientific Inc., USA
RiboRuler High Range RNA Ladder	Thermo Fisher Scientific Inc., USA
RIPA Buffer (10X)	Cell Signaling Technology Inc., USA
SDS (Sodium dodecyl sulfate)	Carl Roth GmbH & Co. KG, Germany
Sodium Chloride	Sigma-Aldrich Co., USA
Sodium Fluoride	Sigma-Aldrich Co., USA
Sodium Pyruvate	PAN-Biotech GmbH, Germany
Taq DNA Polymerase (5U/µl)	Ampliqon III, Denmark
TaqMan gene expression master mix	Life Technologies, USA
TaqMan gene expression master mix	Life Technologies, USA
TEMED (Tetramethylethylendiamin)	Carl Roth GmbH & Co. KG, Germany
Tris-base	Carl Roth GmbH & Co. KG, Germany

Tris-HCL	Carl Roth GmbH & Co. KG, Germany
Triton™ X-100 solution	Sigma-Aldrich Co., USA
Trypanblau 0, 5 %	Biochrom AG, Germany
Tween-20	Sigma-Aldrich Co., USA
β-mercaptoethanol	Carl Roth GmbH & Co.KG, Germany

Table 3. List of chemical substrates used in the present study.

3.1.4. Commercial kits

Kit description	Manufacturer
Annexin V binding buffer (10x)	BD Pharmingen ™, BD Biosciences, USA
AEC 2 component Kit (Peroxidase)	DCS Innovative Diagnostik-Systeme, Germany
Amersham™ ECL™ prime western blot detection reagent	GE Healthcare, UK
Cancer pathway finder RT^2 PCR Array	Qiagen N.V., Germany
DNeasy® Blood and Tissue Kit	Qiagen N.V., Germany
Human IFN-β ELISA Kit	R&D Systems, USA
Human IL-1β/IL.1F2 quantikine	R&D Systems, USA
Human TNF-α quantikine	R&D Systems, USA
QIAshredder™	Qiagen N.V., Germany
RevertAid First Strand cDNA synthesis Kit	Thermo Fisher Scientific Inc., USA
Rnase-free DNase set	Qiagen N.V., Germany
RNeasy Plus Mini Kit	Qiagen N.V., Germany
VenorGeM-Mycoplasmen detection kit	Minerva Biolabs GmbH, Germany

Table 4. Commercial kits used in the present study.

3.1.5. List of Antibodies

FACS antibodies

Antibody description	Volume per reaction	Manufacturer	Catalog No.
Annexin V-APC	1µl	BD Pharmingen ™	550474
Propidium iodide staining solution	1µl	BD Pharmingen ™	556463

Table 5. List of antibodies used for FACS analysis.

Immunofluorescence staining antibodies

Antibody description	Working concentration	Manufacturer	Catalog No.
Anti-TLR3 antibody	1:100	Abcam	ab62566
Anti-TLR4 antibody [76B357.1]	1:50	Abcam	ab22048

Table 6. List of antibodies used for Immunofluorescence staining.

Western hybridization antibodies

Antibody description	Working Concentration	Manufacturer	Catalog No.
Anti-alpha Tubulin antibody [DM1A]-Loading Control	1:8000 in 5%BSA/TBST	Abcam	ab7291
Anti-BTK (phospho Y223) antibody	1:10000 in 1%BSA/TBST	Abcam	ab68217
Anti-BTK (phospho Y551) antibody [EP267Y]	1:2000 in 1%BSA/TBST	Abcam	ab40770

Anti-BTK antibody [7F12H4, 6G5F6]	1:500 in 1%BSA/TBST	Abcam	ab54219
Anti-ERK1&2 [pTpY$^{185/187}$] phospho specific antibody	1:1000 in 3%BSA/TBST	Invitrogen; Life technologies	44680G
Anti-Mouse IgG-peroxidase antibody produced in goat	1:50000	Sigma-Aldrich	A9044
Anti-MyD88 antibody [1B4]	1:1000 in 1%Milk/TBST	Abcam	ab119048
Anti-Rabbit IgG-peroxidase antibody produced in goat	1:50000	Sigma-Aldrich	A0545
Anti-TLR3 antibody	1:500 in 1%Milk/TBST	Abcam	ab62566
Anti-TLR4 antibody [76B357.1]	1:500 in 1%Milk/TBST	Abcam	ab22048
Anti-TRIF antibody [1G7]	1:1000in 1%Milk/TBST	Abcam	139281
ERK1 + ERK2 antibody	1:1000 in 3%BSA/TBST	Invitrogen; Life technologies	44654G
GAPDH (14C10) antibody	1: 1000 in 5%BSA/TBST	Cell Signalling	2118S
p38 MAPK antibody	1:1000 in 5%BSA/TBST	Cell Signalling	9212
Phospho-p38 MAPK antibody	1:1000 in 5%BSA/TBST	Cell Signalling	9211
Phospho-SAPK/JNK (Thr183/Tyr185) antibody	1:1000 in 5%BSA/TBST	Cell Signalling	9251
SAPK/JNK antibody	1:1000 in 5%BSA/TBST	Cell Signalling	9252

Table 7. List of antibodies used for western hybridization.

3.1.6. Primers and TaqMan assays

Primers

Gene name	Sequence	Tm (°C)
Fw β-Actin	5' GAGAAGATGACCCAGATCATGT 3'	58.4
Rev β-Actin	5'CATCTCTTGCTCGAAGTCCAG 3'	59.8
Fw GAPDH	5' CAAGGTCATCCATGACAACTTTG 3'	58
Rev GAPDH	5’ GTCCACCACCCTGTTGCTGTAG 3'	58

Table 8. List of primers used for PCR.

TaqMan assays

Gene name	Gene symbol	Manufacturer	Catalog No.
Actin, beta	β-actin	Life technologies	Hs99999903_m1
Toll like receptor-3	TLR3	Life technologies	Hs01551078_m1
Toll like receptor-4	TLR4	Life technologies	Hs01060206_m1
Interleukin 1, beta	IL-1β	Life technologies	Hs01555410_m1
Interleukin 6	IL-6	Life technologies	HS00985639_m1
Interleukin 8	IL-8	Life technologies	Hs00174103_m1
Interleukin 10	IL-10	Life technologies	Hs00961622_m1
Tumor necrosis factor	TNF-α	Life technologies	Hs00174128_m1
Interferon, beta 1	IFN-β	Life technologies	Hs01077958_s1

Table 9. List of TaqMan assays used for real time (RT)-PCR.

3.1.7. List of Software's

Name of the Software	Source
Adobe Photoshop CS6	Adobe Systems Inc., USA
AxioVision Rel. 4.8.2	Carl Zeiss Jena GmbH, Germany
BD FACS Diva™ 6.1.1	BD Biosciences, USA
CedexXS	Innovatis, Germany
EndNote X5	Thomson-Reuters cooperation, USA
FacsDiva 6.0	BD Biosciences, USA
GraphPad Prism 5	GraphPad Software Inc., USA
ImageJ 1.44p	National Institutes of Health, USA
LightCycler® 96 SW1.1	F. Hoffmann-La Roche AG, Switzerland
Microsoft Office 2010	Microsoft Corporation, USA
NanoDrop 2000	Thermo Scientific, USA
Quantity One 1D Analysis	Bio-Rad Laboratories, Inc., Germany
RTCA DP SW 1.2.1	ACEA Bioscience, USA

Table 10. List of software used in the present study.

3.2. Mammalian cell culture methods

3.2.1 Mammalian HNSCC Cell lines and Media

Cell lines

Cell line	Gender(yrs)	Source	Type	Classification
UT-SCC-16A	Female (77)	Tongue	Primary	$T_3N_0M_0$, G3, Phase III
UT-SCC-16B	Female (77)	Neck	Metastase	$T_3N_0M_0$, G3, Phase III
UT-SCC-60A	Male(59)	Tonsil	Primary	$T_4N_1M_0$, G1, Phase IV
UT-SCC-60B	Male(59)	Neck	Metastase	$T_4N_1M_0$, G1, Phase IV

Table 11. Permanent human HNSCC cell lines used in the present study.

Culture medium, 500ml

Components (Stock conc.)	Amount added	Final concentration.
DMEM (4.5gm/L glucose)	450 ml	NA
Fetal bovine serum	50ml	10%
Sodium pyruvate	5ml	1mM

Stored at +4°C.

Freezing medium, 70ml

Components	Amount added	Final concentration
DMEM	49ml	70%
FBS	14ml	20%
DMSO	7ml	10%

Freshly prepared.

3.2.2. Culturing of adherent cells

All the cell culture works were carried out under the aseptic laminar airflow work station. Four established adherent human head and neck squamous cell carcinoma (HNSCC) cell lines UT-SCC-16A, -16B, -60A and -60B, gifted by Reidar Grénmann from University of Turku (UT), Finland were used in the present study. These cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% sodium pyruvate (PAN-Biotech GmbH, Germany) and 10% Fetal Bovine Serum (FBS (PAA Laboratories GmbH, Austria) and cultured at 37°C with 5%CO_2. Once the cells attain 70-80% confluency, sub-culturing was performed briefly by washing twice with 1× Dulbecco's phosphate buffered saline (DPBS, PAA Laboratories GmbH, Austria) and treated with accutase (PAA Laboratories GmbH, Austria) and incubated at 37°C for approximately 10mins. The proteolytic and collagenolytic enzymes present in the accutase, detach the adherent cells from the flask. To stop the enzymatic reaction, the floating cells were suspended in DMEM and centrifuged at 200g for 5mins at 25°C. The pellet was resuspended with fresh DMEM and diluted according to the requirement into a fresh cell culture flask/ tissue culture plate (Sarstedt AG & Co., Nümbrecht, Germany).

3.2.3. Detection of Mycoplasma

To verify a contamination with Mycoplasma, the cells were screened with PCR based Mycoplasma detection kit (Minerva Bio labs GmbH, Germany) at regular interval which amplifies Mycoplasma (multispecies) DNA and validated by agarose gel electrophoresis. The DNA extraction from the cell culture pellet and PCR setup of the samples was carried out according to the instructions detailed in the manual.

3.2.4. Cryopreservation and Resuscitation

Healthy, viable (>90%) and microbial contamination free cell lines were washed and detached from the flask as mentioned in section 3.2.2 .Centrifuged the cells at 500x g for 5mins and resuspended the cell pellet into the freezing medium containing 70% DMEM, 20% FBS and 10% dimethylsulfoxide (DMSO) (Sigma-Aldrich Co., USA) and

transferred into sterile cryo tubes. The vials were placed gently into a slow freezing container filled with isopropanol (Carl Roth GmbH & Co. KG, Germany). Once the cryo tubes were cooled, they were stored at -80°C.

To defrost the cells, the cryo tubes were quickly placed in a warm water bath at 37°C for 3-4mins and immediately suspended into 10ml of culture medium to dilute the DMSO concentration. The cells were then centrifuged at 500x g for 5mins and the pellet was resuspended into culture medium and allowed to grow at 37°C with 5% CO_2 by transferring the cell suspension into a culture flask. The cell survival and proliferation was examined after 24hrs.

3.2.5. Quantification and harvesting of cells

The viable cell number was determined by using trypan blue exclusion method. The cell suspension was diluted 1:2 with 0.2% trypan blue (Biochrom AG, Germany), 10µl was added into the cedex smart slide and loaded into cedex XS system (F. Hoffmann-La Roche AG, Switzerland) to count the viable cells. Trypan blue selectively stain the dead cells by penetrating through its permeable membrane, whereas the viable cells cannot absorb. Hence, dead cells are shown distinctive blue colour and are recognized using digital imaging technology from cedex XS analyzer (F. Hoffmann-La Roche AG, Switzerland) and determines the unstained viable cell number.

The treated cells, according to the experimental setup, were harvested after the incubation/stimulation time period. Hence, the cell monolayer was washed immediately twice with ice-cold DPBS. Later, the adherent cells were detached using accutase as mentioned in the earlier section 3.3.2. The cells suspended with DMEM were collected into sterile ice-cold 2ml microfuge and centrifuged at 300x g for 3mins at 4°C. The pellet thus obtained was washed with 1ml ice-cold DPBS. After final spun at 300x g the pellet was stored at -20°C/-80°C for further analysis.

3.3. Cell Based Assays

3.3.1. Buffers and Reagents

MTT solution, 3ml

Components	Amount added	Final concentration
MTT	15mg	5mg/ml
1x PBS	3ml	NA

Stored at -20°C.

MTT solubilizing solution, 50ml

Components	Amount added	Final concentration
Triton X-100	5ml	10%
HCL (2N)	2.5ml	0.1N
Isopropanol	42.5ml	100%

Stored at +4°C.

3.3.2. Treatment with Ibrutinib

In the present study, HNSCC cells were treated with pharmacological inhibitor Ibrutinib (PCI-32765) (Selleckchem.com, USA) prepared as.10mM stock solution by dissolving in DMSO and used at working concentrations of 1µM, 5µM, 10µM. 1×10^6 cells per T25 flask (Sarstedt AG & Co., Germany) were cultured at 37°C with 5% CO_2 for overnight followed by 24h treatment with culture medium containing different working concentrations of Ibrutinib. The treated cell pellets were prepared as indicated in section 3.2.5 and stored at -20°C or processed immediately for proceeding experiments.

3.3.3. Stimulation with TLR ligands

The cells were treated with high molecular weight Poly (I:C) (10µg/ml) (InvivioGen, USA) and LPS (2µg/ml) (Sigma-Aldrich Co., USA) to stimulated the TLR3 and TLR4 signaling cascade for respective time points according to the experimental setup after overnight culturing at 37°C with 5% CO_2. The treated cell pellets were either preserved at -20°C as indicated in section 3.2.5 or processed immediately for further analysis.

3.3.4. Transfection with Poly (I:C)

In order to elucidate endosomal TLR3 receptor signals, transfection of Poly (I:C) was performed using Lipofectamine® 2000 reagent (Life Technologies, USA). 1.5×10^5 cells per well were seeded for overnight into 6-well plates (Sarstedt AG & Co., Germany) and the adherent cells were washed twice with DPBS (PAA Laboratories GmbH, Germany). The transfection medium was prepared according to the manufacturer's instructions. Briefly, by diluting the lipofectamine 2000® reagent (optimum amount is 8µl/well) and 10µg/ml of Poly (I:C) in Opti-MEM® reduced serum medium (Life Technologies, USA) and incubated for 15mins at room temperature followed by addition of this Poly (I:C) and reagent complex to the cells and incubated for 6h at 37°C with 5% CO_2. The cell pellets were further processed accordingly.

3.3.5. Cell viability assay

The viability of the cells was determined using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (Sigma-Aldrich Co., USA) tetrazolium reduction assay. 5×10^3 cells per 200µl were seeded into 96well plates for overnight and treated with either Ibrutinib or TLR ligands accordingly for 0-72h. After every 24h, the viable cell activity was measured by incubating 2h at 37°C with MTT solution (100µl) which is yellowish in colour when dissolved in DPBS or phenol red in culture medium. The mitochondrial dehydrogenase from the viable cells with active metabolism cleaves the tetrazolium ring and converts the MTT into undissolved purple coloured formazan crystals. This was dissolved in equal volume of acidified MTT solubilising solution (100µl) for 24h at room temperature and the resulting purple solution was measured

using benchmark plus microplate spectrophotometer (Bio-Rad Laboratories Inc., Hercules (CA), USA) with a maximum absorbance at 570 nm.

3.3.6. Cell proliferation assay

To quantitatively monitor the HNSCC cell proliferation in response to Ibrutinib treatment, xCELLigence Real-Time Cell Analyzer (RTCA) (F. Hoffmann-La Roche AG, Basel, Switzerland) was used. 5×10^3 cells/well were cultured overnight into the proliferation E-Plate 16 (ACEA, BioScience, Inc., USA) and treated with different concentrations (1µM, 5µM, 10µM) of Ibrutinib for 96h respectively. The data were measured and acquired by RTCA integrated automated cell-imaging system and the results were analyzed using RTCA v.1.2 software.

3.3.7. Wound healing assay

Wound healing assay was performed to measure HNSCC cell migration *in vitro* using ibidi culture insert in µ-dishes (ibidi GmbH, Germany). The culture insert is designed with a 500µm width of cell-free gap. Approximately 2.45×10^4 cells in 70µl of culture medium were placed in to each well and the outer area in the µ-dish was filled with 200µl of culture medium. After overnight culturing at 37°C containing 5%CO_2, the culture-insert was gently removed using sterile tweezers and the adherent cells were washed twice with sterile PBS. To the adherent cells, 2ml of culture medium, with or without TLR ligands (Poly(I:C), LPS) or Ibrutinib (IBT) was added accordingly and incubated at 37°C until 72h respectively. The migration of cells was captured at the beginning (0h) of the treatment and at regular intervals (12h, 24h, 30h, 48h and 72h) using bright field mode on Axiovert 200M fluorescence microscope (Carl Zeiss Jena GmbH, Germany). The close proximity of the cell-free gap and the migration rate was analyzed using AxioVision Rel. 4.8.2 software.

3.4. Molecular Methods

3.4.1. Buffers and Reagents

10x MOPS, 1L

Components	Amount added	Final concentration
MOPS	41.8gm	0.2M
Sodium acetate	4.1gm	50mM
EDTA	3.72gm	10mM
Water	make up to 1L	NA

Stored at room temperature.

50x TAE, 1L

Components	Amount added	Final concentration
Tris base (pH 8.0)	242gm	2M
Glacial acetic acid	57.1ml	100%
EDTA	37.2gm	0.05M
Water	make up to 1L	NA

Stored at room temperature.

3.4.2. RNA extraction and DNA digestion

RNA extraction was performed using RNeasy plus Mini Kit (Qiagen N.V., Germany), according to the manufacturer's instructions. Briefly, the cells were lysed using RLT buffer (350µl) containing freshly added β-mercaptoethanol and loaded onto the QIAshredder homogenizer and centrifuged. The homogenized mixture was then spun through the gDNA eliminator column to remove genomic DNA. To this mixture equal volume of 70% ethanol (350µl) was added and centrifuged after loading into the RNeasy spin column. RNA pellet was then washed with RW1 buffer and RPE buffer as

per instructions and allowed to air dry before eluting by centrifugation with nuclease free-water.

After the initial washing with 350µl of RW1 buffer, the RNA pellet was treated with 10µl of RNase-free DNase-I enzyme (Qiagen N.V., Germany) together with 70µl of RDD buffer provided within the kit for 15mins at room temperature followed by washing with RW1 buffer and RPE buffer as per RNeasy plus Mini Kit instructions.

3.4.3. Quantification of RNA

The total RNA concentration and purity was determined using the NanoDrop2000 (Thermo Fisher Scientific Inc., USA). The RNA concentration was measured at a wavelength of 260nm and 280nm and the purity was considered to be good if the absorbance of 260/280 range between 1.9-2.1. NanoDrop calculates the RNA concentrations according to the modified Beer-Lambert equation as given below

$$C=(A\times\varepsilon)/b$$

Where, C=nucleic acid concentration in ng/µl; A=Absorbance in AU; ε=wavelength dependent extinction coefficient in ng-cm/µl; b=path length in cm.

3.4.4. First strand cDNA synthesis

The isolated RNA was reverse transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., USA) according to manufacturer's protocol. 0.5-2µg of RNA was used for each reaction and the following mixture was prepared in RNase free PCR tubes.

Components (Stock concentration)	**Amount added**
RNA (0.5-2µg)	n µl
Random hexamer primer (100µM)	1µl
RNase free water	up to 12µl

This mixture was incubated for 5mins at 65°C to break the GC-rich sequence or secondary structure of the RNA and placed on ice for 1min. To this mixture, following freshly prepared cDNA synthesis mix was added.

Components (Stock concentration)	**Amount added**
5x Reaction buffer	4µl
RiboLock RNase inhibitor (20U/µl)	1µl
10mM dNTP Mix	2µl
RevertAid M-MuLV Reverse transcriptase (200U/µl)	1µl

The final 20µl mixture containing above mentioned components was shortly centrifuged and incubated at 25°C for 5mins to activate the reverse transcriptase followed by cDNA synthesis at 42°C for 60mins and termination of the reaction at 70°C for 5mins and the cDNA was stored at -20°C or used for PCR.

