Synopsis of the thesis on

Studies On Pro-oncogenic Role Of Toll-Like Receptor Signaling In Head and Neck Cancer

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Introduction

Head and neck cancers are a heterogenous group of malignancies arising in the upper aero-digestive tract. Majority of the head and neck cancers are squamous cell carcinomas (SCCs). Head and Neck Squamous Cell Carcinomas (HNSCCs) are the sixth most common cancer worldwide. In 2018, 890,000 new cases and 450,000 deaths were reported globally [1]. Common risk factors are tobacco, alcohol consumption or Human Papillomavirus (HPV) infection [2]. Early-stage tumors are conventionally treated with surgery and radiotherapy. For advanced tumors, surgery combined with adjuvant radiotherapy/chemoradiotherapy is the standard approach. Commonly used chemotherapeutic agents for the treatment of HNSCCs include Docetaxel, Cisplatin and 5-fluorouracil (5-FU). Patients with advanced or recurrent tumor are treated with combinations of these chemotherapeutic agents [3]. Combination treatments are effective against HNSCC as they show better survival and organ preservation as compared to single agent treatment. However, only a small percentage of patients are responsive to it due to various tumor escape mechanisms adapted by them. These conventional agents have considerable toxicity, hence, the patient survival rate ranges not more than 40-50% [4,5]. There is need of new treatment modalities for HSNCC with better efficacy and reduced toxicity either as standalone drugs or in combination with conventional chemo-drugs.

Pattern Recognition receptors (PRRs) are proteins expressed conventionally on innate immune cells for the detection of Pathogen Associated Molecular Patterns (PAMPs) present on infectious agents and Damage Associated Molecular Patterns (DAMPs) derived from our own cells under stress or death. Upon binding of PAMP/DAMP based ligands to PRRs, the downstream signaling pathways are activated leading to production of various inflammatory cytokines and type I interferons [6, 7]. Toll-Like receptors (TLRs) are the first and best characterized PRRs which detect a wide range of PAMPs and DAMPs based ligands [8]. TLRs (TLR 1- 11) have been identified in humans. TLR 1, 2, 4, 5, 6, and 10 are located on the cell surface while TLR 3, 7, 8 and 9 are located in the endosome. These TLRs detect various PAMP ligands such as bacterial lipopolysaccahride, flagellin, lipoteichoic acids, bacterial or viral DNA, RNA and DAMP ligands such as heat shock protein and high mobility group box-1(HMGB-1) [9,10].

The TLRs engage Toll/IL-1R receptor (TIR) domain which mediates downstream signaling upon TLR activation. TLRs engage TIR adaptors MyD88 (Myeloid differentiation primary response 88) and TRIF **(**TIR-domain-containing adapter-inducing interferon-β**)** which mediate downstream signaling and activation of NF- κB (nuclear factor kappa-light-chain-enhancer of activated B cells), MAPK (mitogen-activated protein kinase) and IRF3 (Interferon regulatory factor 3) pathways. All TLRs except TLR-3, utilizes the MyD88 dependent pathway for signaling. The MyD88 adaptor forms a myddosome complex with two kinases known as Interleukin-1 Receptor Associated Kinase (IRAKs) -1 and -4. Upon myddosome formation, MyD88 activates IRAK-4 which further activates IRAK-1 and auto phosphorylates it. IRAK-1 further activates TRAF-6, which induces activation of transforming growth factor-β-activated kinase 1 (TAK1). TAK1 further activates two pathways: IκB kinase (IKK)-complex-NF-κB pathway and MAPK pathway inducing the expression of pro-inflammatory genes [11,12,13].

Apart from immune cells, TLRs and its downstream signaling proteins have also been shown to be expressed in various types of cancers. Studies suggest their role in tumor progression, growth and chemo-resistance in Triple Negative Breast Cancer (TNBC), melanoma, myeloma and lung cancer [10]. Downstream kinases IRAK 1 and IRAK 4 have been reported to be over expressed in non-squamous cell lung cancer (NSCLC), TNBC, Hepatocellular carcinoma (HCC), melanoma, and colorectal carcinoma (CRC). Over expression of IRAK-1 and IRAK-4 has also been reported to promote tumor proliferation, survival, migration and inflammation in TNBC, HCC, melanoma and colorectal carcinoma and shown to be associated with poor prognosis and survival of patients [14].

