1. INTRODUCTION

Head and neck cancers are the seventh most common cancers worldwide with head and neck squamous cell carcinomas (HNSCCs) being the most common histologies among them. These develop in the mucosal epithelium of the mouth, nose, pharynx and larynx (Cohen *et al.*, 2018; Sun *et al.*, 2022). Asia contributes for 58% of HNSCCs worldwide with South Asian countries accounting for a major proportion (Khosla *et al.*, 2021). In India, HNSCCs account for 30% of all the cancers countrywide (Mishra *et al.*, 2021).

Predominant risk factors involved in the generation of HNSCCs are alcohol consumption, cigarette smoking, drinking, betel quid chewing, inadequate diet, infection by Human papillomavirus (HPV) and Epstein Barr virus (EBV). Apart from this, genetic mutations inactivating the tumor suppressor genes and over-activating the tumor promoter genes that leads to proliferation and migration of cells also contribute to the development of HNSCCs (Sun *et al.*, 2022).

Treatment of head and neck cancer requires a multidisciplinary approach including surgery, radiotherapy and chemotherapy/systemic therapy (Cognetti *et al.*, 2008). Conventional chemotherapeutics used for the treatment of HNSCC are docetaxel (T), cisplatin (P) and 5-FU (F). A combination of these three drugs, known as the TPF-triplet regimen, for the treatment of HNSCC has also been approved by the Food and Drugs Administration (FDA) in 2006 (Blasco *et al.*, 2017). Resistance to these chemotherapies is still significant indicating its inefficacy in HNSCC and subsequently leading to local recurrence of the cancer (Sher *et al.*, 2016). Cetuximab, a monoclonal antibody against Epidermal growth factor receptor (EGFR) is an FDA approved (in 2011) immunotherapy for HNSCC in combination with cisplatin/5-FU, but is also associated with limited efficacy (Cohen *et al.*, 2013; Alsahafi *et al.*, 2019). In 2019, FDA has also approved pembrolizumab (anti-PD-1 antibody) in combination with cisplatin/5-FU for the treatment of HNSCC (Cohen *et al.*, 2019). This highlights the limited therapeutic options available apart from an urgent need to develop effective therapies and discovering clinically relevant biomarker for early patient stratification and diagnosis of the diseases.

Toll-like receptors (TLRs) are Pattern-recognition receptors (PRRs) conventionally expressed on immune cells and a main component of the innate immunity. These receptors recognize pathogen associated molecular patterns (PAMPs) such as Lipopolysaccharide (LPS),

Zymosan and Flagellin from microbes, and damage associated molecular patterns (DAMPs) such as heat shock proteins (HSPs), Fibronectin and high mobility group box 1 protein (HMGB1) released from stressed, dying or dead cells. Till date, 10 functionally active TLRs in human (TLRs 1-10) and 12 TLRs (TLRs 1-9,11-13) have been identified in mice (Isaza-Correa *et al*., 2014; Schaefer 2014; El-Zayat *et al.*, 2019). Activation by PAMPs and DAMPs induces the downstream signaling cascade of the TLR signaling pathway, leading to the production of cytokines and inflammatory mediators (Kawai & Akira, 2006).

The TLR pathway comprises of adaptor molecules that play a key role in the signaling. Activated TLRs transfers signal to the Myeloid-differentiation primary response 88 gene (MyD88), the earliest adaptor molecule common to all TLRs, except TLR 3. Activation of MyD88 by the TLRs leads to the recruitment and activation/phosphorylation of the family members of Interleukin-1 receptor associated kinases (IRAKs) forming the Myddosome complex. This event allows IRAKs to further activate TNF receptor–associated factor 6 (TRAF6) and Transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1). TAK1 then mediates the activation of the Mitogen-activated protein kinases (MAPK), the nuclear factor-kappa B (NF-κB) pathway and Interferon regulatory factors (IRFs) leading to the induction of the pro-inflammatory gene expression. Almost all TLRs transduce downstream signaling through the MyD88 protein, except TLR 3 that completely, and TLR 4 that partially utilizes TIR-domain-containing adapter-inducing interferon-β (TRIF) for MyD88 independent signaling. TRIF further activates TRAF3 and IRFs that induces the secretion of Type 1 and Type 3 interferons **(Figure 1.1)** (Kawasaki & Kawai, 2014; Farooq *et al.*, 2021).