3.4.5. Polymerase Chain Reaction (PCR)

By using thermo cycling method, Polymerase chain reaction (PCR) enables specific DNA-sequence to amplify into millions of copies. This reaction was carried out using heat-stable TaqDNA polymerase enzyme (Ampliqon III, Denmark).
The following mixture was prepared to run the reaction:

Components(stock concentration)	**Amount added**
DNA template (100ng/µl)	1.00µl
β-actin primer mix	1.00µl
TaqMan polymerase (5U/µl)	0.25µl
dNTP mix (10mM)	0.50µl
10x Reaction buffer	2.00µl
$MgCl_2$	2.00µl
RNase free water	13.25µl

The components were mixed gently and centrifuged briefly before thermal cycling was performed. The three basic steps which were performed to amplify the specific DNA sequence were as follows: Denaturation of the DNA at 94°C to break the hydrogen bonds between the double stranded DNA and to yield a single stranded DNA template. Annealing of the DNA template at 57°C to specifically hybridize the two oligonucleotides (primers) to the complementary part of the DNA template. Elongation of the DNA template at 72°C to bind the TaqDNA polymerase to the primer-template hybrid for the new complementary DNA strand synthesis from 5'→3' direction by adding specific deoxyribonucleotide triphosphates (dNTP's) and magnesium ions. The PCR reaction was always carried out in PCR master cycler gradient S (Eppendorf AG, Hamburg, Germany) using the following thermal profile depicted below:

Step	Description	Temperature	Time	Cycles
1	Initial denaturation	94°C	5 mins	1
	Denaturation	94°C	30 s	
2	Annealing	57°C	30 s	35
	Extension	72°C	45 s	
3	Final extension	72°C	10 mins	1
4	Cooling	4°C	Infinite time	

3.4.6. Quantitative real-time PCR

From the synthesized cDNA as indicated in above section 3.4.4, quantitative real-time PCR (qRT-PCR) was conducted to detect the gene expression profiles of TLR3, TLR4, β-actin, IL-1β, IL-6, IL-8, TNF-α and IFN-β on LightCycler1.5 (F. Hoffmann-La Roche AG, Switzerland) using following components for each reaction:

Components	Amount added
cDNA (25ng)	0.25µl
TaqMan probe	1.00µl
TaqMan gene expression master mix	10.0µl
Nuclease free water	up to 20µl

During the extension phase of PCR, the specifically hybridized TaqMan probe with a reporter FAM (i.e., 6-carboxyfluorescein) at the 5' end and the quencher, TAMRA (i.e., 6-carboxy-tetramethylrhodamine) at the 3' end was cleaved by the 5'-3' exonuclease activity of AmpliTaq Gold DNA polymerase from the TaqMan gene expression master mix (Life Technologies, USA) and release the FAM fluorescent emission. The fluorescent spectrum obtained was monitored in real time (Heid*, et al.*, 1996).

Following thermal profile was used to perform qRT-PCR reaction:

Step	Temperature	Time	Cycles
Incubation	50°C	02 mins	1
Initialization	95°C	10 mins	1
Denaturation	95°C	15 s	50
Annealing	60°C	01 min	

The obtained C_T value data was analyzed by quantifying the relative changes in gene expression using $2^{-\Delta\Delta C}{}_T$ method, which indicates the fold change in gene expression of the treated samples relative to the untreated control. The difference in threshold cycles was normalized to standard internal housekeeping gene β-actin for all the samples.

$$\Delta C_T = (C_{T,\ Target} - C_{T,\ \beta\text{-actin}})$$

$$\Delta\Delta C_T = (C_{T,\ Target} - C_{T,\ \beta\text{-actin}})_{\ Treated} - (C_{T,\ Target} - C_{T,\ \beta\text{-actin}})_{\ Untreated}$$

Where, $\Delta\Delta C_T$ is the difference in ΔC_T of treated with ΔCT of untreated control; ΔC_T is

the difference in threshold cycles and CT is threshold cycles.

For the amplicons which designed to be less than 150bp, the efficiency is close to one. Therefore the relative amount of target is given by $2^{-\Delta\Delta C}{}_T$ (Livak, *et al.*, 2001).

3.4.7. RT^2 Profiler PCR Array

For enabling reliable gene expression analysis of 84 genes representing 9 different biological pathways, the human cancer pathway finder RT^2 Profiler PCR array (Qiagen N.V., Germany) was used. UT-SCC-60A and -60B cell lines were seeded in 6 well plates at 6×10^5 cells per well for overnight and treated with 5µM Ibrutinib for 24h. Total RNA was prepared as indicated in section 3.4.2 and 3.4.3. 1µg of cDNA was synthesized according to the RT^2 first strand cDNA synthesis kit (Qiagen N.V., Germany) to perform one human cancer pathway finder RT^2 Profiler PCR Array. The obtained C_T value data was analyzed by quantifying the relative changes in gene expression using $2^{-\Delta\Delta C}{}_T$ method.

3.4.8. Agarose gel electrophoresis

For the efficient separation of the nucleic acid fragments that differ in conformation, agarose gel electrophoresis was used. Due to the net negative charge of the sugar-phosphate backbone, the nucleic acids migrate according to their size through the three dimensional (3D) agarose gel matrix towards the anode upon applied electric field at 100V for 1h. The ethidium bromide added to the gel intercalates with the DNA/RNA fragments and fluoresce under excited UV light. The size of the fragments will be determined using the GeneRuler DNA Ladder (100bp, 1kb) (Thermo Scientific, USA).

The following components were used to separated DNA and RNA:

For DNA, 100ml

Components	Quantity	Final concentration
Agarose	1gm	1% (w/v)

TAE	100ml	1x
Ethidium bromide	10µl	1µg/ml

The DNA samples were prepared by diluting 10µl of control PCR product with 2µl of 6x loading dye.

For RNA, 100ml

Components	Quantity	Final concentration
Agarose	1.2gm	1.2% (w/v)
MOPS	94.8ml	1x
Formaldehyde	5.2ml	1.90%
Ethidium bromide	10µl	1µg/ml

The RNA samples were prepared by diluting 1:1 of RNA (1µg) in 2xRNA loading dye.

3.5. Molecular and Cellular Immunology Methods

3.5.1. Buffers and Reagents

10x Binding buffer, 50ml

Components	Amount added	Final concentration
HEPES	5ml	0.1M
NaCl	4.09gm	1.4M
$CaCl_2$	0.13gm	25mM
Water	up to 50ml	NA

Filtered and stored at +4°C.

10x PBS, 1L

Components	Amount added	Final concentration
NaCl (pH 7.4)	80gm	1.4M
KCl	2gm	0.03M
Na_2HPO_4	14.4gm	0.1M
KH_2PO_4	2.4gm	0.02M
Water	up to 1L	NA

Stored at room temperature.

3.5.2. Enzyme-Linked Immunosorbent Assay (ELISA)

For measuring the cytokine production in Ibrutinib and TLR ligand treated cells, enzyme-linked immunosorbent assay (ELISA) was performed. 8×10^4 cells/well were seeded into 24 well plates (Sarstedt AG & Co., Nümbrecht, Germany) for overnight at 37°C with 5% CO_2. The adherent cells were then treated for 24h with culture medium containing Ibrutinib (5µM) followed by 6h incubation with addition of Poly (I:C) (10µg/ml) and LPS (2µg/ml) respectively. The supernatant from all the conditions were instantly frozen with liquid nitrogen and preserved at -80°C. The protein concentrations of the human IL-1β, TNF-α and IFN-β was determined from the supernatants according to the protocol given by the commercial ELISA kits (R&D Systems, USA).

3.5.3. Flow Cytometry (FC)

To determine the apoptotic cells, annexin-V and propidium iodide (PI) staining was performed. 2×10^5 cells cultured in each well of 6 well plates and incubated for overnight at 37°C with 5% CO_2. The adherent cells were then treated for 24h with Ibrutinib (5µM) followed by addition TLR agonists (LPS/Poly (I:C)) for 72h and 96h respectively. The whole cell supernatant and cells were centrifuged for 5 mins with 700x g at 4°C. The cell pellet was washed twice with ice cold DPBS and re-suspended in 50µl of 1x binding buffer containing APC conjugated annexin-V (1µl) and Propidium iodide (PI) staining solution (1µl). This mixture was incubated for 15mins in dark and

diluted with 200µl of 1x binding buffer. Annexin-V and PI positive cells were measured using BD FACS Canto^{TM} Flow cytometer (BD Biosciences, USA) within an hour. The data obtained was further analyzed to obtain the apoptotic cell percent using BD FACS Diva^{TM} 6.1.1 software.

3.5.4. Immuno Fluorescence (IF) staining

For the immunofluorescence staining, 4×10^4 cells/ml were seeded in each well of the culture slides (BD Biosciences, USA) for overnight at 37°C with 5% CO_2. The adherent cells were washed twice with 1x PBS and fixed for 15mins at room temperature with ice cold acetone and again washed twice in 1x PBS. The fixed cells were in 1x PBS containing 0.1% Triton X-100 for 15mins. Cells were washed thrice in 1x PBS and incubated for 2h at room temperature with antibody dilution buffer containing primary antibodies, which include rabbit anti-TLR3 (1:100, Abcam), mouse anti-TLR4 (1:50, Abcam). Cells were again washed thrice with 1x PBS and incubated with Cy2-conjugated goat anti-rabbit (1:100) and goat anti-mouse (1:100) antibodies for 1h at room temperature. Finally, the cells were incubated for 1min in DAPI (4′, 6-Diamidin-2-phenylindol; F. Hoffmann-La Roche AG, Switzerland) (1:50,000) as a nuclear counter-stain and washed in 1x PBS. The slides were then mounted gently with cover slip (Thermo Fisher Scientific, Germany) using Fluoromount-G®, Slide mounting medium (Southern Biotech, USA) and let dried for overnight at room temperature.

3.6. Protein Methodology

3.6.1. Buffers and Reagents

1% SDS, 500ml

Components	Amount added	Final concentration
SDS	5gm	1%
Water	up to 500ml	NA

Stored at room temperature.

10% APS, 1ml

Components	Amount added	Final concentration
APS	1gm	228.2M
Water	up to 1ml	NA

Alliquoted and stored at -20°C.

1x Cell lysis buffer, 1ml

Components	Amount added	End concentration
Lysis buffer (10x)	100µl	1x
Water	900µl	NA
Aprotinin	30µl	30µg/ml
PMSF	1µl	1ml
Pepstatin A	10µl	1µg/ml
Sodium fluoride	20µl	10ml
Phosphatase Inhibitor Cocktail	10µl	1%

Freshly prepared on ice.

4x SDS loading buffer, 50ml

Components	Amount added	Final concentration
Tris-HCL (pH6.8)	12.5ml	0.25mM
SDS	4gm	8%
Glycerol	20ml	40%
β-Mercapto ethanol	10ml	20%
Bromophenol blue	NA	0.004%
Water	up to 50ml	NA

Stored at room temperature.

10x Running buffer (SDS-PAGE), 1L

Components	Amount added	Final concentration
Glycin	144.13gm	200mM
SDS	10gm	1%
Tris base (pH 8.3-8.8)	30.3gm	25mM
Water	up to 1L	NA

Stored at room temperature.

10x TBS buffer, 1L

Components	Amount added	Final concentration
Tris base (pH 7.6)	24.22gm	0.2M
NaCl	80gm	1.37M
Water	up to 1L	NA

Stored at room temperature.

10x Transfer buffer, 1L

Components	Amount added	Final concentration
Tris base (pH 8.0-10.5)	30.28gm	25mM
Glycin	144.13gm	192mM
Methanol	200ml	20%
Water	up to 1L	NA

Stored at room temperature.

3.6.2. Protein isolation and quantification

The adherent cells were washed twice with ice cold DPBS and scraped gently after adding 100µl (T25 flask) of 1x cell lysis buffer. The cell homogenates were shortly vortexed and incubated for 90mins on ice before centrifugation at 13,000rpm for 15mins at 4°C. The supernatants were recovered for protein quantification by Bradford assay. 5µl of the cell homogenate was incubated with 250µl of Quick Start™ Bradford 1x dye reagent (Bio-Rad Laboratories Inc., Hercules (CA) USA) for 10mins at room temperature. The coomassie brilliant blue G-250 dye present in the bradford reagent forms a complex with proteins and converts to a stable unprotonated blue form (A_{max}=595 nm) (Fazekas de St Groth*, et al.*, 1963; Sedmak*, et al.*, 1977) which was detected by spectrophotometer. Based upon the protein standard extinction coefficient values, the concentration of the protein lysate was quantified photometrically.

The quantified protein lysates were stored at -20°C after adding the 4x SDS sample buffer and denaturing at 95°C for 5mins for further analysis.

3.6.3. Western Hybridization

To detect the specific proteins listed in table 7, 30µg of the whole cell lysate was electrophoresed in SDS-Polyacrylamide (SDS-PAGE) gel at 140V and transferred onto equilibrated nitrocellulose membrane (2µM or 4.5µM) (Bio-Rad Laboratories Inc., Hercules USA) by wet blot transfer method for 1h at 100V. To confer the quality of the protein transfer, the membrane was stained with non-specific dye Ponceau S (Sigma-

Aldrich Co., St. Louis (MO) USA) for a minute and washed thoroughly with 1x tris buffered saline (TBS). The membrane was then blocked with either 5% non-fat milk (Carl Roth GmbH & Co. KG, Karlsruhe) or 5% BSA (Sigma-Aldrich Co., St. Louis (MO) USA) in 1x TBS containing 0.1% tween 20 (Sigma-Aldrich Co., St. Louis (MO) USA) for 1h at room temperature to prevent the non-specific binding, and incubated with the specific antibody solution against the protein of interest for overnight at 4°C on gentle agitation. Protein bands were detected after the incubation with horseradish peroxidase-coupled secondary antibodies for 1h at room temperature, under the enhanced chemiluminescence detection system (Vilber Lourmat Deutschland, GmbH, Germany). The protein expression results were quantified using ImageJ 1.44p software (National Institutes of Health, Bethesda) and the pixel intensity was normalized to the corresponding housekeeping protein bands GAPDH or α-tubulin. The Protein expression in response to the stimulation or inhibition was expressed as fold increase in intensity over the control samples.

3.7. Statistical Analysis

Statistical analysis was performed using paired student's t-test and the significant *P* values were marked with an asterisk * as following: "n.s. if $P>0.05$; * if $P \leq 0.05$; ** if $P \leq 0.01$; and *** if $P \leq 0.001$.

4. RESULTS

4.1. Characterization of TLR3, TLR4 Signaling in HNSCC cells

It has been known that Toll like receptors (TLRs) are expressed on immune cells as well as on normal epithelial cells and cancer cells. TLRs expressed on cancer cells can upregulate inflammatory and anti-apoptotic signaling cascades that can contribute to the immunosuppression and tumor cell proliferation. To characterize the TLR3 and TLR4 signal transduction process in permanent HNSCC cells, the cells were treated with TLR agonists Poly (I:C) and LPS and the series of events occurred in response to the stimuli in four permanent HNSCC cell lines UT-SCC-16A, -16B, -60A and -60B were studied and are detailed below:

4.1.1. Protein expression of TLR3 and TLR4 receptors

As a first measure, immunofluorescence staining of acetone-fixed permanent HNSCC cells was performed to detect the expression of TLR3 and TLR4. As shown in figure 8a and 8b, all permanent HNSCC cells resulted in pronounced expression of TLR3 and TLR4 according to their different phenotypical characters. The primary tumor cell line UT-SCC-16A and its corresponding metastasis related cell line UT-SCC-16B established from tongue and neck are found to be larger in size and grow more as a colony, whereas UT-SCC-60A and its corresponding metastasis cell line UT-SCC-60B established from tonsils and neck were found to be smaller in size and grow more as idividual cells representing differences in the expression patterns of TLR3 and TLR4.

Further analysis to detect the basal expression of TLR3 and TLR4 in untreated HNSCC cells was performed using immunofluorescence staining and western hybridization was performed to detect the protein expression from cells treated with Poly (I:C) (10µg/ml) and LPS (2µg/ml) for 24hrs. Constitutive expression of TLR3 and TLR4 was found in all the four HNSCC cell lines in both experiments. The expression levels of TLR3 and TLR4, differed from one cell line to other, and it could be attributed to the differences in phenotypical as well as cell growth characteristics of HNSCC cells. And there was no distinguishable difference in expression pattern of TLR3 and TLR4 was noticed in treated to untreated HNSCC cell lines.

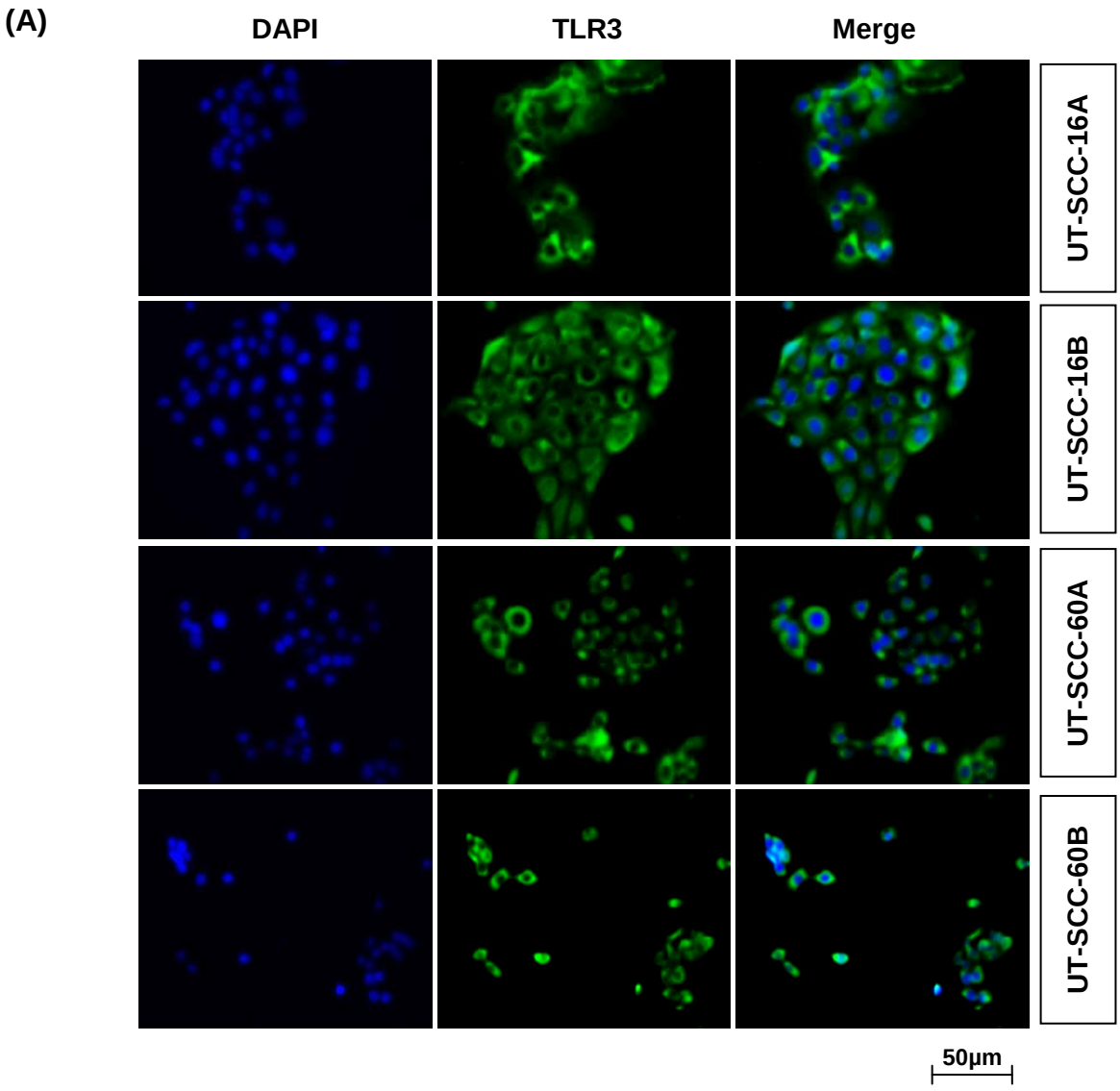


Figure 8a. Constitutive expression of TLR3 in HNSCC cells illustrated by Immunofluorescence and western hybridization analysis; (A) Immunofluorescent stainings showing expression of TLR3 (green fluorescence, cy2) and nucleus (blue, DAPI) (scale bar, 50µm). (B) Expression profile of TLR3 and housekeeping control α-tubulin by Western blotting analysis in HNSCC cell lines stimulated with TLR agonists Poly (I:C) (10µg/ml) and LPS (2µg/ml) for 24h.