Various studies have also highlighted the role of TLR signaling in chemo-resistance of cancers. IRAK-1 activation regulated the production of pro-inflammatory cytokines IL-6, IL-8 and CXCL-1 which were required for mammosphere growth in TNBC. Chemotherapeutic treatment using paclitaxel induced significant IRAK-1 phosphorylation along with production of IL-6, IL-8, CXCL-1 and cancer stem cells (CSCs) formation thus suggesting the role of TLR signaling in acquiring chemo-resistance in TNBC [15]. Treatment with sorafenib induced IRAK-4 phophorylation in HCC resulting in chemo-resistance [16]. Chemotherapy using 5-fluorouracil and oxaliplatin induced acquired chemo-resistance via DNA damage initiating TLR signaling which led to IRAK-4 activation and NF-κB signaling in CRC [17]. Pharmacological inhibition of

TLR signaling has also been tested in solid cancers such as breast cancer, melanoma and HCC. Significant improvement in tumor phenotype along with sensitization of tumor to chemotherapy has been observed [18].

Expression of TLR 2, 3, 4, 5 and 9 have been reported in head and neck cancer of different origins [19]. Expression of IRAK-1 is reported in tongue origin cancer cell lines and its inhibition has been shown to increase apoptosis [20]. There is limited data available on the role of Toll like receptor signaling in progression of HNSCC and its impact on chemo-resistance.

Rationale

Cancer cells release DAMPs in the tumor microenvironment under various conditions like stress, spontaneous or therapy induced cell death etc. These DAMPs can bind to the TLRs present on the head and neck cancer cells, activating downstream signaling. Such signaling event may be advantageous to the cancer cells, imparting pro-oncogenic effects such as increased cell survival, proliferation, metastasis, cancer stem cells (CSCs) formation etc. TLR signaling can also have impact on therapeutic responses and have scope to serve as biomarker of therapeutic resistance or disease progression. If such phenomenon holds true than inhibiting TLR signaling could be an attracting strategy for treating HNSCC.

Aim

To understand the pro-oncogenic role of Toll-Like Receptor Signaling in Head and Neck Cancer

Objectives

1. Expression profile of TLRs 1-10 (TLR 1, 2, 3, 4, 5, 6, 7, 8, 9, 10)
2. Evaluation of constitutive TLR signaling
3. To study the effect of TLR signaling on oncogenic properties
4. To study the role of TLR signaling in chemo-resistance
5. Evaluation of therapeutic potential of IRAKs based TLR inhibitor as combination therapy with conventional chemo-drugs

Experimental system

HEp-2 - Human laryngeal carcinoma cell line which is an epithelial and adherent cell line was used for the study. It was cultured in DMEM (Dulbecco's modified eagle medium) supplemented with 10% FBS (Fetal bovine serum) and a mix of antibiotic/antimycotic in a humidified incubator at 37°C with 5% CO_2.

Results

Objective 1: Expression profile of TLRs

Total RNA was isolated from HEp-2 cells by TRIzol RNA extraction method. The concentration and purity of RNA was checked. RNA was reverse transcribed to cDNA. Quantitative PCR was performed for Human TLRs 1-10. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression was used as a reference for normalization of results. All the TLRs were found expressed on HEp-2 cells at mRNA level, except TLR 2 and 10 (C_t value greater than 35).

Objective 2: Evaluation of constitutive TLR signaling

To know whether the TLRs expressed on HEp-2 are functionally intact and constitutively activated or not, expression of major converging downstream kinases IRAK-1,-4 and their phosphorylation status were evaluated. Intracellular flow cytometry was carried out to detect the expression of IRAKs and p-IRAKs in HEp-2 cells using commercially available antibodies. IRAK-1, IRAK-4 and their respective phosphorylated forms were detectable in HEp-2.

HEp-2 cells expressed IRAK-1 and IRAK-4 but most of the IRAKs were found to be in phosphorylated state. Data suggest that TLR signaling is constitutively ongoing in HEp-2 cells.

Objective 3: To study the effect of TLR signaling on oncogenic properties

To know the effect of TLR signaling on oncogenic properties of HEp-2 cells, TLR signaling was blocked using IRAK-1and IRAK-4 dual inhibitor, which are downstream kinase of the pathway effective against all TLRs except TLR3. Commercially available pharmacological and selective small molecule based inhibitor of IRAK-1 and -4 was used in the study.