Downstream molecules-Interleukin-1 receptor associated kinases (IRAKs) are serine-threonine kinases, key signal transducers and critical components of the TLR and Interleukin 1-receptor (IL-1R) signaling pathway. The structure of IRAKs consists of a Proline-Serine-Threonine domain and a kinase domain between an N-terminal and a C-terminal domain (Jain *et al.*, 2014). The family of IRAK proteins comprises of 4 members – IRAK-1, IRAK-2, IRAK-3/M and IRAK-4. IRAK-1 and IRAK-4 are the only active kinases amongst all and are known to positively regulate the TLR signaling pathway. IRAK-2 and IRAK-3/M are pseudo-kinases, lacking catalytic activity. IRAK-3/M is also reported to negatively regulate the TLR signaling pathway (Singer *et al.*, 2018).

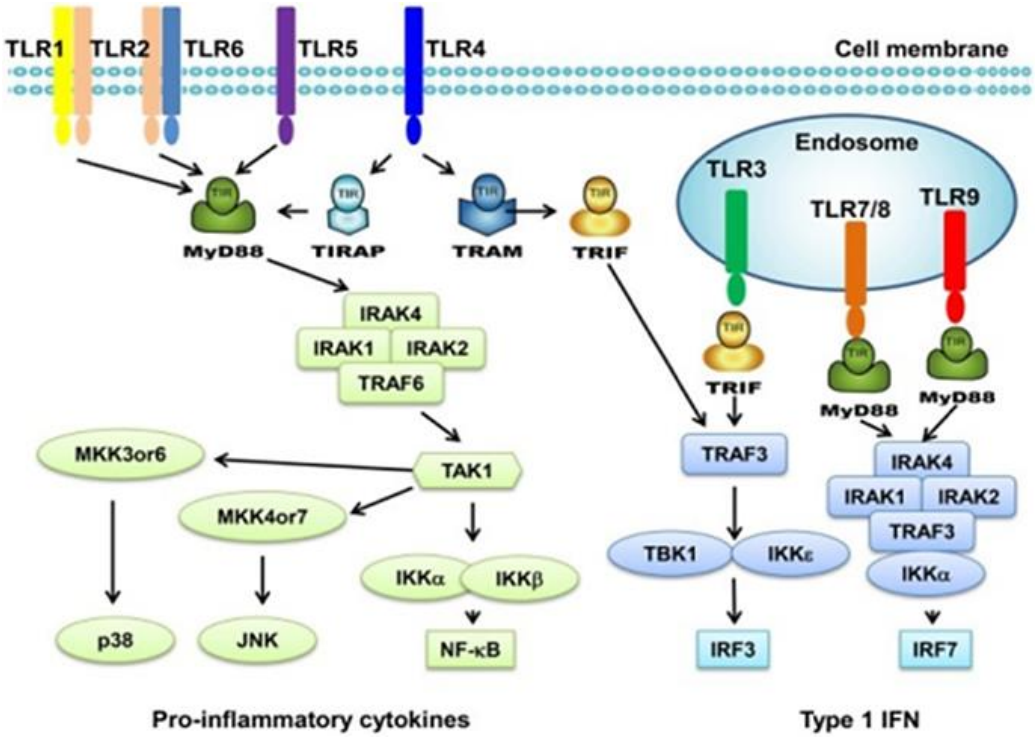

Figure 1.1: Toll-like receptor signaling pathway. TLRs signal through MyD88 dependent and MyD88 independent pathway involving various adaptor molecules **(**Source: El-Zayat *et al.*, 2019)