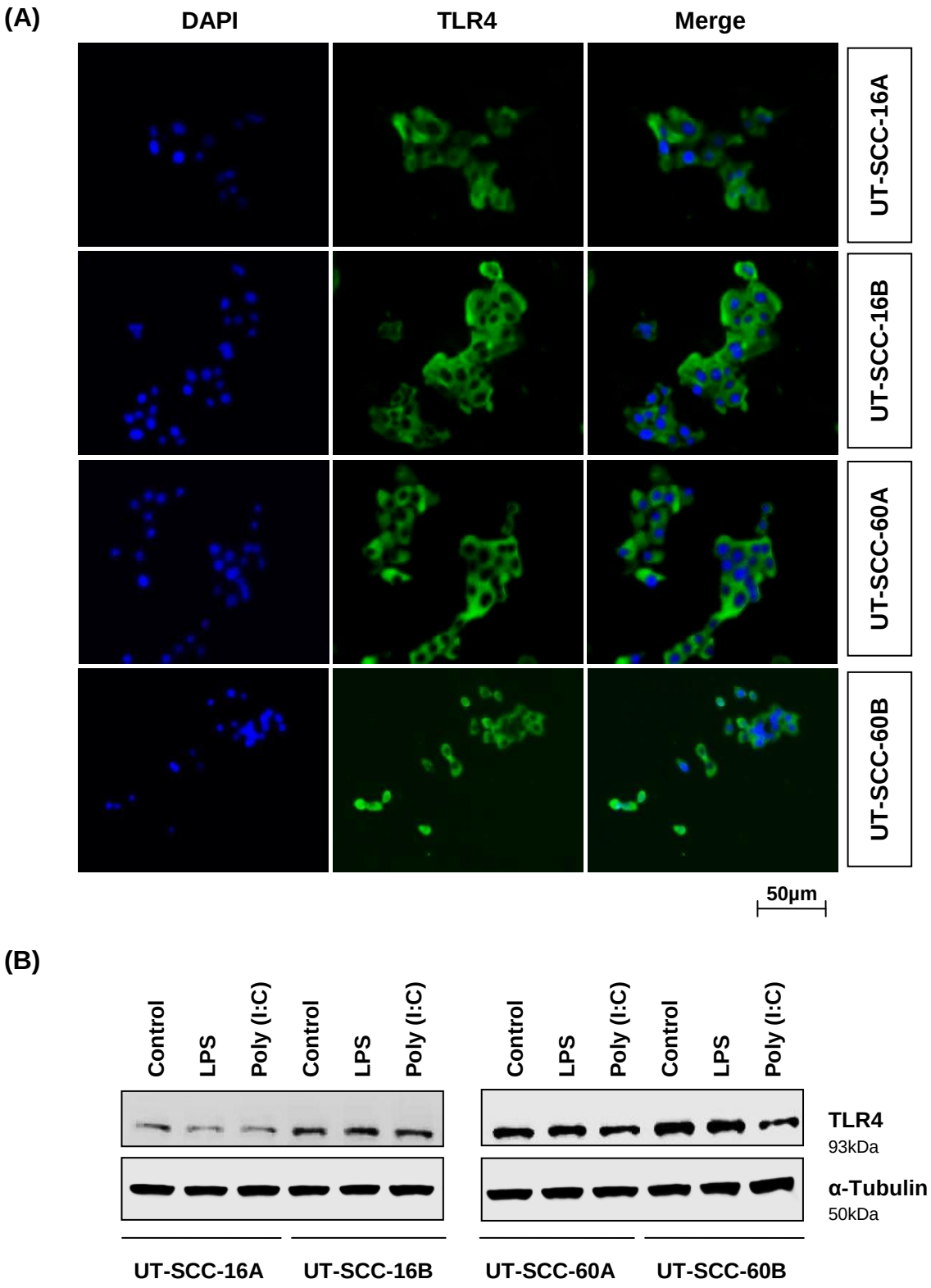


Figure 8b. Constitutive expression of TLR4 in HNSCC cells illustrated by Immunofluorescence and western hybridization analysis. (A) Immunofluorescent stainings showing expression of TLR4 (green fluorescence, cy2) and nucleus (blue, DAPI) (scale bar, 50µm). (B) Expression profile of TLR4 and housekeeping control α-tubulin by Western blotting analysis in HNSCC cell lines stimulated with TLR agonists Poly (I:C) (10µg/ml) and LPS (2µg/ml) for 24h.

4.1.2 Protein expression of TLR adaptor molecules, TRIF and MyD88

To assess the expression pattern of actively mediating downstream signaling molecules of TLR3 and TLR4 in HNSCC, the cells treated with Poly (I:C) and LPS were analyzed by western blotting to detect TRIF and MyD88 adaptor molecules. Basal expression of TRIF and MyD88 was noticed in all four cell lines with differences in the expression pattern from one cell line to the other. Similarly, when the expression profile of these molecules in treated and untreated cells were compared, the effect of treatment with Poly (I:C) and LPS did not lead to apparent change in protein expression.

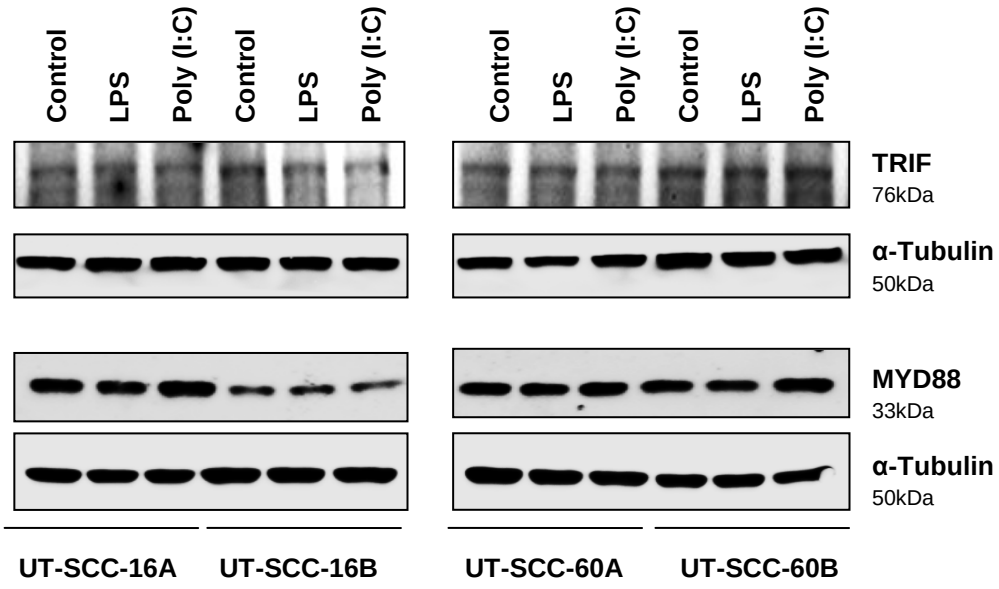


Figure 9. Western hybridization analysis on cell extracts from HNSCC cell lines stimulated with TLR agonists Poly (I:C) (10µg/ml) and LPS (2µg/ml) for 24h illustrating the basal expression of TLR adaptor molecules TRIF, MYD88 and the house keeping control α-tubulin.

4.1.3. Protein expression of Bruton's Tyrosine Kinase (BTK)

Several investigations of non-canonical pathways activated downstream of TLR signaling have detected Bruton's tyrosine kinase (BTK) as a key non-receptor tyrosine kinase required for the activation of TLR-induced immune responses. In order to understand the expression pattern of BTK and its active form in HNSCC cells, western hybridization analysis was performed on the cells treated with Poly (I:C) and LPS for 24hrs. It could be observed that the BTK (Phosphorylation at Y551) was actively turned on in all permanent HNSCC cells, irrespective of stimulation representing vigorous activation of inflammatory and immune responses in all analyzed HNSCC cell lines at baseline.

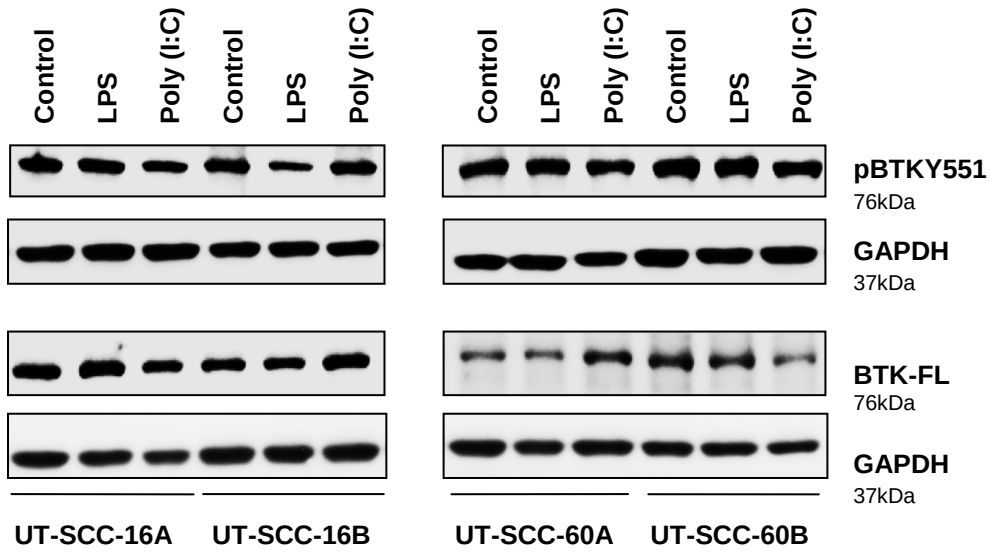


Figure 10. Western hybridization analysis on cell extracts from HNSCC cell lines stimulated with TLR agonists Poly (I:C) (10µg/ml) and LPS (2µg/ml) for 24h, illustrating the constitutive expression of phosphorylated BTK (pBTKY551), full length BTK (BTK-FL) and the house keeping control GAPDH.

4.1.4. Gene profiling of TLR3-, 4-induced pro-inflammatory cytokines

In order to determine the activation of TLR signaling in HNSCC cells more definitively, the gene expression profiling of pro-inflammatory cytokines including, Interleukin (IL)-1β, IL-6, Tumor necrosis factor (TNF)-α and Interferon (IFN)-β in response to TLR3 and TLR4 stimulation by Poly (I:C) and LPS at different time intervals (1hr, 2hrs and 6hr) was analyzed using real-time (RT)-PCR. The pro-inflammatory cytokines were induced, in response to stimulation for different time intervals, in all four HNSCC cell lines included in the present study (Figure 11). Although, the induction of inflammatory cytokines was evident in all the cell lines, their level of expression differed significantly with respect to the cell line and the time of stimulation. However, the patterns of expression profile of these cytokines remained similar in all the cell lines analyzed. It was apparent that all the cell lines significantly showed enhanced expression of inflammatory cytokines with a maximum fold change after 2h stimulation. The expression levels of cytokines in cell treated for 6h stimulation was markedly reduced compared to cells with 2h stimulation. Therefore, the 2h stimulation time point was used for further analysis of inflammatory cytokines in HNSCC cells.

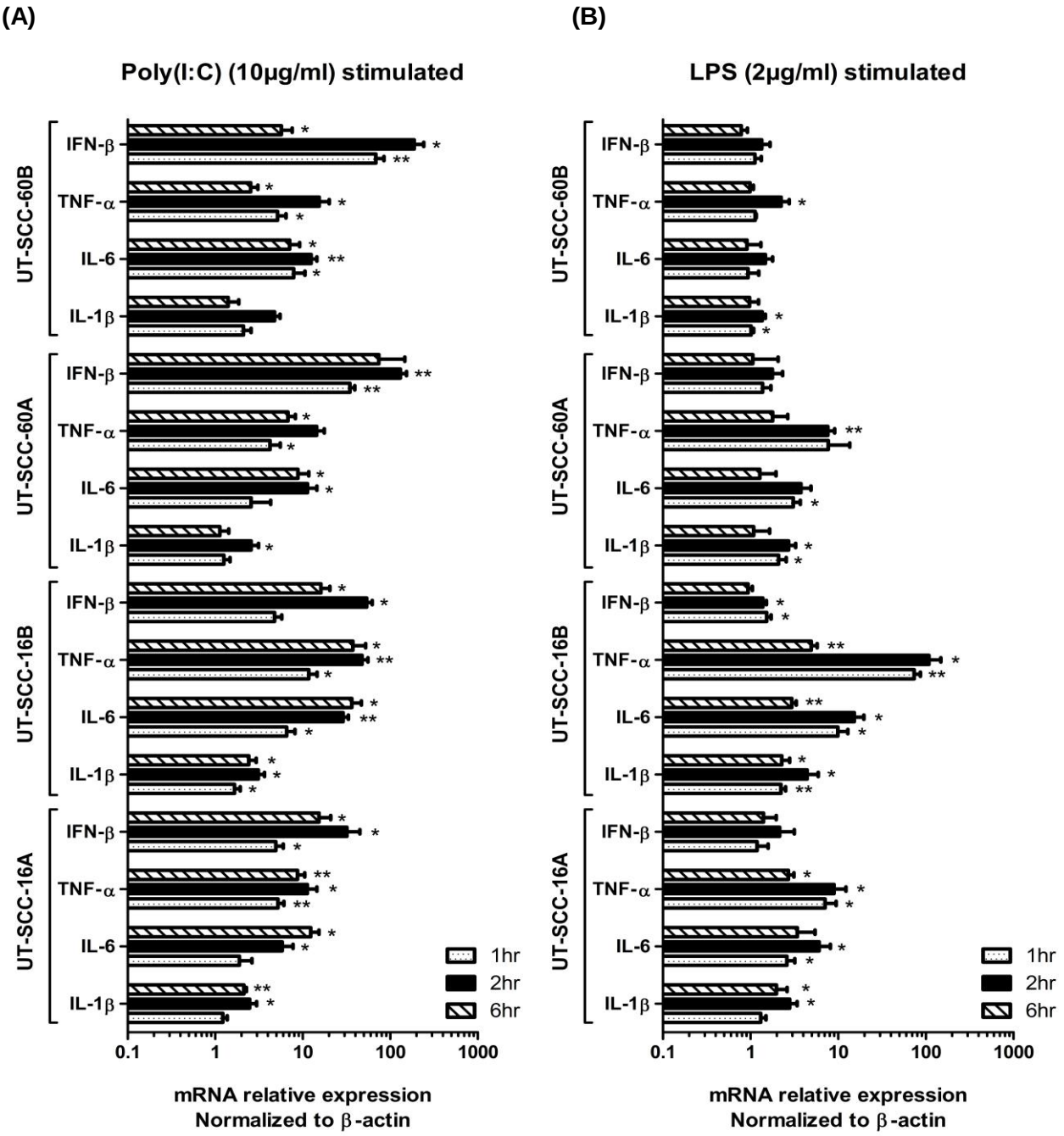


Figure 11. mRNA expression levels for pro-inflammatory cytokines IL-1β, IL-6, TNF-α and IFN-β in HNSCC cell lines stimulated with TLR3 and TLR4 agonists for 1h, 2h and 6h (A)Pro-inflammatory cytokine's expression profile in cells treated with Poly (I:C) (B) Pro-inflammatory cytokine's expression profile in cells treated with LPS. A significant upregulation of all the genes was observed after 6h stimulation in both cases. Results depicted were calculated according to $2^{-\Delta\Delta C}{}_{T}$ method and represented as mean with standard deviation from three independent experiments. *P ≤ 0.05, **P ≤ 0.01 compared with untreated controls analyzed using paired student's t-test.

4.1.5. Migration analysis in response to TLR3, 4 agonists

Tumor cell migration is a key event of different pathologic and physiologic processes such as wound healing, cancer, inflammation, cell growth and differentiation. To study the involvement of TLR agonists Poly (I:C) and LPS on HNSCC tumor cell migration, UT-SCC-60A and -60B cells were treated with Poly (I:C) (10µg/ml) and LPS (2µg/ml), the levels of migration occurred in response to the treatment at different time intervals (12h, 24h, 30h) monitored and compared to the untreated control cells. As shown in figure 12 (only UT-SCC-60A cell line was presented), the average area closure achieved within 12h demonstrate inhibition of cell migration in response to Poly (I:C) treatment. However, the percentage of average area closure achieved after 24h and 30h was rational and comparable to untreated cells, and it may be accredited to few unaffected populations which may contribute to the cell growth and migration during 24h and 30h incubation. LPS treatment had no apparent change in migration of HNSCC cells with respect to the control cells. These results indicated the contribution of TLR induced mechanisms in HNSCC tumor cell migration.

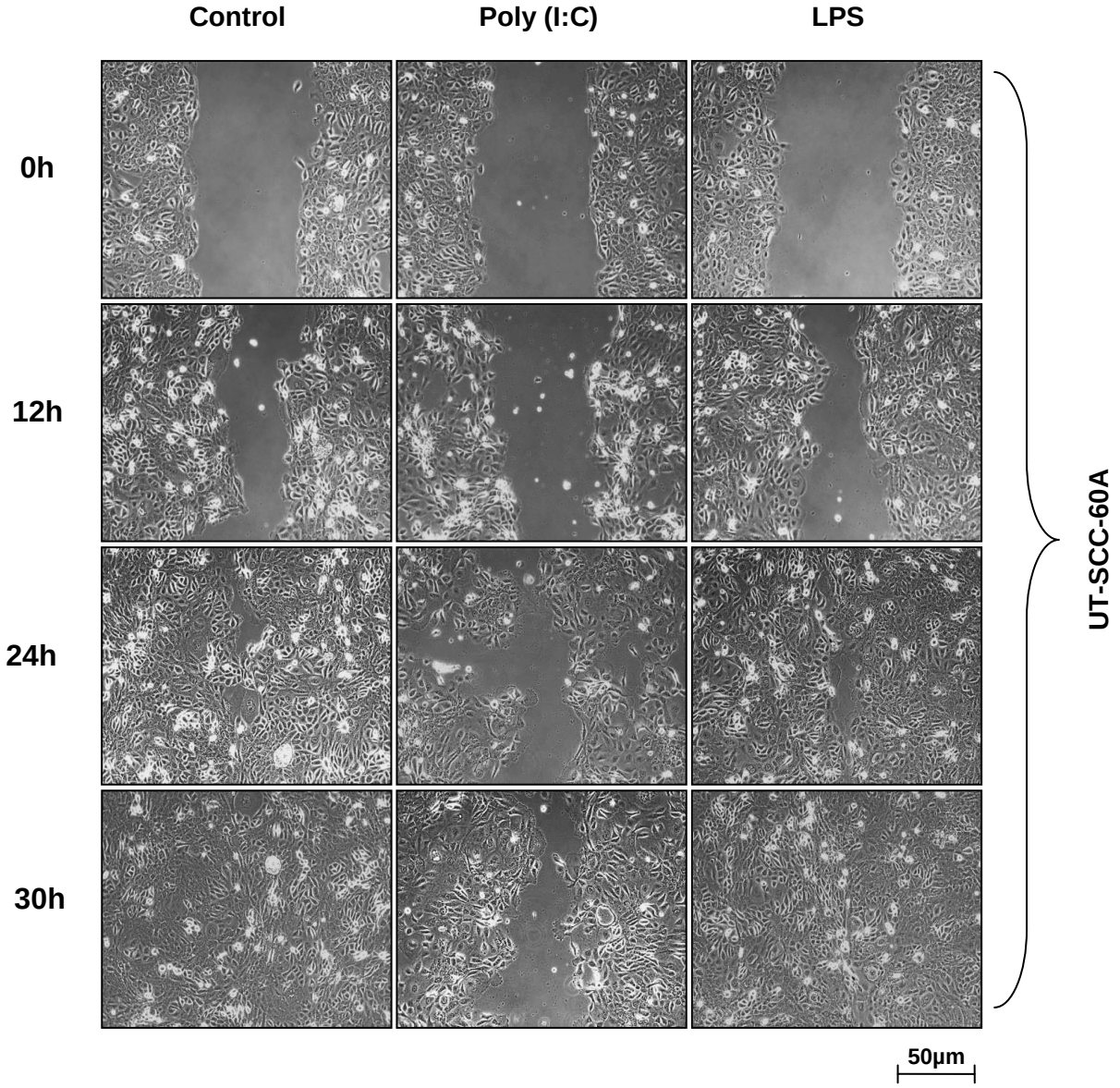


Figure 12. Wound healing assay for analysing the level of migration of UT-SCC-60A cell line treated with TLR agonists Poly (I:C) and LPS incubated for different time time intervals 0h,12h,24h and 30h. Results obtained from three independent experiments, and captured using the bright field mode on Axiovert 200M fluorescence microscope (50µm scale bar). Significant migration of cells in response to TLR agonists was noticed after 12h of incubation in different manner. LPs promotes faster migration than that of Poly (I:C) treatment.