HEp-2 Cells were treated with IRAK-1 & -4 dual inhibitor for 72 hours and expression of following markers were evaluated for the listed oncogenic properties-

i. **Cell Survival:** Viability by resazurin dye based colorimetric cell assay

ii. **Proliferation**: Ki-67 (nuclear proliferation antigen) by intracellular flow cytometry

iii. **Cancer stem cells (CSCs) formation**:
-CD44 (cell surface glycoprotein): by flow-cytometry
-Nanog (regulator for embryonic stem cell pluripotency): by intracellular flow-cytometry
-ALDH1(Aldehyde dehydrogenase 1) by Western Blotting

iv. **Metastasis:**
-MMP2 (matrix metalloproteinase 2): by quantitative PCR
-IL-6 (Interleukin-6): by commercial ELISA

v. **Epithelial to Mesenchymal Transition (EMT):**
-E-Cadherin: by flow cytometry
-Vimentin: by flow cytometry

Using cell viability assay, the IC_{50} value of IRAK-1 & -4 dual inhibitor was observed as 21.58 ± 1.77 uM. IRAK-1 & -4 dual inhibitor showed cytotoxic effects on HEp-2 cells suggesting TLR signaling is required for HEp-2 cells survival. No statistically significant change in the expression levels of any of the others markers were observed upon treatment with IRAK-1 & -4 dual inhibitor. Except cell survival, no other above listed oncogenic properties of HEp-2 cancer cells were impacted by inhibition of TLR signaling through IRAKs.

Objective 4: To study the role of TLR signaling in chemo-resistance

Induction chemotherapy by Docetaxel, Cisplatin and 5-FU, commonly known as TPF triplet regimen is used for the clinical management of HNSCC patients. To study the role of TLR signaling in chemo-resistance, an *in vitro* model to mimic chemo-resistance to TPF using HEp-2 was developed.

HEp-2 was subjected to individual chemo-drugs namely Docetaxel, Cisplatin and 5-FU for 72 hours and a dose response curve was generated. IC_{50}, $IC_{3.125}$ and $IC_{6.25}$ of the each chemo-drug were determined. $IC_{3.125}$ and $IC_{6.25}$ were used to develop a model for chemo-resistance. In first cycle, HEp-2 cells were incubated with $IC_{3.125}$ concentrations of all three chemo-drugs together for 72 hours followed by second cycle of $IC_{6.25}$ with incubation in drug free medium for 72 hours in between the two cycles [21]. The triple chemo-drug resistant HEp-2 cell line was maintained in drug free medium onwards.

To validate the acquired chemo-resistance, IC_{50} of each chemo-drug was also determined on the chemo-resistant cell line. The IC_{50} values for Docetaxel, Cisplatin and 5-FU were 1.45 ± 0.7 uM, 53.04 ± 4.88 uM and 2.8 ± 0.8 mM respectively. An increase of approximately 1686, 4 and 12 folds was observed in IC_{50} of Docetaxel, Cisplatin and 5-FU, respectively for the chemo-resistant HEp-2 cell line as compared to the Parent HEp-2 cell line. The increase in IC_{50} values validated the acquisition of chemo-resistance to all three chemo-drugs.

Further, mRNA expression profiling of TLRs 1-10 was performed for chemo-resistant HEp-2 and compared with Parent HEp-2 and all TLRs were found expressed in the chemo-resistant cell line compared to parent cell line with exception of TLR 2 and TLR 10. TLR 4 was relatively higher expressed compared to all TLRs. Although we did not find any significant difference in the expression of all TLRs between the parent and resistant cells.

Fraction of total cells expressing IRAK-1, IRAK-4, p-IRAK-1 and p-IRAK-4 were higher in chemo-resistant HEp-2 as compared to the parent line. A significantly higher over-all expression (Mean Fluorescence Intensity) of IRAK-1, -4, p-IRAK-1 and p-IRAK-4 was also observed in the chemo-resistant HEp-2 as compared to Parent HEp-2. IRAK-1 was approximately 40% more phosphorylated and IRAK-4 was 12% more phosphorylated in chemo-resistant HEp-2 as compared to Parent HEp-2. Data suggest enhanced TLR signaling in chemo-resistant HEp-2 cells as compared to parent cells.

Again, to know the impact of such signaling on chemo-resistant HEp-2 oncogenic profile, TLR signaling was blocked using IRAK-1 & -4 dual inhibitor. The IC_{50} of IRAK-1 & -4 dual inhibitor was observed as 121.31 ± 22.53 uM on the chemo-resistant HEp-2 cell line. There was a 5 fold increase in the IC_{50} of IRAK-1 & -4 dual inhibitor on the chemo-resistant HEp-2 cell line as compared to the Parent HEp-2 cell line. chemo-resistant HEp-2 was further treated with a suboptimal concentration IC_{25} of IRAK-1 & -4 dual inhibitor for 72 hours and the various oncogenic markers were evaluated as listed in Objective 3.