Considerable number of studies suggests aberrant signaling of TLRs is associated with the development of diseases like autoimmune disorders, chronic inflammation, infectious diseases and cancer (El-Zayat *et al.*, 2019). Recent studies indicate expression of TLRs on cancerous cells, apart from their regular expression on immune cells. Over-expression of the TLRs could lead to dysregulated downstream signaling leading to the generation of cytokine storm in the tumor microenvironment (Sato *et al.*, 2009). Signaling of TLRs could further worsen the cancerous state by promoting tumor progression, metastasis and drug resistance as observed in gastric (Schmaußer *et al.*, 2005; West *et al.*, 2017), melanoma (Goto *et al.*, 2008), breast (Xie *et al.*, 2009) and many other cancers (Merrell *et al.*, 2006). IRAK-1 and IRAK-4 also contribute significantly in the development of diseases mediated by the dysregulated TLR signaling. Development and progression of autoimmune diseases and cancer have been linked to the upregulation and increased phosphorylation of IRAK-1 and IRAK-4. Upregulation of IRAK-1 and IRAK-4 enhancing pro-tumor responses have been observed in haematological malignancies such as Myelodysplastic syndrome (MDS), Acute myeloid leukemia (AML) and T-cell acute lymphoblastic leukemia (T-ALL) and in solid tumors such as Triple negative breast cancer (TNBC), Hepatocellular carcinoma (HCC), Colorectal carcinoma (CRC) and many other cancers (Jain *et al.*, 2014; Singer *et al.*, 2018; Bennet and Starczynowski, 2022). In

this context, IRAK-1 and IRAK-4 acts as rational biomarkers and candidate drug targets (Singer *et al.*, 2018).

In HNSCCs, upregulation of TLRs 2, 3, 4 and 9 have been reported and enhance the pro-tumor effects such as proliferation, invasion and production of cytokines (Mäkinen *et al.*, 2016; Szczepanski *et al.*, 2009; Chuang *et al.*, 2018). Further, over-expression of IRAK-1 has been detected in HNSCC patients and expression of IRAK-1 regulated the survival and metastasis of HNSCC cell lines (Adams *et al.*, 2015; Meng *et al.*, 2020). Increased IRAK-1 phosphorylation is reported to contribute to paclitaxel resistance in HNSCC (Liu *et al.*, 2021).

There is a deficiency of data describing the wider role of TLR signaling in the development and progression of pro-oncogenic effects in HNSCC. Through this work, we investigated the state of the TLR signaling in HNSCC by determining the expression and phosphorylation of IRAK-1 and IRAK-4. We also analyzed alterations in their status upon development of chemo-resistance towards the FDA-approved combination of conventional chemo-drugs -the TPF-triplet regimen for HNSCC.

As a number of evidences indicate the role of uncontrolled TLR signaling in the development of cancer, TLRs and TLR-signaling pathway related molecules appear as potential therapeutic targets for the treatment of cancer. In this view, many immunoadjuvant therapies have been designed and developed for the treatment of cancer. These therapies comprise of diverse drug candidates such as TLR agonists and antagonists, blocking antibodies and small molecule inhibitors (Anwar *et al.*, 2019). The limitations of TLR agonists/antagonists is their off-site activity, i.e., targeting and killing of normal cells. This persuades for the need of using specific inhibitors against relevant TLRs and related proteins on cancer. Small molecule inhibitors of IRAK-1 and IRAK-4 have been designed and used for the treatment of sepsis, auto-immune disease and haematological malignancies (Singer *et al.*, 2018; Bennet & Starczynowski, 2022). The benefit of using these inhibitors is that it allows the therapeutic targeting of most TLRs as they converge on the MyD88 dependent signaling pathway. Pre-clinical studies, testing IRAK-1 and IRAK-4 inhibitors in TNBC, CRC and Pancreatic ductal adenocarcinoma (PDAC) have exhibited notable anti-tumor effects (Singer *et al.*, 2018). There is limited data available on the use and effect of IRAK-1 and IRAK-4 inhibitors for the treatment of HNSCC.

In this study, we targeted TLR signaling in HNSCC using a commercially available small molecule IRAK-1 &-4 dual inhibitor. We evaluate the effect of such treatment on a range

of pro-oncogenic features of chemotherapy-naïve and -resistant HNSCC cells as stand-alone therapy as well as in combination with the conventional chemo-drugs. This study can help in designing of targeted therapy for HNSCC along with reducing the toxic side effects mediated by chemo-drugs through an anticipated reduction in effective drug doses used for the treatment of cancer.