4.2. Molecular Profiling of Ibrutinib treated HNSCC cells

Given its critical role in TLR signaling, Bruton's tyrosine kinase (BTK) has become an appealing therapeutic target. As Ibrutinib is an established irreversible inhibitor of BTK with potential clinical activity with broad therapeutic utility, it was used in the present study to target the BTK and to evaluate its effect on both molecular and cellular events occurring in permanent HNSCC cell lines. Different concentrations of Ibrutinib (1µM, 5µM and 10µM) for the following studies:

4.2.1 Pharmacological inhibition of BTK activation

In the first preference, the pharmacological inhibition of the BTK phosphorylation by Ibrutinib was analyzed. Cell lysates obtained from the cells treated with Ibrutinib (1µM and 5µM) for 24hrs, were analyzed by western hybridization using the anti-phospho BTKY551 and anti-BTK antibodies. It was observed that Ibrutinib at 5µM effectively inhibited phosphorylation in UT-SCC-16A, 60A and -60B cells. In converse, no effect on the phosphorylation of BTK was observed in UT-SCC-16B cells, indicating their resistance towards Ibrutinib treatment. Hence, Ibrutinib at 5µM concentration was considered as ideal molar concentration for further analysis.

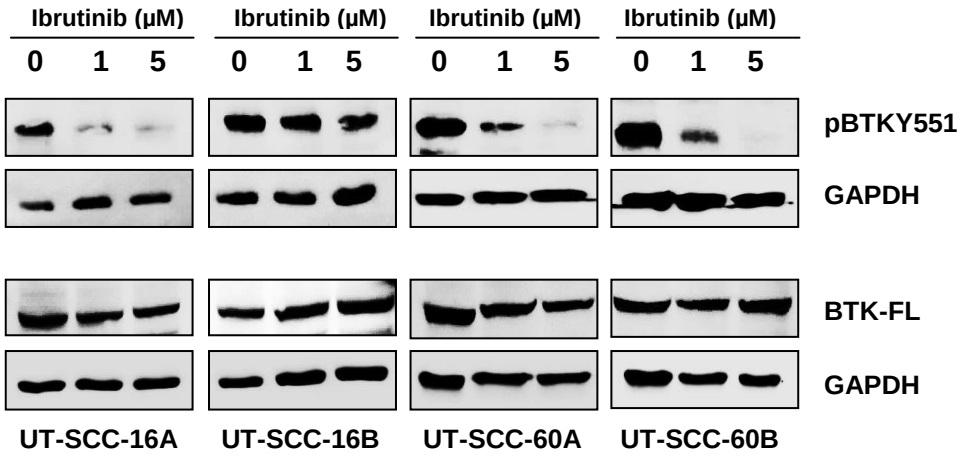


Figure 13. Western hybridization analysis illustrating the significant inhibitory effect of Ibrutinib on BTK phosphorylation (pBTKY551): Blots depicts the phosphorylated BTK (pBTKY551), full length BTK (BTK-FL) and housekeeping control GAPDH in HNSCC cell lines incubated with Ibrutinib at 1µM and 5µM concentrations for 24h.

4.2.2. Analysis of cancer pathway gene array

It is well known that several signaling pathways are involved in the pathogenesis of HNSCC. Ibrutinib has been shown to alter microenvironment survival signals and block the protective effect of stromal co-cultures *in vitro* (Herman*, et al.*, 2011). Hence to understand the additional pathways altered by Ibrutinib, HNSCC cell lines UT-SCC-60A and -60B were treated with Ibrutinib (5µM) for 24hrs and analyzed for the mRNA expression levels of 94 cancer pathway-related genes using RT^2 Profiler PCR array. Out of 94 genes studied by relative expression of mRNAs, 9.57% (9 genes) were upregulated (fold change > 1.5) (Table 10) whereas, 5.31% (5 genes) were down regulated (fold change < 0.5) (Table 11) following Ibrutinib treatment in comparison to untreated UT-SC-60A and -60B cell lines. The detailed analysis of the influenced genes showed an association with induction of apoptosis, cell invasion, migration and proliferation which were discussed in the following sections.

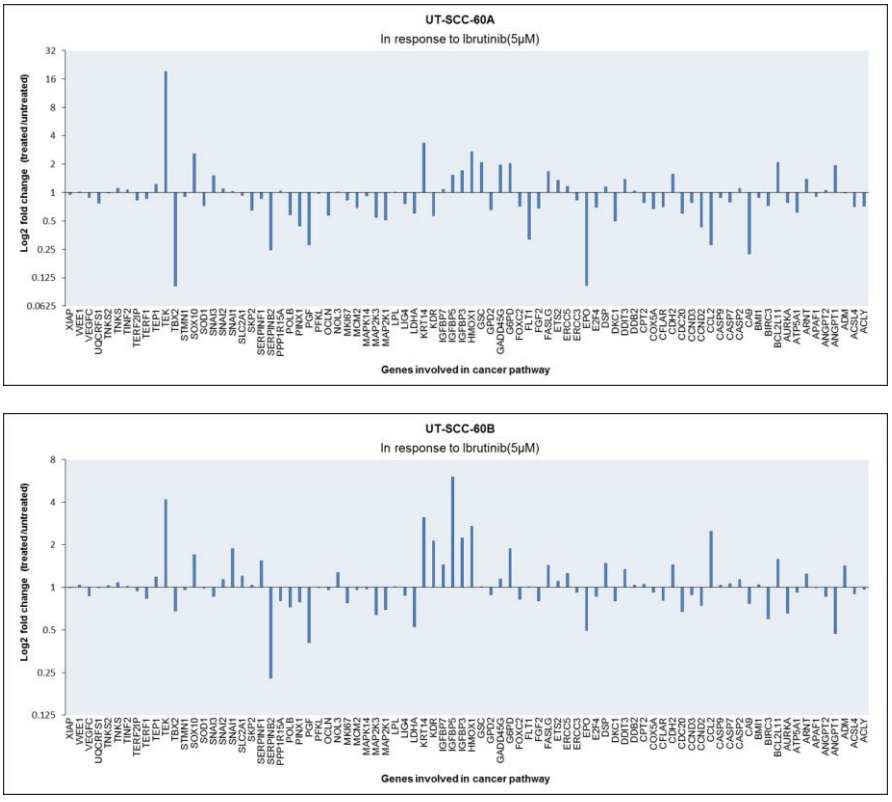


Figure 14. mRNA expression levels of 94 genes involved in cancer pathway in UT-SCC-60A and -60B cell lines treated with Ibrutinib (5µM) for 24h. Results were calculated according to $2^{-\Delta\Delta C}{}_T$ method and represented as relative to untreated control, normalized to housekeeping control β-actin.

List of genes upregulated

Gene	Name	Relative fold increase	
		UT-SCC-60A	UT-SCC-60B
TEK	TEK tyrosine kinase, endothelial	19.3131	4.1581
BCL2L11	BCL2-like 11 (apoptosis facilitator)	2.0727	1.5756
FASLG	Fas Ligand (TNF superfamily, member 6)	1.6604	1.4299
IGFBP3	Insulin-like Growth Factor Binding Protein 3	1.7189	2.2438
IGFBP5	Insulin-like Growth Factor Binding Protein 5	1.5279	6.0458
KRT14	Keratin 14	3.3438	3.1079
SOX10	SRY (Sex determining region Y)-box 10	2.5874	1.7005
HMOX1	Heme Oxygenase (decycling) 1	2.6973	2.7056
G6PD	Glucose-6-Phosphatase Dehydrogenase	2.0442	1.8868

Table 12. List of genes up-regulated in response to Ibrutinib (5μM) treatment after 24h. Relative fold change was calculated according to $2^{-\Delta\Delta C}{}_{T}$ method and normalized to the internal control β-actin.

List of genes down regulated

Gene	Name	Relative fold decrease	
		UT-SCC-60A	UT-SCC-60B
PGF	Placental Growth Factor	0.2796	0.405
MAP2K3	Mitogen-Activated Protein Kinase Kinase 3	0.5477	0.6399
SERPINB2	Serpin Peptidase Inhibitor, cladeB, member 2	0.2451	0.2278
EPO	Erythropoietin	0.1038	0.4951
LDHA	Lactate Dehydrogenase A	0.6035	0.5234

Table 13. List of genes down-regulated in response to Ibrutinib (5μM) treatment after 24h. Relative fold change was calculated according to $2^{-\Delta\Delta C}{}_{T}$ method and normalized to the internal control β-actin.

4.2.3. Analysis of tumor cell viability

As the results from section 4.2.2 suggest an effect of Ibrutinib on the expression of genes involved in cell proliferation, further analysis was performed using MTT assay to study the cell vitality and bioactivity in response to Ibrutinib treatment. The absorbance obtained in the MTT assay performed on Ibrutinib (1µM, 5µM and 10µM) treated HNSCC cell lines of the present study suggested a reduction in their viability compared to the untreated (control) cells. The viability of cells differed significantly after 72h and the pattern of reduction in the viability was predominant in the cells treated with higher concentration (10µM) than in cells treated with lower concentration (1µM), demonstrating the dose and time dependent effect of Ibrutinib on HNSCC cell lines.

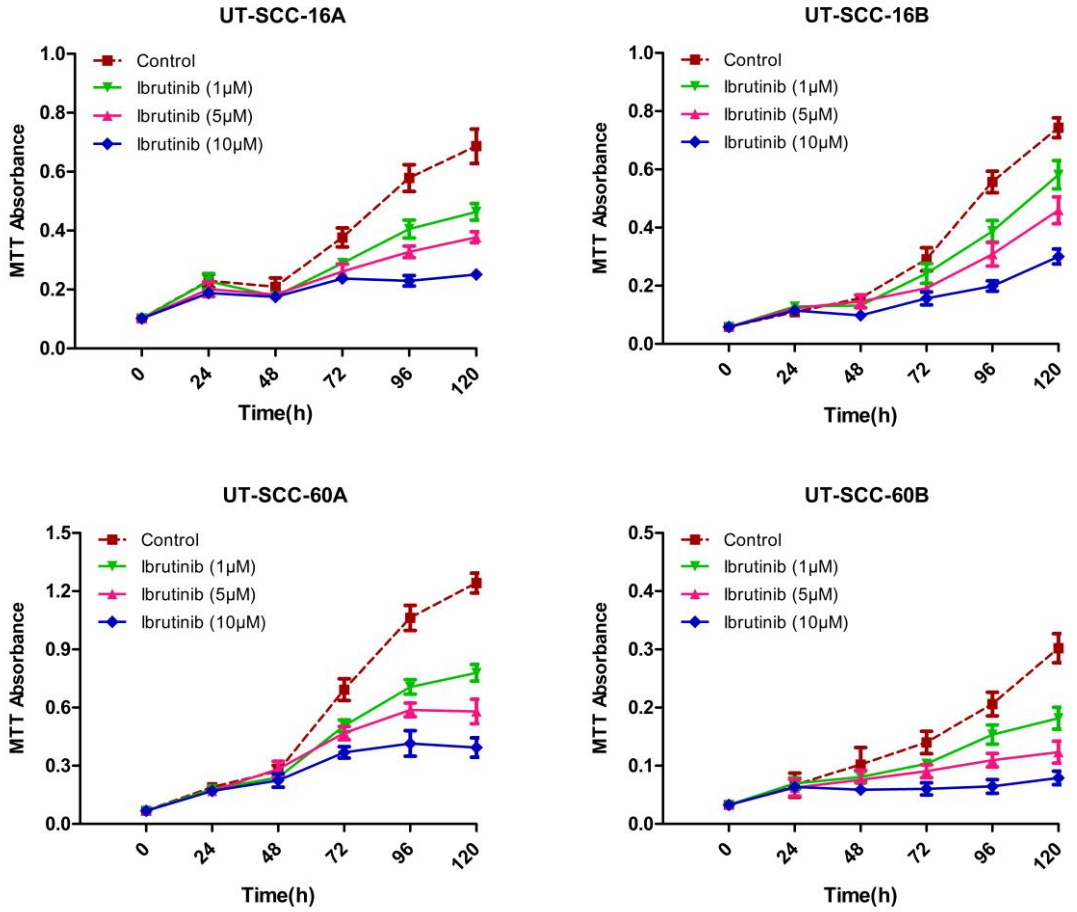


Figure 15. Dose- and time- dependent inhibitory effect of Ibrutinib on HNSCC cells viability. Cells were incubated with 1µM, 5µM and 10µM of Ibrutinib for 24h, 48h, 72h, 96h and 120h and cell growth was determined by MTT assay. The MTT absorbance at 570-690nm was used to determine the viability of cells and the graphs were represented as mean and standard deviation from three independent experiments. *P ≤ 0.05, **P ≤ 0.01, compared to untreated control and analyzed using paired student's t-test.

4.2.4. Analysis of tumor cell proliferation

In the further analysis, tumor cell proliferation assays were performed with above mentioned concentration of Ibrutinib using real-time cell analysis (RTCA). In support to the results obtained in the cell viability assays, the inhibition of tumor cell proliferation was observed in cells when treated with Ibrutinib and the inhibition pattern observed was dose and time dependent. Effective inhibition of proliferation at higher concentration of Ibrutinib (10µM) was noticed when compared to 1µM and 5µM conforming the potential role of Ibrutinib in inhibiting HNSCC cell viability and progression to minimal concentrations.

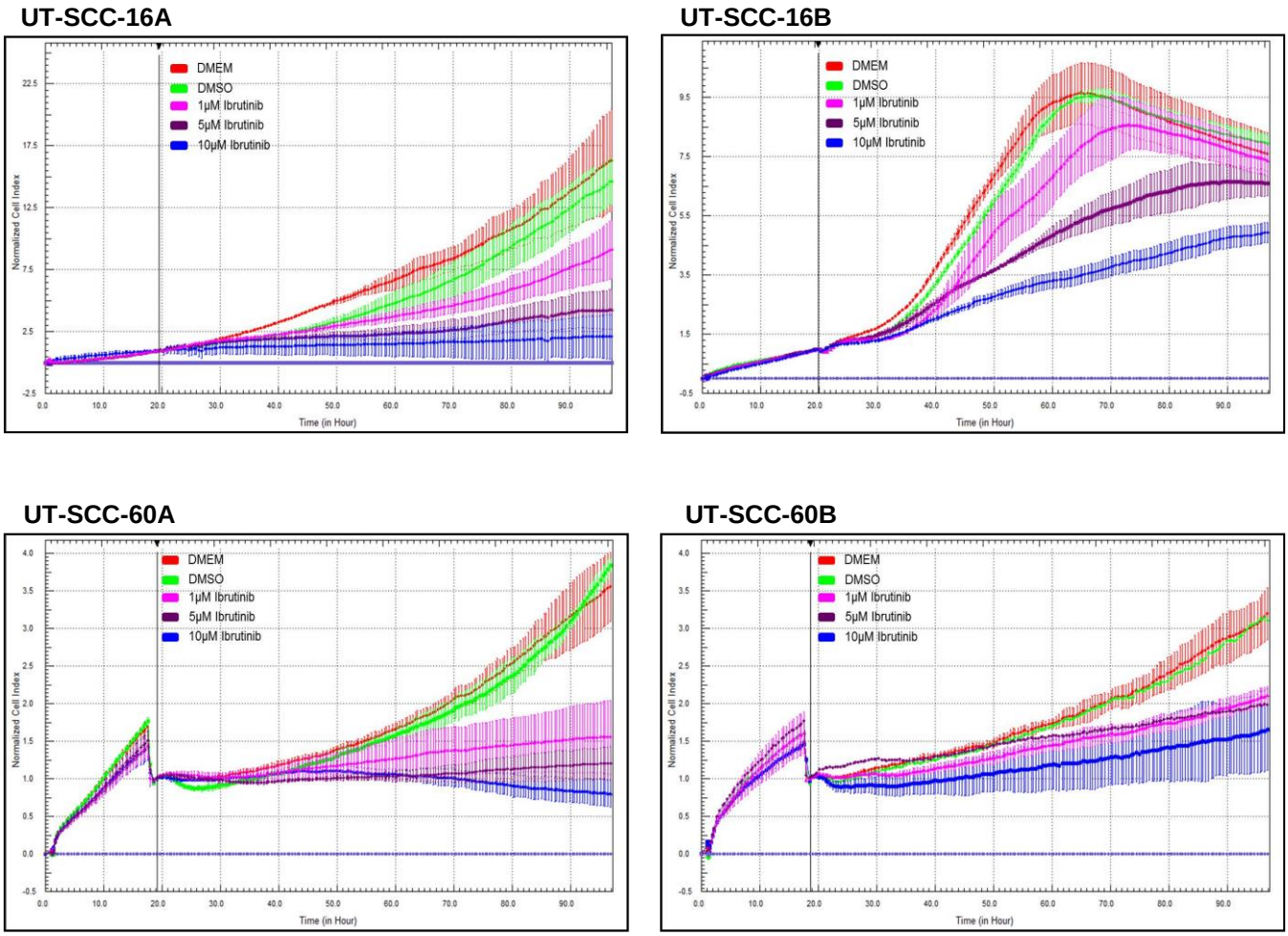


Figure 16. Dose- and time-dependent inhibitory effect of Ibrutinib on HNSCC cell proliferation. Normalized cell index results obtained after 96h incubation with Ibrutinib (1µM, 5µM and 10µM) on RTCA-DP analyzer.

4.3. Role of Ibrutinib in regulating TLR3 induced inflammation

TLR3 is known to mediate antiviral responses by phosphorylating its cytoplasmic domain and initiating the downstream signaling. There are studies stating Bruton's tyrosine kinase (BTK) as a responsible phosphorylating enzyme of TLR3 cytoplasmic domain. In this study, to understand the critical role of BTK in activating TLR3 induced signaling in HNSCC cell lines, initially BTK of HNSCC cells was inhibited by pharmacological inhibitor Ibrutinib and followed by stimulation with TLR-3 agonist Poly (I:C). Later to the treatment, the TLR3 induced inflammation was analyzed by measurement of pro-inflammatory cytokines.

4.3.1. Gene expression analysis of pro-inflammatory cytokines

After a 24h pretreatment of the cells with Ibrutinib (5µM) and consecutive 2h stimulation with Poly (I:C) (10µg/ml), gene expression profiles of TLR3 induced pro-inflammatory cytokines including interleukin (IL)-1β, IL-6, IL-8 and tumor necrosis factor (TNF)-α in UT-SCC-16A, -16B, -60A and -60B cells were performed. The relative gene expression of Ibrutinib treated pro-inflammatory cytokines induced by TLR3 indicated significantly reduced expression of IL-1β in all analyzed cell lines. The expression of IL-6 in UT-SCC-60A, IL-8 in UT-SCC-16A, -60A and TNF-α in UT-SCC-60A, -60B were found to be significantly reduced in UT-SCC-60A cell line (Figure 17). Whereas, in other cell lines, the results obtained were not statistically significant.

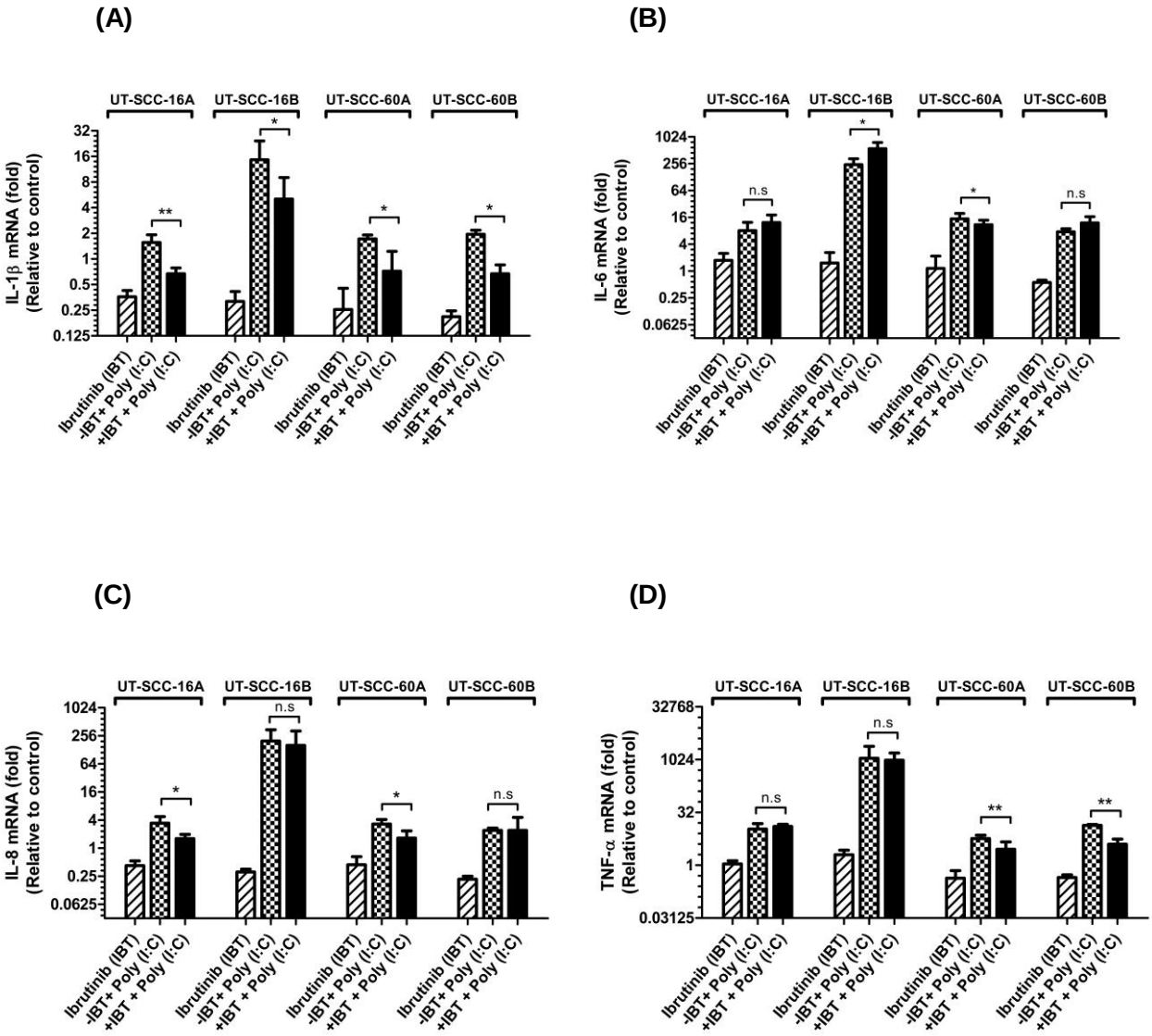


Figure 17. mRNA expression levels for pro-inflammatory cytokines: (A) IL-1β, (B) IL-6, (C) IL-8 and (D) TNF-α in HNSCC cell lines treated with Ibrutinib for 24h and stimulated with TLR3 agonist Poly (I:C) (10µg/ml) for 2h. Significant reduction in the gene expression pattern of IL-1β, IL-8 (-16A,-60A) and TNF-α (-60A, -60B) was observed. Relative expression calculated according to $2^{-\Delta\Delta C}{}_{T}$ method and represented as mean with standard deviation from four independent experiments. Non-significant (n.s) P > 0.05, *P ≤ 0.05, **P ≤ 0.01 compared to Poly (I:C) treated without Ibrutinib and Poly (I:C) treated with Ibrutinib treated, analyzed using paired student's t-test.

4.3.2. Detection of IL-1β and TNF-α cytokine secretion

The mRNA expression levels of pro-inflammatory cytokines were verified on the protein level by enzyme linked immuno sorbent assay (ELISA) which was performed on fresh supernatants of HNSCC cells which were treated with Ibrutinib (5µM) for 24h prior to Poly (I:C) stimulation for 6h. The amounts of TLR3 induced cytokines secreted were found to be strongly reduced in Ibrutinib treated cells when compared to untreated UT-SCC-60A and -60B cells. Whereas in UT-SCC-16A and -16B cells the cytokine secretion was under the detection range and hence cannot be plotted.

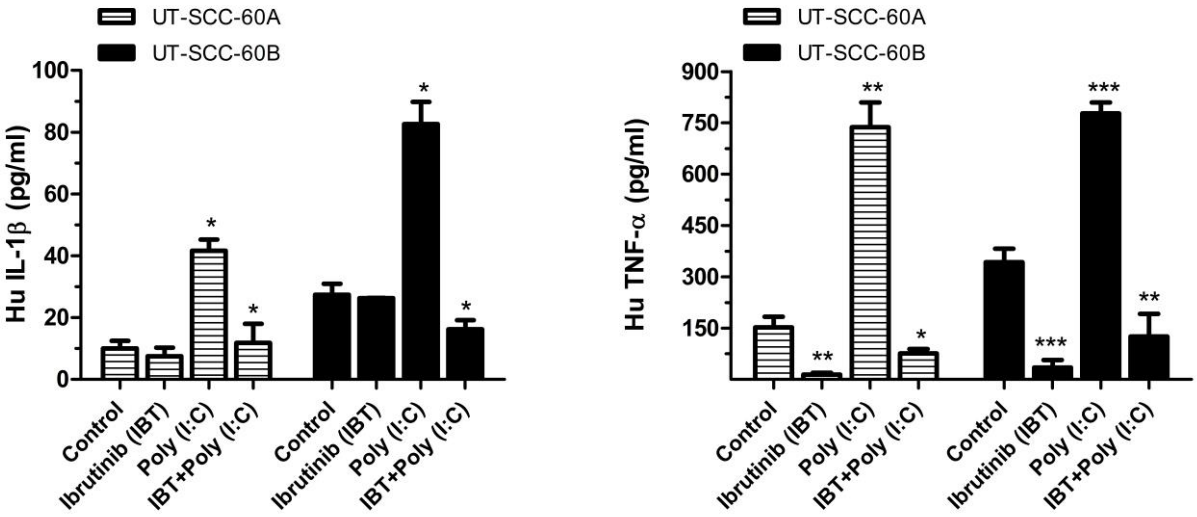


Figure 18. Significant reduction of human IL-1β and TNF-α secretion in the supernatants of Ibrutinib and Poly (I:C) treated HNSCC cell lines (UT-SCC-60A, -60B) detected by ELISA. Each data bar represents the mean and standard deviation of three independent experiments. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 compared to untreated control and analyzed using paired student's t-test.

4.3.3. Gene profiling of intracellular TLR3 induced pro-inflammatory cytokines

As TLR3 is predominantly localized in the endosomal compartments, and Poly (I:C) can be applied either through direct addition to culture medium or more effectively through transfection. Hence, further analysis was performed by transfection of Ploy (I:C) using lipofectamine to conform the direct influence of Ibrutinib on TLR3 induced signaling. Transfection of Poly (I:C) (10µg/ml) in parallel to addition to the cell culture medium was performed for 6h to the Ibrutinib treated cells. As expected the relative

gene expression of all transfected cells was found to be elevated to that of direct stimulation on the adherent cells. The gene expression of TLR3 induced pro-inflammatory cytokines IL-1β in all cell lines, IL-8 in three cell lines except in -16B and IL-6, TNF-α in UT-SCC-60A and -60B cells lines were significantly reduced in response to Ibrutinib treatment confirming the effect of Ibrutinib on TLR3 signaling.

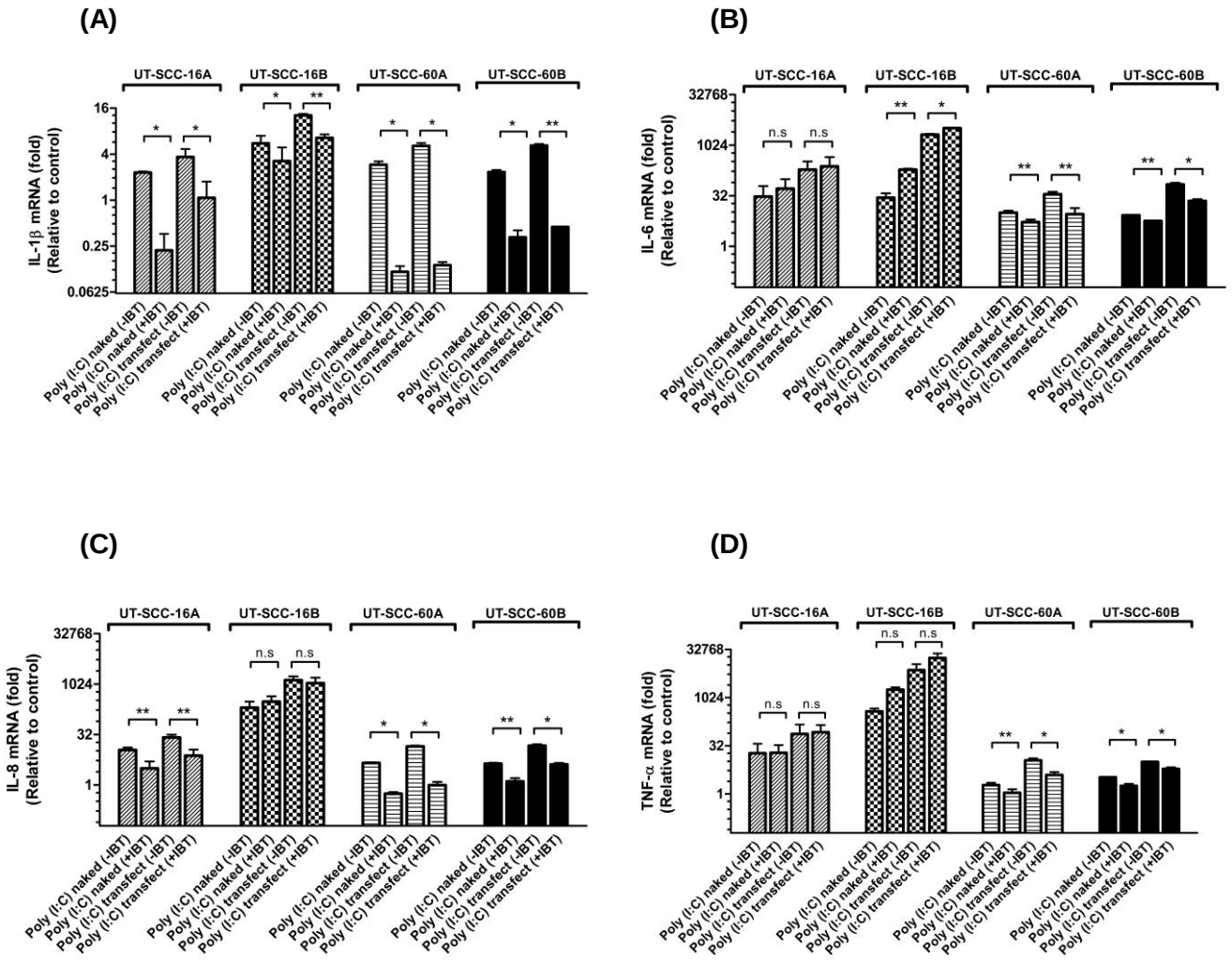


Figure 19. Significant reduction in the mRNA gene expression pattern of (A) IL-1β, (B) IL-6 (except in UT-SCC 16A, -16B) (C) IL-8 (except in UT-SCC-16B) and (D) TNF-α (except in UT-SCC 16A, -16B) in Ibrutinib treated cells for 24h and stimulated the cells with either Poly (I:C) transfected or direct treatment for 6h.Relative expression was calculated according to $2^{-\Delta\Delta C}{}_{T}$ method and represented as mean with standard deviation from three independent experiments. Statistical analysis using paired student's t-test, represents non-significant (n.s) $P > 0.05$, *$P \leq 0.05$, **$P \leq 0.01$.

4.4. Role of Ibrutinib in regulating TLR4 induced inflammation

There are several studies indicating the critical role of BTK in TLR-4 signaling in different cell types. To understand these mechanisms involved in HNSCC cells. BTK inhibited HNSCC cells by Ibrutinib were stimulated with LPS (2µg/ml) to activate TLR4 dependent signaling and the results were elucidated as following:

4.4.1 Gene expression analysis of pro-inflammatory cytokines

HNSCC cells were treated for 24h with Ibrutinib (5µM) and stimulated for 2h with LPS (2µg/ml) and the TLR4 induced pro-inflammatory cytokine gene profiling analysis was performed by real-time PCR. Relative expression of pro-inflammatory cytokine presented in figure 20 indicated that the expression of IL-1β was significantly down regulated in UT-SCC-16A, -16B, 60A and -60B cells. IL-8 and Tumor necrosis factor (TNF)-α were reduced in UT-SCC-60A cell lines. The outcome of analysis performed to determine the effect on IL-6, IL-8 and TNF-α expression in the HNSCC cells of the present study was not significant, and hence the effect of Ibrutinib on TLR4 induced pro-inflammatory cytokine expression cannot be established.

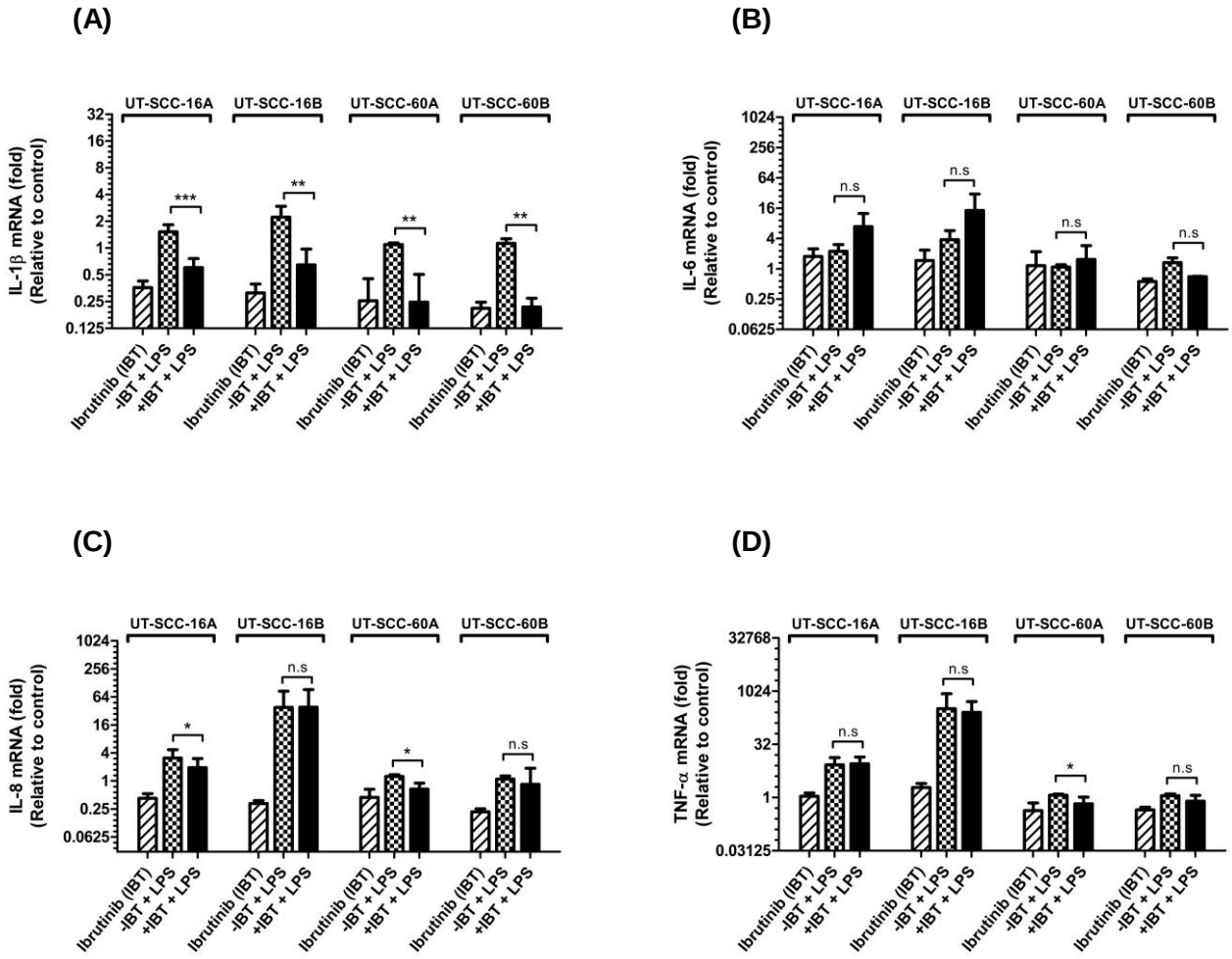


Figure 20. mRNA expression levels for pro-inflammatory cytokines: (A) IL-1β, (B) IL-6, (C) IL-8 and (D) TNF-α in HNSCC cell lines treated with Ibrutinib for 24h and stimulated with TLR4 agonist LPS (2µg/ml) for 2h. Significant reduction in the gene expression pattern of IL-1β, IL-8 (-16A, -60A) was observed. Relative expression calculated according to $2^{-\Delta\Delta C}{}_{T}$ method and represented as mean with standard deviation from four independent experiments. Non-significant (n.s) $P > 0.05$, *$P \leq 0.05$, **$P \leq 0.01$ compared to LPS treated without Ibrutinib and LPS treated with Ibrutinib treated, analyzed using paired student's t-test

4.4.2. Detection of IL-1β and TNF-α cytokine secretion

For better understanding, ELISA was performed on TLR4 stimulated fresh supernatants of Ibrutinib treated HNSCC cell lines. Pro-inflammatory cytokines IL-1β and TNF-α secretion levels were analyzed after 6hrs of TLR4 stimulation with LPS on Ibrutinib treated (24h) HNSCC cell lines. The secretion of TLR4 induced IL-1β and TNF-α were found to be significantly inhibited in Ibrutinib treated UT-SCC-60A and -60B cell lines. While the UT-SCC-16A and -16B cells secreted to low amounts of cytokines to be detected by ELISA and hence cannot be presented.

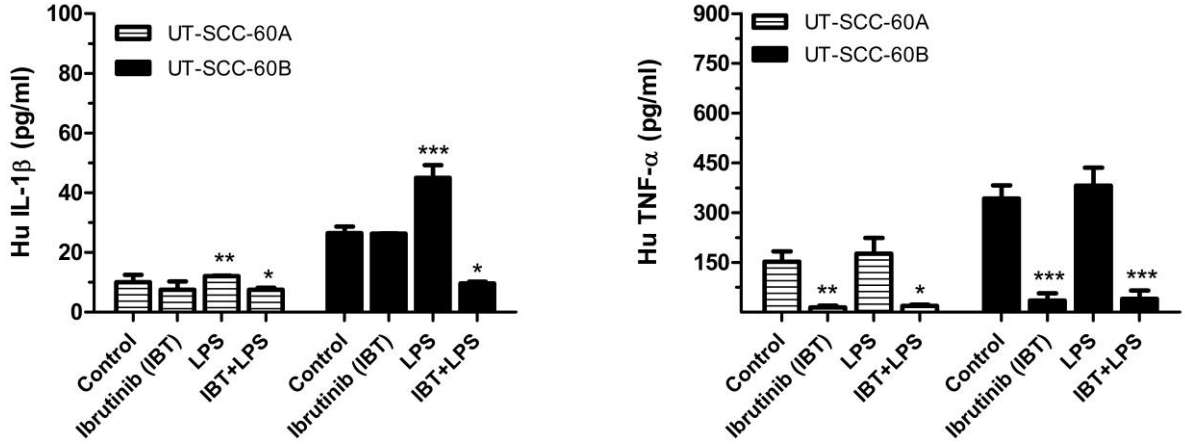


Figure 21. Significant reduction of human IL-1β and TNF-α secretion in the supernatants of Ibrutinib and LPS treated HNSCC cell lines (UT-SCC-60A, -60B) detected by ELISA. Each data bar represents the mean and standard deviation of three independent experiments. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 compared to untreated control and analyzed using paired student's t-test.

4.5. Role of Ibrutinib in regulating the activation of MAP Kinases

Activation of mitogen-activated protein kinases (MAPK) is a well-known key event in TLR signaling and inflammation. In order to examine whether the MAPK activation is TLR3 and TLR4 specific, activation of these two signaling pathways in BTK inhibited HNSCC cell lines was performed. The degree of three MAPK, the JNK, the ERK1/2 and the P38 activation was analyzed by western blotting from the cells stimulated for 1h with Poly (I:C) and LPS after 24h inhibition with Ibrutinib (5µM). As given below Poly (I:C) and LPS stimulation resulted in activation of all three MAPKs. In contrast, the activation of JNK and ERK1/2 was impaired, whereas the activation of P38 was not influenced in Ibrutinib treated TLR3 and TLR4 stimulated HNSCC cells indicating activation of JNK and ERK1/2 is TLR3 and TLR4 specific and the influence of Ibrutinib in TLR signaling.

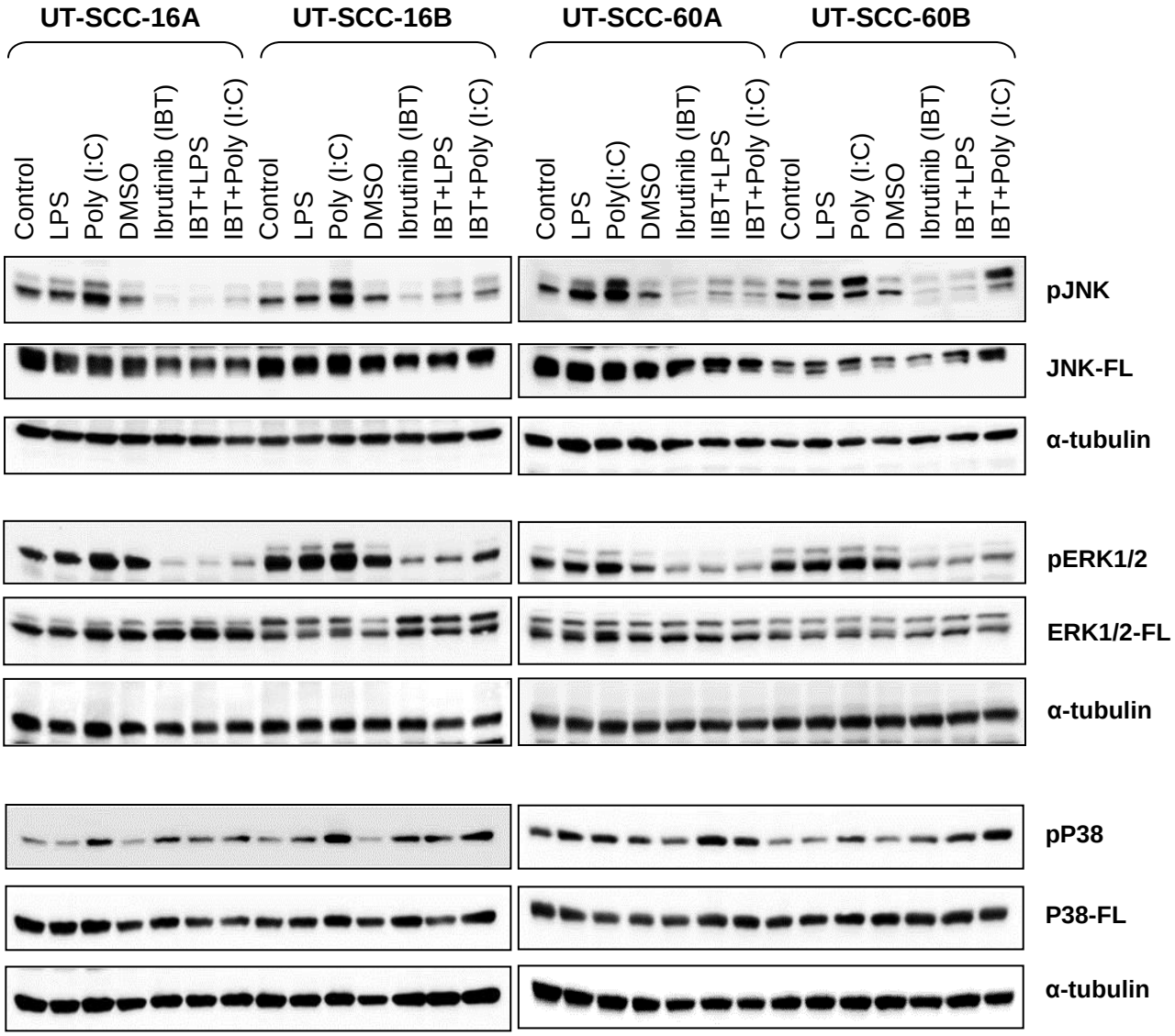


Figure 22. Western hybridization of phosphorylated MAPK (pJNK, pERK1/2, pP38), full length MAPK (JNK, ERK1/2, P38) and the house keeping control α-tubulin expression in HNSCC cell lines treated with Ibrutinib(5μM) for 24h and stimulated with TLR agonists Poly (I:C) (10μg/ml) and LPS (2μg/ml) for 1h. The phosphorylation of JNK (pJNK) and ERK1/2 (pERK1/2) was significantly inhibited in response to stimulation with Poly (I:C) or LPS in Ibrutinib treated HNSCC cells. Results obtained from three independent experiments.

4.6. Anti-tumor potential of Ibrutinib and TLR agonists

Aberrant TLR signaling was believed to initiate or add to the pathological behavior of cancer cells resulting in malfunction of immune response and apoptosis. There are findings suggesting the advantage of combining TLR agonists with other targeted therapies producing moderate success in activating anti-tumor potential and apoptosis. In the present study from section 4.2.3 and 4.2.4, it is evident that Ibrutinib inhibits cell viability and cell proliferation of HNSCC cells. Therefore, to further identify if the combined treatment of Ibrutinib with TLR3 and TLR4 agonists in HNSCC cell lines in inducing anti-tumor potential, cell viability assay and apoptosis assay was conducted and the results were as followed:

4.6.1. Analysis of Ibrutinib and Poly (I:C) effect on cell viability and apoptosis

HNSCC cells were treated with Ibrutinib at an optimal concentration of 5µM for initial 24h followed by the treatment with TLR3 agonist Poly (I:C) (10µg/ml), performing cell viability assays for 96h and apoptosis assay for 72h. In the cell viability assay results (Figure 23) it was clearly evident that combined treatment of Ibrutinib with Poly (I:C) increasingly inhibited cell viability compared to treatment with Ibrutinib or Poly (I:C) alone.

Similar effects were noticed in the apoptosis assay, where induction of apoptosis measured applying Ibrutinib and Poly (I:C) alone or as a combination. The percentage of apoptosis represented in figure 24 indicates increased apoptosis induction in combined treatment with Ibrutinib-Poly (I:C) compared to the Ibrutinib or Poly (I:C) treatment alone. These results demonstrated the potential of Ibrutinib as a strong inhibitor of tumor cell proliferation and its capacity to induce anti-tumor potential in successful combination with TLR3 agonist Poly (I:C) in HNSCC cell lines.

Results from cell viability assay:

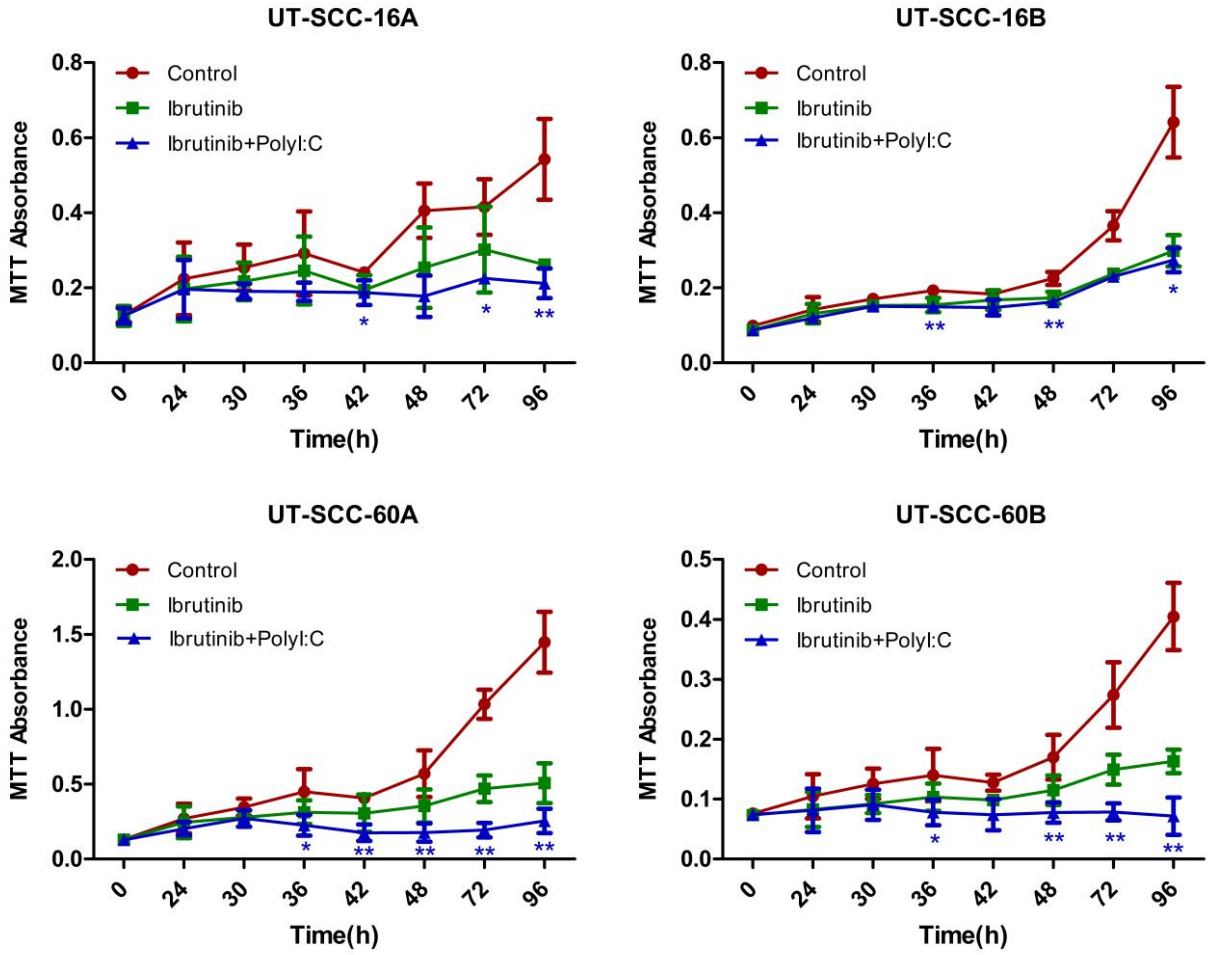
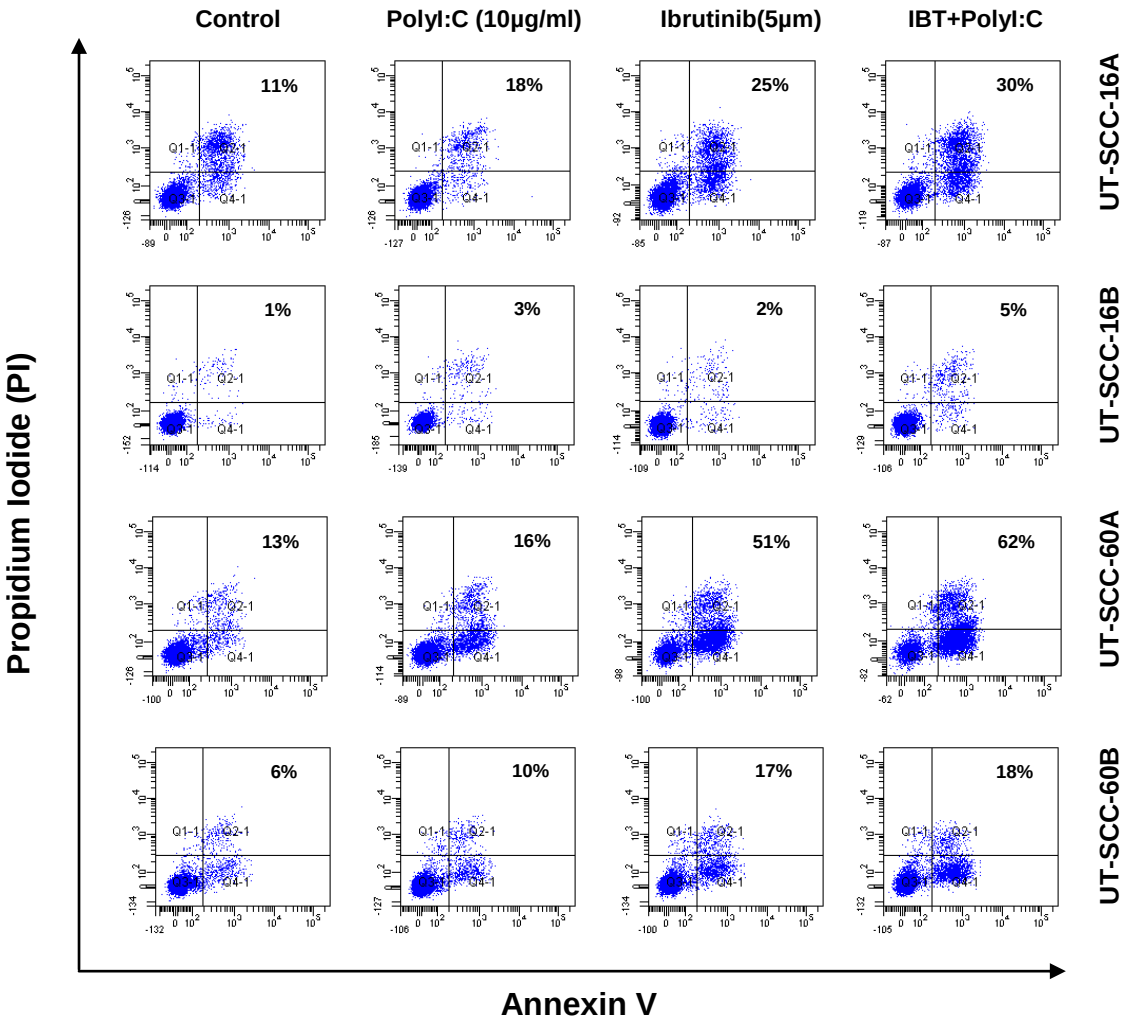


Figure 23. Dose- and time- dependent effects of Ibrutinib with Poly (I:C) on the viability of HNSCC cells determined by MTT assay. Significantly increased inhibition of cell viability was noticed in combined treatment with IBT+ Poly(I:C) than that of IBT treatment level. The MTT absorbance at 570-690nm was used to determine the viability of cells and the graphs were represented as mean and standard deviation from three independent experiments. *P ≤ 0.05, **P ≤ 0.01, compared to untreated control and analyzed using paired student's t-test.

Results from apoptosis assay:

(A)



(B)

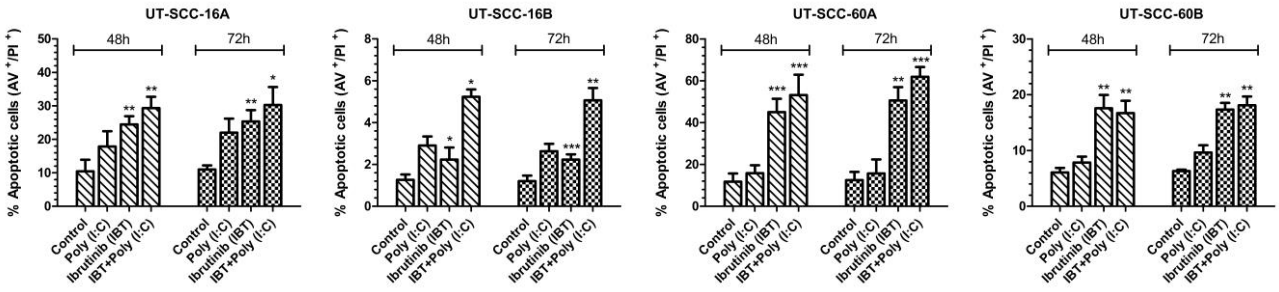


Figure 24. Effect of Ibrutinib with Poly (I:C) on the apoptosis of HNSCC cells, representing increased apoptosis in combined treatment with IBT+Poly (I:C) than that of Poly(I:C) or IBT alone. (A) Annexin V/PI double staining assay of cells incubated with or with our Ibrutinib and Poly (I:C). (B) Statistical analysis indicating the percent of apoptotic cells in response to treatment. The mean data of each condition were the results of three independent experiments and represented as mean with standard deviation. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 compared to untreated control and analyzed using paired student's t-test.

4.6.2. Analysis of Ibrutinib and LPS effect on cell viability and apoptosis

To further evaluate the effect of Ibrutinib in combination with TLR4 agonist LPS, HNSCC cells were pretreated with 5µM Ibrutinib for 24hrs and incubated with 2µg/ml LPS for 96h for cell viability assay and 72h for apoptosis assay. The combination of Ibrutinib with LPS did not show any increase in the inhibition levels of cell viability to that of Ibrutinib treatment alone. Similarly, very little effect was noticed in the induction of apoptosis in the combined treatment. These results suggested additional treatment with the TLR4 agonist LPS was not adding to anti-tumor effect of Ibrutinib in HNSCC cells.

Results from cell viability:

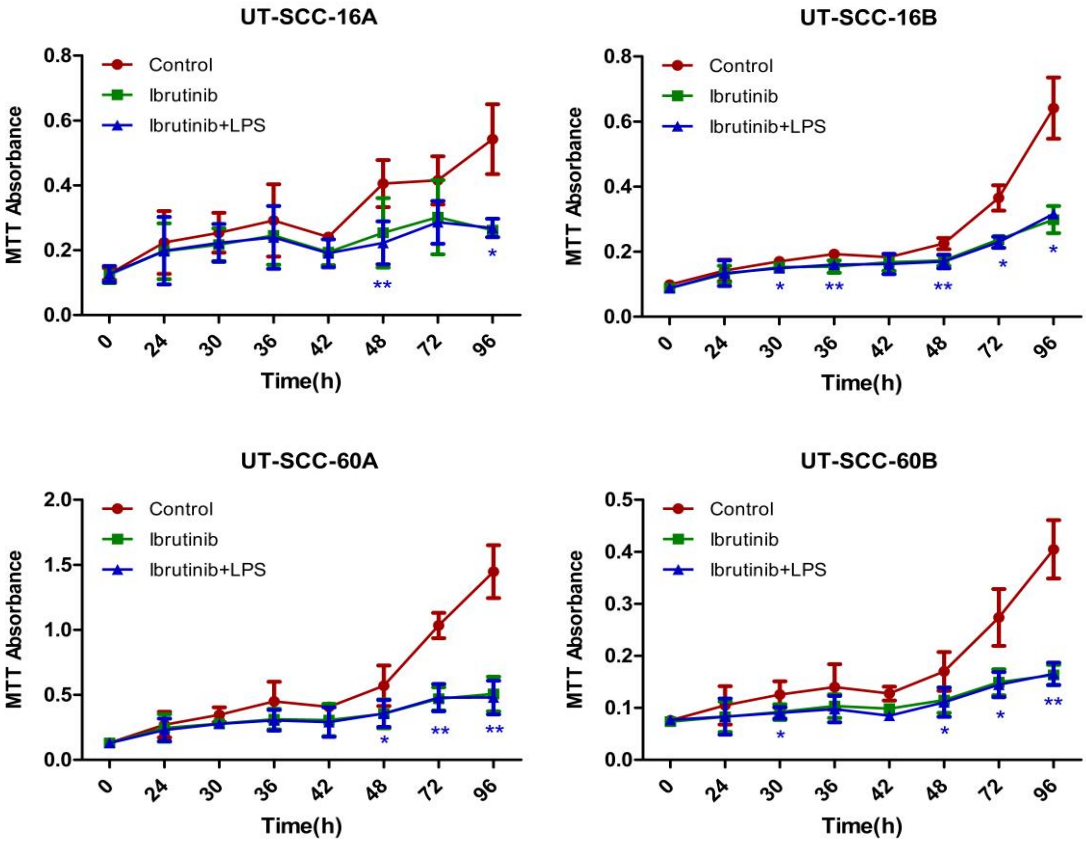
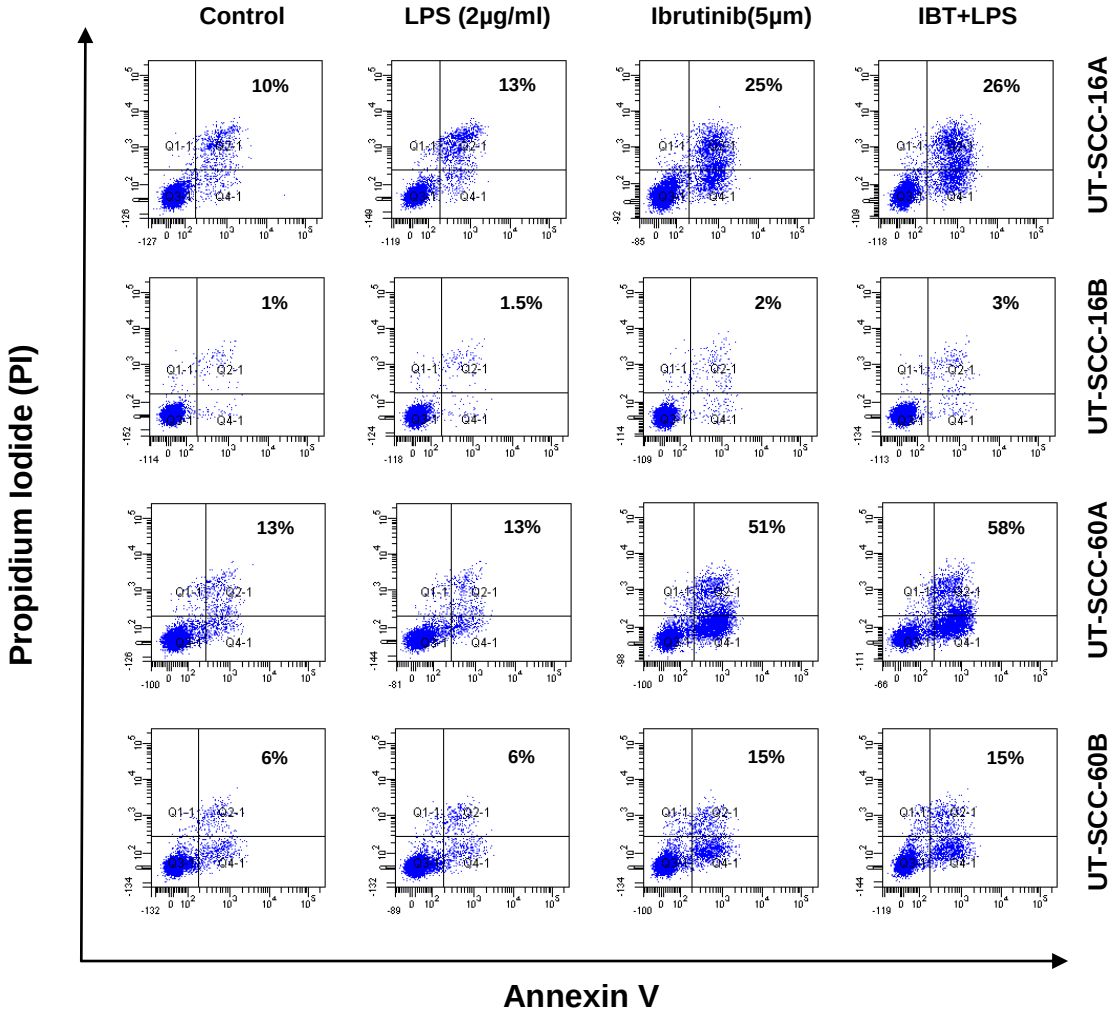


Figure 25. Dose- and time- dependent effects of Ibrutinib with LPS on the viability of HNSCC cells determined by MTT assay. No significant reduction of cell viability was noticed in combined treatment with IBT+LPS than IBT treatment alone. The MTT absorbance at 570-690nm was used to determine the viability of cells and the graphs were represented as mean and standard deviation from three independent experiments. *P ≤ 0.05, **P ≤ 0.01, compared to untreated control and analyzed using paired student's t-test.

Results from apoptosis assay:

(A)



(B)

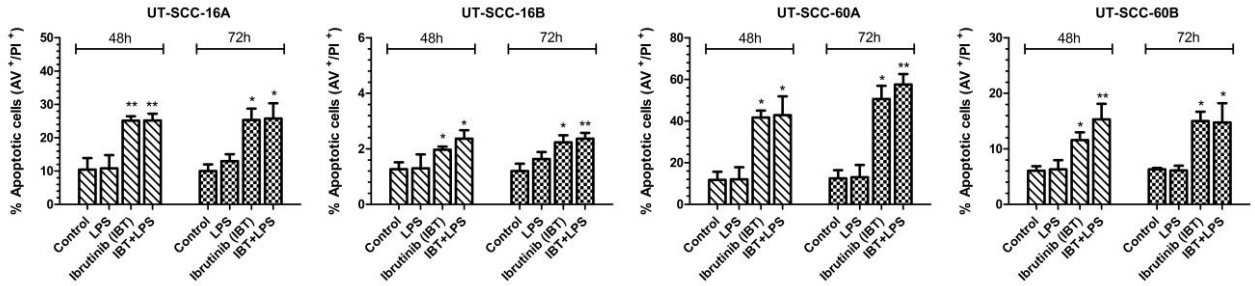


Figure 26. Effect of Ibrutinib with LPS on the apoptosis of HNSCC cells, representing very little effect in the induction of apoptosis in combined treatment with IBT+LPS than that of LPS or IBT alone. (A) Annexin V/PI double staining assay of cells incubated with or with our Ibrutinib and Poly (I:C). (B) Statistical analysis indicating the percent of apoptotic cells in response to treatment. The mean data of each condition were the results of three independent experiments and represented as mean with standard deviation. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 compared to untreated control and analyzed using paired student's t-test.

4.7. Migration analysis in response to Ibrutinib co-treatment with TLR agonists

As indicated in section 4.1.5, malignant HNSCC cells were found to contribute continuous migration in response to TLR3 and TLR4 stimulation. Therefore, to analyze if Bruton's tyrosine kinase (BTK) is a common molecule that is involved in promoting this mechanism, wound healing assay was performed to study the migration of UT-SCC-60A and -60B cells in the presence of BTK inhibitor Ibrutinib (IBT) alone and in combination with TLR3, TLR4 ligands respectively. The level of migration occurred in response to the treatment at different time intervals (0h, 12h, 24h, 48h and 72h) was monitored and compared to the controls (untreated cells).In Figure 27, 28 the results from UT-SCC-60A cell line were represented in respect to TLR3 and TLR4 stimulation.

After 12h treatment, the control cells and the cells treated with either Poly (I:C) or LPS were found to migrate by reducing the width of cell-free gap. In Ibrutinib treated cells there was a clear cell-free gap indicating strong inhibition of HNSCC cell migration, and similar effect was also noticed in combined treatment with Ibrutinib and TLR ligands (Poly(I:C), LPS). After 48h, few cells were observed to start migrate in IBT treated cells and the average area closure achieved in combined of IBT with LPS was rational and comparable to control cells. Whereas, in cells treated with IBT and Poly (I:C) together, a clear inhibition was noticed even after 72h. These results indicate that BTK is a key mediating molecule that strongly contributes to the TLR3 and TLR4 induce migration in malignant HNSCC cells.

Migration of HNSCC cells in response to Ibrutinib co-treatment with Poly (I:C)

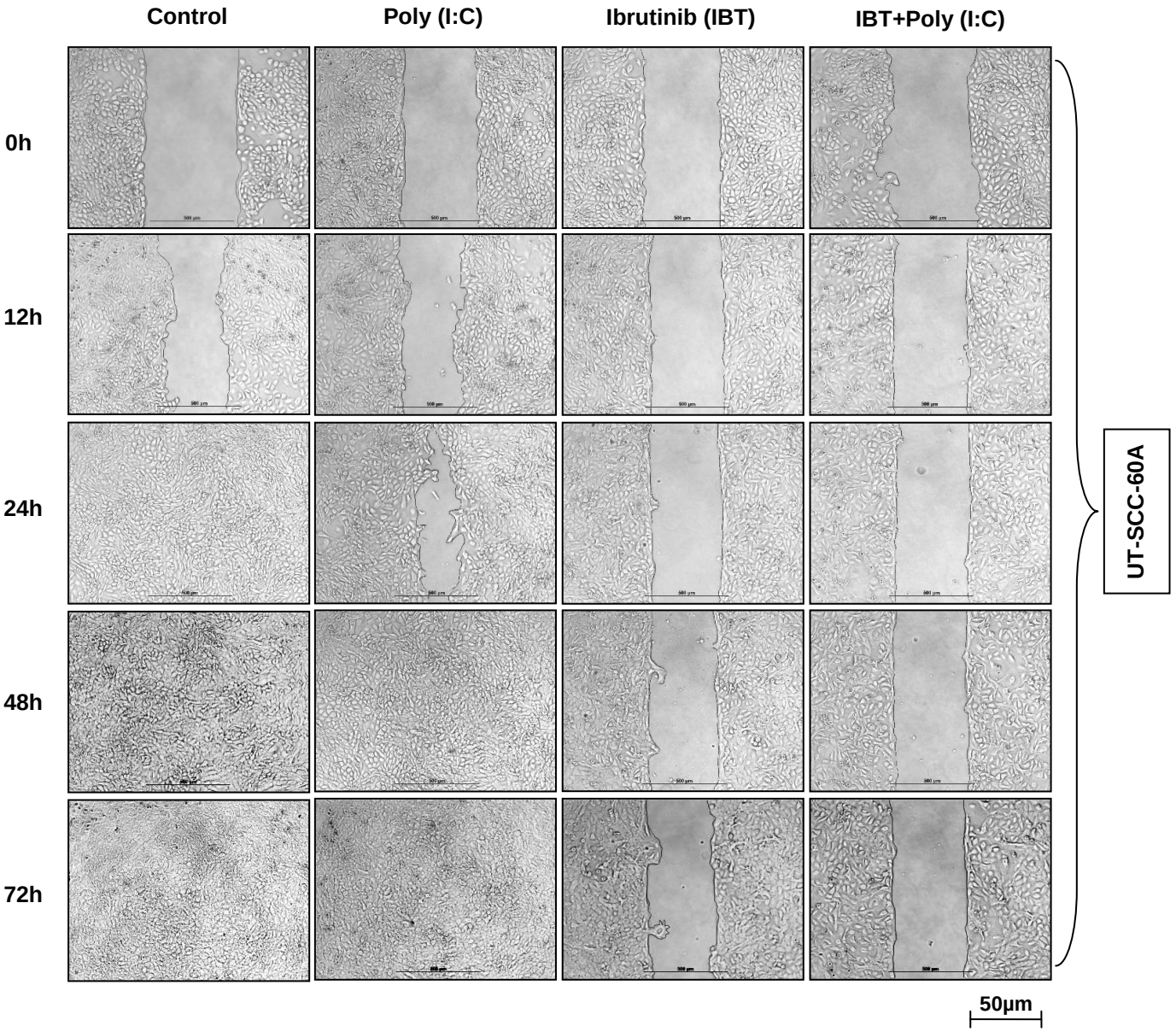


Figure 27. Wound healing assay for analysing the level of migration of UT-SCC-60A cell line treated with BTK inhibitor Ibrutinib (IBT) alone and in combination with TLR3 agonist Poly (I:C) at different time intervals 0h,12h,24h,48h and 72h. Results obtained from three independent experiments, and captured using the bright field mode on Axiovert 200M fluorescence microscope (50µm scale bar). A significant inhibition of migration was noticed in combined treatment than that of control (untreated) cells or Poly (I:C) treatment alone.

Migration of HNSCC cells in response to Ibrutinib co-treatment with LPS

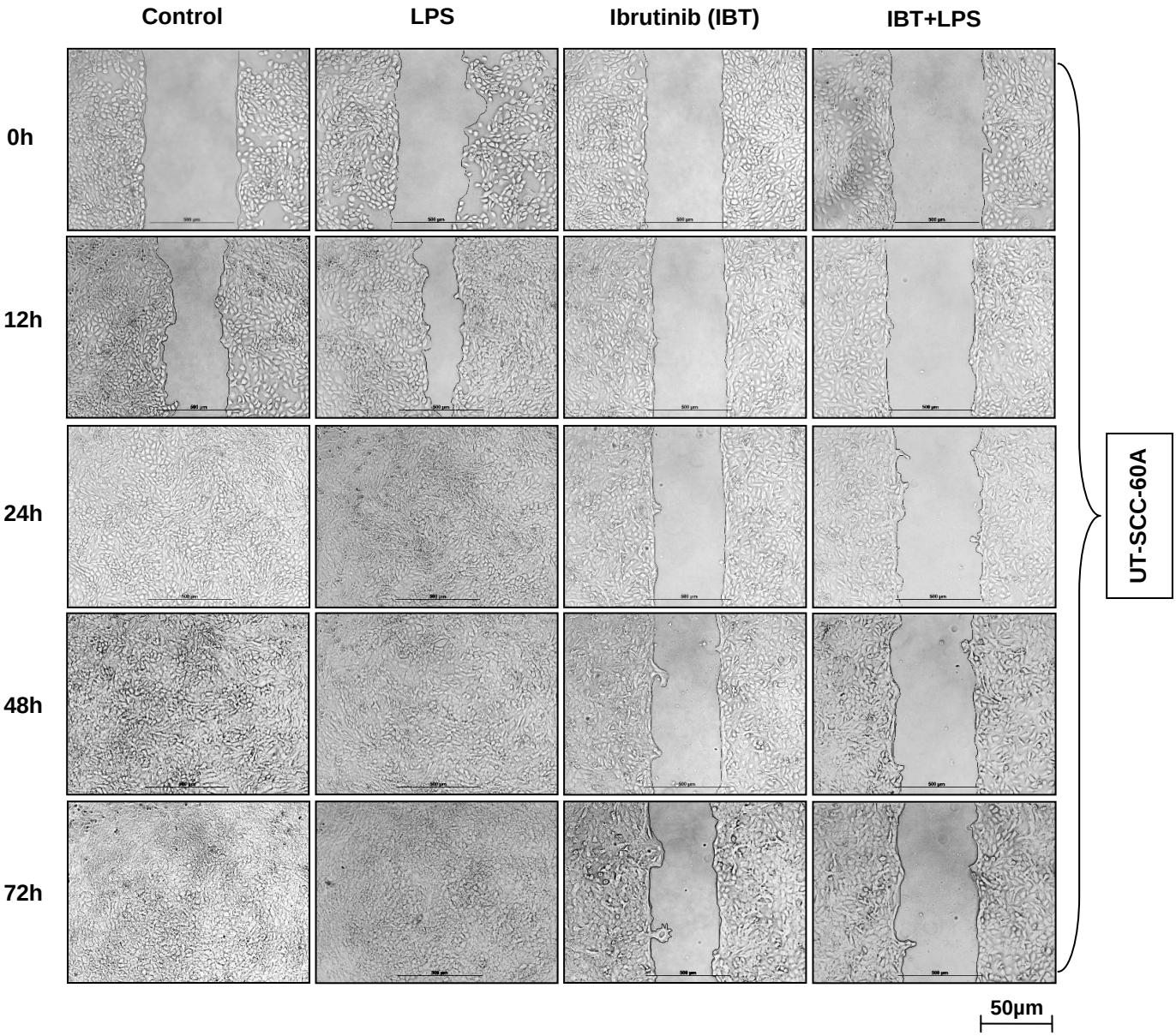


Figure 28. Wound healing assay for analysing the level of migration of UT-SCC-60A cell line treated with BTK inhibitor Ibrutinib (IBT) alone and in combination with TLR4 agonist LPS at different time intervals 0h,12h,24h,48h and 72h. Results obtained from three independent experiments, and captured using the bright field mode on Axiovert 200M fluorescence microscope (50µm scale bar). A significant inhibition of migration was noticed in combined treatment than that of control (untreated) cells or LPS treatment alone.

5. Discussion

HNSCC is one of the most frequent and aggressive malignancy worldwide. It uses diverse immuno suppressive strategies (Duray*, et al.*, 2010) to activate high incidence of locoregional recurrence or distant metastasis (Carvalho*, et al.*, 2005) leading to poor prognosis and has limited the overall survival rate of the patients (Chin*, et al.*, 2005). Growing evidences on inflammation at the tumor-microenvironment support its strong association with tumor progression by fostering several molecular mechanisms through immune receptors like TLRs (Bhatelia*, et al.*, 2014; Wang*, et al.*, 2014). Beside the known mechanisms of TLRs in active innate immunity, they are also found as a major sensors to induce prolonged inflammation, immunosuppression and tumor progression in various cancer cell types (Goto*, et al.*, 2008; Gray*, et al.*, 2006; He*, et al.*, 2007; Meyer*, et al.*, 2011; Xie*, et al.*, 2007; Yoneda*, et al.*, 2008). Extended knowledge on the molecular networks that regulate the immune response in the tumor microenvironment may enable the identification of novel therapeutic targets that would keep a check to the immune evasion strategies and control the tumor progression of HNSCC. Recently Bruton's Tyrosine Kinase (BTK) has emerged as a significant molecule involved in TLR signaling (Gray*, et al.*, 2006; Jefferies*, et al.*, 2003; Lee*, et al.*, 2012) In the present study, attempts were made to discover the critical role of BTK in TLR3 and TLR4 signaling and inflammation in malignant HNSCC cells. Here the potent inhibitor of BTK, Ibrutinib as a candidate pharmacological molecule was used to study the HNSCC cell behavior in the presence and absence of TLR agonists. To the best of our knowledge, the study presented here may be the first report in this regard.

5.1. TLRs as key players in inflammation associated cancer

TLRs, the most evolutionarily conserved receptors regulating the immune function, has been extensively studied in the last several years. Although the roles of TLRs are well described in immune defense mechanisms, growing evidences indicate that major chronic inflammatory diseases are associated with TLRs, leading to cancer development (Balkwill*, et al.*, 2004; Wolska*, et al.*, 2009). It was found that TLRs are not only expressed by immune cells, but also by several cancer cells and the TLR downstream signaling molecules are often involved in the tumorigenic inflammatory

responses (So*, et al.*, 2010). From the earlier studies, it was evident that various molecular patterns released from necrotic cancer cells or adjacent injured epithelial cells, act as PAMPS and promotes aberrant TLR activation during tumor expansion leading to prolonged inflammation, activation of host immune escape mechanisms, anti-apoptotic activity and cancer progression (Fukata*, et al.*, 2007; Goto*, et al.*, 2008; He*, et al.*, 2007; Ilvesaro*, et al.*, 2007; Kelly*, et al.*, 2006; Kim*, et al.*, 2008; O'Neill, 2008b; Rich*, et al.*, 2014; Xie*, et al.*, 2009; Yoneda*, et al.*, 2008). In HNSCC, it was found that there has been an increased expression of TLR3 (Pries*, et al.*, 2008; Xie*, et al.*, 2007) and TLR4 (Szczepanski*, et al.*, 2009). And it was also found that HNSCC cells constitutively activate the transcription factor NF-κB (Meyer*, et al.*, 2011) leading to active TLRs-induced inflammation, immune escape mechanisms and tumorigenesis. (Szczepanski*, et al.*, 2009). In agreement, we found constitutive expression of receptors TLR3, TLR4, their downstream signaling molecules MYD88, TRIF and the TLR-induced expression of inflammatory cytokines Il-1β, IL-6, IL-8, TNF-α and IFN-β in malignant HNSCC. We also found that TLR3 and TLR4 stimulation drives migration of malignant HNSCC cells. Thus these results aid in hypothesising that activation of TLR3 and TLR4 are involved in inducing tumorigenic inflammatory responses and the progression of malignant HNSCCs. Contemporaneous results from other studies reports that the TLR3 activation on head and neck cancer as pro-tumorigenic by enhancing tumor invasion and metastasis through cell migration (Chuang*, et al.*, 2012).

5.2. Cell survival and proliferation of HNSCC is associated with BTK activity

Out of numerous crossroads of cell signaling pathways, BTK has been coined as an essential activator downstream molecule of several receptors thereby involved in diverse signaling cascades and cellular processes such as regulation of B-cell proliferation, apoptosis, differentiation and inflammation (Bolen, 1993; Khan*, et al.*, 1995). Most studies were carried on BTK in BCR signaling pathway which is critical for B-cells and hence considered as a promising target for B-cell malignancies (de Rooij*, et al.*, 2012). From the present study, it became evident that BTK was constitutively

expressed and has been active (pBTK) in different malignant HNSCC cell lines. It appeared to be critical for the tumor cell proliferation and survival of HNSCCs.

Hence, to understand the role of BTK in HNSCC cell proliferation and survival, a selective molecular inhibitor Ibrutinib was used, which covalently binds to a cysteine residue (Cys-481) leading to irreversible inhibition of BTK enzymatic activity (Cameron*, et al.*, 2014; Honigberg*, et al.*, 2010). In line with the evidence from the studies on B-cell malignancies, Ibrutinib has been known to inhibit BCR-signaling, chemokine controlled adhesion and migration in chronic lymphocytic leukemia (CLL) (de Rooij*, et al.*, 2012). In Similar effects were reported in mantle cell lymphoma (MCL) (Chang*, et al.*, 2013). Also reports on acute myeloid leukemia (AML) showed that Ibrutinib effectively inhibits blast proliferation (Rushworth*, et al.*, 2014). In malignant HNSCC cells, upon Ibrutinib treatment we observed reduced gene levels of PGF, MAP2K3, both of which are selectively associated with tumor invasion and progression. In human gliomas and breast tumors, an upregulation of MAP2K3 was found to be involved in invasion and progression (Demuth*, et al.*, 2007). Its expression was shown to be regulated by mutant p53 through involvement of NF-κB, thereby inducing proliferation and survival of diverse human tumor cells (Gurtner*, et al.*, 2010). Similarly, in Ewing sarcomas, G-protein coupled receptor 64 (GPR64) was found to promote invasiveness and metastasis through expression of placental growth factor (PGF) and matrix metalloproteinase (MMP) 1 (Richter*, et al.*, 2013). It is also known to enhance breast cancer cell motility by mobilising ERK1/2 phosphorylation (Taylor*, et al.*, 2010) and the inhibition of PGF activity reduces severity of inflammation in cirrhotic mice (Van Steenkiste*, et al.*, 2011). In addition we also found over expression of HMOX-1, which is known to inhibit the xenograft tumor growth and tumor cell migration in hepatocellular carcinomas (Zou*, et al.*, 2011), induce apoptosis and suppress tumor proliferation and invasion in the breast cancer cells (Hill*, et al.*, 2005; Lee*, et al.*, 2014; Lin*, et al.*, 2008).These findings suggest that presumably, BTK is associated in regulating malignant HNSCC cell survival, proliferation and also cell invasion and metastasis.

Our further examinations on malignant HNSCC in response to Ibrutinib treatment provided significant evidence that BTK mediates tumor cell survival and proliferation. In support to the earlier observations, Ibrutinib treatment has suppressed the tumor

cell viability and tumor cell proliferation in dose dependent manner. The enhanced expression of BCL2L11/Bim, and FasL genes in response to Ibrutinib treatment observed in the present study would also support the earlier statement, as these genes were either known to inhibit tumor progression or induce apoptosis in many cancer types. BCL-2 like 11 (BCL2L11/BIM) is a proapoptotic member that activates the intrinsic apoptotic signaling (Youle*, et al.*, 2008). It has emerged as a key modulator of apoptosis for many cancer types and the deficiencies in BCL2L11 expression result in targeted therapy resistance (Faber*, et al.*, 2012). FASL is a type-II transmembrane protein which is known to induce a caspase-mediated apoptosis in many cell types upon binding with Fas receptor (Zhao*, et al.*, 2012). In human lung cancer cells, it was reported that demethoxycurcumin (DMC) induces apoptosis via promoting the expression of FASL and Fas and inhibits cell growth (Ko*, et al.*, 2015). Similarly in wild type and long-term estrogen deprived (LTED) breast cancer cells; estradiol (E2) induces apoptosis by increasing the transcriptional activity of FoxO3 which was in turn demonstrated by upregulation of FoxO3 target genes FASL and BCL2L11/Bim. (Chen*, et al.*, 2015). Therefore, our findings demonstrated that BTK plays an important role in malignant HNSCC cell survival, proliferation and a wide range of cellular processes. Inhibition of BTK induces apoptosis and it was likely through the activation of BCL2L11/Bim and FasL genes in malignant HNSCC cells.

5.3. BTK regulates TLR induced inflammation in HNSCC

Previous investigations on BTK reported its apparent involvement in regulating NFκB activation (Petro*, et al.*, 2001) and innate immune responses (Gagliardi*, et al.*, 2003) via multiple receptors including the TLRs (Jefferies*, et al.*, 2003; Lee*, et al.*, 2012; Liljeroos*, et al.*, 2007). Based on earlier studies, there are reports stating the potential role of TLR3 and TLR4 in immune escape mechanisms in HNSCC. Hence we further analyzed the role of BTK in TLR signaling in malignant HNSCC cells in detail (Szczepanski*, et al.*, 2009; Xie*, et al.*, 2007). Although Ibrutinib is a highly potent and specific target for BTK, so far no investigations have utilized Ibrutinib as a tool to understand the role of BTK in TLR synergy. Here we showed that Ibrutinib treated malignant HNSCC cells had defective production of proinflammatory cytokines IL-1β, IL-8 and TNF-α in responses to extracellular and intracellular Poly (I:C), as it can be

recognized predominantly by TLR3 when added directly to the culture medium (naked) or as liposome complexed Poly (I:C) (transfected). Our finding corroborate recent data indicating that BTK is critical for TLR3 signaling and is required for the secretion of inflammatory cytokines in macrophages (Lee*, et al.*, 2012).

Consistent with other reports from XID mice (BTK defective), which shows impaired secretion of LPS induced IL-1β and TNF-α by macrophages/monocytes (Doyle*, et al.*, 2002; Gray*, et al.*, 2006; Horwood*, et al.*, 2006; Jefferies*, et al.*, 2003; Mukhopadhyay*, et al.*, 2002), we found that Ibrutinib treated malignant HNSCC cells showed reduced production of LPS-induced proinflammatory cytokines IL-1β, TNF-α and IL-8 expression. Similar effect was noticed in response to Poly (I:C) induction as well. In contrast, the expression of IL-6 in either Poly (I:C) or LPS-stimulated HNSCC cells treated with Ibrutinib did not show any significant down regulation indicating that BTK is not required for the TLR-induced IL-6 expression which correlate well with the observations performed on XLA PBMCs (Horwood*, et al.*, 2006). Taken together the present results indicate that Ibrutinib has strong influence on TLR singling indicating the critical role of BTK in driving TLR3 and TLR4 induced inflammatory process in malignant HNSCC cells. However, the role of BTK in induction of inflammation through cytokines is selective, as it is does not have any apparent effect on IL-6 expression. These results would also suggest that BTK acts through a common signaling mechanism, apparently through activation of NFκB (Mukhopadhyay*, et al.*, 2002) leading to enhanced TLR3 and TLR4 triggered production of proinflammatory cytokines in malignant HNSCC cells.

5.4. TLR induced MAPK signaling is dependent on BTK activation

To further extend our analysis on mechanisms of BTK with regards to the aspects of TLR biology and production of proinflammatory cytokines in HNSCC cells, the influence of BTK on the activation of MAPKs was determined. Here we found rapid increase in the phosphorylation of three major MAPK: the ERK1, the JNK and the P38 when stimulated with Poly (I:C) or LPS in malignant HNSCC cells. Earlier studies on mouse macrophages and RAW 264.7 cells demonstrates that Poly (I:C) induces activation of the ERK, JNK, and p38 which regulates COX-2 expression (Steer*, et al.*,

2006), while in human monocytes, LPS was found to activate ERK1/2 pathway which regulates TNF-α expression (Guha*, et al.*, 2001) and production (Shinohara*, et al.*, 2005). Further we noticed significant reduction in the phosphorylation of ERK1/2 and JNK MAPK in HNSCC cells primed for TLR3 and TLR4 signalling, when BTK was inhibited using Ibrutinib indicating direct influence of BTK for active TLR mediated signaling and IL-1β and TNF-α cytokine production. A similar observation on ERK1/2 was reported in BCR signaling in BTK deficient DT40 cells (Jiang*, et al.*, 1998), and also in TREM-1/DAP12 signaling in BMDCs from BTK deficient mice, PBMCs from XLA patients (Ormsby*, et al.*, 2011). It was also well demonstrated that active JNK pathway induces pro-survival effects on LPS-induces activation of microglial BV-2 cells (Svensson*, et al.*, 2011). In contrast, we found increase in the phosphorylation of P38 MAPK which could be a control mechanism to regulate the TLR ligand mediated cytotoxicity and induce cell death as indicated by Pisegna (Pisegna*, et al.*, 2004) in human NK cells. This findings correlate well with reports on human monocytes/macrophages suggesting the involvement of BTK in the activation of LPS induced P38 MAPK (Horwood*, et al.*, 2006). Together, our results demonstrate that BTK is a positive regulator of active TLR3 and TLR4 signaling and activation of MAPKs. Inhibition of BTK in TLR signaling also suggests improvement in the pro-apoptotic effect on HNSCC cells.

5.5. BTK empower TLR-induced tumorogenesis in HNSCC

Besides TLR induced inflammation, their aberrant expression also involved in diverse signaling elements and other mechanisms, implying tumor growth and resistance to apoptosis, treatment resistance and immune evasion and tumor recurrence (Kelly*, et al.*, 2006; Rich*, et al.*, 2014). In line, the levels of TLR3 and TLR4 expression in prostate cancer cells levels were highly associated with tumor recurrence (Gonzalez-Reyes*, et al.*, 2011). So far, the explicit mechanism involved in HNSCC cell resistance to TLR induced cell survival, migration and apoptosis remained unclear. In this context, we hypothesize that BTK could possibly involved in regulating the anti-tumor strategies induced by TLR stimulation. Our findings provide novel insight into the regulation of pro-tumor strategies induced by TLR3 and TLR4 agonists. Ibrutinib with TLR agonist showed reduced cell viability and also inhibits the tumor cell migration

indicating that BTK is a selective molecule required for the tumor cells to obtain TLR induced pro-survival mechanisms and tumor migration. Correlating to our findings it was shown that Ibrutinib inhibits BCR- and chemokine- mediated adhesion and migration of mantle cell lymphoma patients (Chang*, et al.*, 2013) and also stromal derived factor 1 (SDF1) mediated migration by inhibiting AKT and MAPK activation in human acute myeloid leukemia (Zaitseva*, et al.*, 2014) and chronic cell lymphoma (de Rooij*, et al.*, 2012). In addition, we also noticed increase in apoptotic levels of malignant HNSCC cells in response to co-treatment with Poly (I:C) which is well supported by the earlier reports on Ibrutinib combined treatment with ACY1215 and a selective histone deacetylase 6 (HDAC6) in MCL tumor cell lines (Vij*, et al.*, 2012) similarly in a study conducted using Ibrutinib plus bendamustine and rituximab (BR) for the treatment in relapsed/refractory CLL patients also showed a profound clinical response (Brown, 2012) Our findings portray that combined treatment with Ibrutinib and Poly (I:C) induces more apoptosis demonstrating BTK as a key molecule involved in promoting anti-apoptotic resistance upon TLR activation, predominantly in response to Poly (I:C) in malignant HNSCC cells. Moreover, we found no rise in the level of apoptosis in Ibrutinib co-treatment with LPS suggesting feeble effect of BTK on TLR4 signaling. This could possibly due to the dual function of BTK either by inhibiting Fas-activated apoptosis and functioning as a pro-apoptotic molecule by down-regulating the anti-apoptotic activity of STAT3 transcription factor which was observed in B-cells (Uckun, 1998), or the LPS mediated resistance to apoptosis is independent of BTK. However, the precise mechanism is unclear and requires further investigations. Therefore, the contribution of BTK seems to have a differential role in TLR induced resistance towards cell viability and apoptosis, most likely in TLR3 and TLR4 but strongly inhibits the tumor cell migration in HNSCC.

6. CONCLUSION AND PRESPECTIVES

Taken together, the present data evidence that BTK is activated in human malignant HNSCC cells *in vivo*. Its function is found to be crucial in diverse signaling cascades implying cell survival, progression and migration. Inhibition of BTK by a clinically potent inhibitor, Ibrutinib, prompts therapeutic response in malignant HNSCC cells and is efficacious in altering the cell viability and proliferation. It also promotes the apoptosis. In addition, BTK function is also found to be effective in modifying the genes that are associated with tumor cell invasion and metastasis.

The inhibition of BTK by Ibrutinib leads to reduced TLR3- and TLR4-induced production of IL-1β, TNF-α and IL-8 expression indicating the crucial role of BTK in TLR induced inflammation. These results also provide support for BTK being a common signaling mechanism for TLR3 and TLR4 induced proinflammatory cytokines. However, the mechanism of action of Ibrutinib seems selective, as the role of BTK was not effective on IL-6 expression. It was also found that activation of BTK regulates the phosphorylation of ERK1/2, JNK MAPK upon TLR3 and TLR4 stimulation. Therefore, our results demonstrate that inhibition of BTK activity impairs TLR signaling which presumably associates with the aberrant signaling mechanisms leading to malignant HNSCC cell progression and recurrence.

Further, in pursuit of co-treatment with Ibrutinib and TLR agonists indicate that BTK as a prominent immuno modulatory molecule involved in TLR-induced resistance towards apoptosis, tumor progression and migration especially in TLR3. Hence, therapeutic targeting of BTK *in vivo* as a selective mediator of TLR signaling would provide an important insight in malignant HNSCC biology. It would also provide a promising and highly efficacious combined therapeutic approach of malignant HNSCC.

Finally to achieve better therapeutic regimens for HNSCC in future, it is more important to understand the adverse immunosuppressive mechanisms. In malignant HNSCC cells, apart from the illustrated immuno-modulatory role of BTK in TLR3 and TLR4 synergy, further understandings on the BTK influenced cytokine functions in HNSCC cells could provide the sequence of events leading to tumor progression, migration, treatment resistance and more immunosuppressive mechanisms. It is also

worthwhile to study the role of BTK in other TLR induced responses. Moreover, it is of prime importance to execute interaction studies in experimental models, which could provide thorough understandings of the BTK role in complex mechanisms of HNSCC tumor biology. And the similar studies in co-treatment with TLR agonist to make a promising and highly efficacious combined therapy, that could possibly resulting in greater benefit for HNSCC patients.

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GERMAN SUMMARY

Maligne Kopf-Hals Tumore (Head and Neck Squamous Cell Carcinoma, HNSCC) gehören zu den häufigsten und aggressivsten Karzinomen weltweit. In den meisten Fällen haben die Patienten mit HNSCC eine schlechte Prognose und Überlebensrate, insbesondere auch aufgrund der Metastasierung und der immunsuppressiven Strategien des Tumors. Toll like Rezeptoren (TLRs) sind zentrale Regulatoren verschiedener Immunfunktionen, wobei jedoch im Tumormilieu abweichende Funktionen hinsichtlich der Regulation von Entzündungsprozessen und der Tumor Progression vermutet werden. Die zugrunde liegenden Mechanismen und Charakteristika dieser möglichen dualen Funktionen sind nach wie vor nicht verstanden. In diesem Zusammenhang wurde die Bruton's Tyrosin Kinase (BTK) zunehmend als wichtiger Mediator innerhalb der TLR Signalkaskaden wahrgenommen. In malignen B-Zell Erkrankungen wurde der BTK Inhibitor Ibrutinib bereits erfolgreich eingesetzt. In HNSCC ist die Bedeutung der BTK für die Regulation TLR abhängiger Biosynthesewege bislang nicht bekannt. Im Fokus dieser Arbeit stand die umfassende Untersuchung der Bedeutung der BTK für die Regulation der TLR3- und TLR4-induzierten Biosynthese des Mikromilieus und der Tumorprogression in HNSCC unter dem Einfluss des Inhibitors Ibrutinib (IBT). *In vitro* Analysen verschiedener permanenter HNSCC Zelllinien zeigten eine konstitutive Expression und Aktivierung der BTK. Eine Inhibierung der BTK durch IBT führte zu deutlichen Veränderungen der Expressionslevel verschiedener Tumor-relevanter Gene im Zusammengang mit Proliferation, Migration und Apoptoseregulation. Des Weiteren führte die BTK Inhibierung zu einer signifikant verminderten Expression der durch TLR3- und TLR4-induzierten proinflammatorischen Zytokine IL-1β, TNF-α und IL-8. Auch zeigte sich eine deutliche Verminderung der TLR-abhängigen Aktivierung der MAP Kinasen ERK1/2 und JNK. Eine kombinierte Inkubation der Tumorzellen mit IBT und dem TLR3 Liganden Poly (I:C) führte zu erhöhter Apoptose und zu einer Reduktion der Zellviabilität und -migration. Zusammenfassend geben die Ergebnisse dieser Arbeit neue Erkenntnisse in die komplexe Bedeutung der BTK in Bezug auf die TLR3- und TLR4-abhängigen Biosynthesewege der Tumorprogression. Somit deuten die dargestellten Ergebnisse darauf hin, dass BTK ein vielversprechendes Zielprotein therapeutischer Behandlungsansätze bei Patienten mit malignen Kopf-Hals Tumoren sein könnte.

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I dedicate this work at the lotus feet of the Lord