Proliferation marker Ki-67 was found over-expressed in the chemo-resistant HEp-2 as compared to Parent HEp-2 which was effectively reduced upon IRAK-1 & -4 dual inhibitor treatment. Expression of CSC markers CD44, Nanog and ALDH1 were also statistically increased in chemo-resistant HEp-2. IRAK-1 & -4 dual inhibitor did not have any significant effect on the expression of CD44 and ALDH1, but marginally reduced Nanog expression in the chemo-resistant HEp-2. Increased expression of MMP-2 mRNA and IL-6 cytokine levels were observed in the chemo-resistant HEp-2 as compared to Parent HEp-2. Treatment with IRAK-1 & -4 dual inhibitor was highly effective on reducing the expression of MMP-2 mRNA and IL-6 levels. Decreased expression of E-Cadherin and increased expression of Vimentin in the chemo-resistant HEp-2 as compared to the Parent HEp-2 was observed indicating that chemo-resistant HEp-2 has higher metastatic potential through enhanced EMT. IRAK-1 & -4 dual inhibitor reduced the expression of E-Cadherin and Vimentin both.

Comparative data of chemo-resistant vs. Parent HEp-2 cells strongly suggest the role of TLR signaling in enhancing oncogenic properties of the chemo-resistant cells. Blocking TLR signaling through inhibition of IRAK-1 and -4 was found to be highly effective against chemo-resistant cells suggesting TLR as a potential drug target in TPF chemo-resistant HNSCC.

Objective 5: Evaluation of therapeutic potential of IRAKs based TLR inhibitor as combination therapy with conventional chemo-drugs

Parent HEp-2 and chemo-resistant HEp-2 were treated with various concentrations of the three chemo-drugs Docetaxel, Cisplatin and 5-FU in combination with IC_{25} dose of IRAK-1 & -4 dual inhibitor. Cell viability was determined by resazurin assay and IC_{50} of each combination treatment was calculated. The results are summarized in the Table 1 and 2 below.

Table 1: IC_{50} of combination treatment on Parent HEp-2 cell line

Chemo-drug	**IC_{50} chemo-drug (n = 4)**	**IC_{50} combination (Chemo-drug + IRAK-1 & -4 dual inhibitor) (n = 4)**	**Fold change in IC_{50}**
Docetaxel	0.864 ± 0.42 nM	0.63 ± 0.19 nM	1.3 fold decrease
Cisplatin	9.73 ± 2.5 uM	6.8 ± 2.73 uM	1.4 fold decrease
5-FU	0.286 ± 0.84 mM	0.151 ± 0.04 mM	1.9 fold decrease

Table 2: IC_{50} of combination treatment on chemo-resistant HEp-2 cell line

Chemo-drug	**IC_{50} chemo-drug(n = 6)**	**IC_{50} combination (Chemo-drug + IRAK-1 & -4 dual inhibitor) (n = 6)**	**Fold change in IC_{50}**
Docetaxel	1.45 ± 0.7 uM	0.00765 ± 0.68 uM	207 fold decrease
Cisplatin	53.04 ± 4.88 uM	25.2 ± 5 uM	2.1 fold decrease
5-FU	2.8 ± 0.8 mM	0.45 ± 0.42 mM	6.2 fold decrease

IC_{50} of the combination treatment was reduced approximately by 50% for the Parent HEp-2 cell line as compared to the IC_{50} of single drug treatment. In chemo-resistant HEp-2, TLR signaling inhibition by IRAK-1 & -4 dual inhibitor in combination with chemotherapy was highly effective (Table 2).

Further, Parent HEp-2 and chemo-resistant HEp-2 were treated with two suboptimal doses of each chemo-drug in combination with IC_{25} of IRAK-1 & -4 dual inhibitor for 72 hours. The effect of combination treatment on oncogenic properties was studied as listed in objective 3.

Single chemo-drug treatment with Cisplatin and Docetaxel induced CSCs formation and increased the proliferative potential in both Parent and Chemo-resistant HEp-2 with higher increment seen in chemo-resistant HEp-2. Combination treatment with IRAK-1 & -4 dual inhibitor and chemo-drugs was effective in reducing the expression of these markers on chemo-resistant HEp-2while the same had no impact on Parent HEp-2.

In chemo-resistant HEp-2, chemo-drugs Docetaxel and 5-FU as standalone therapy effectively reduced ALDH1 expression which could not be further improved by addition of IRAK-1 & -4 dual inhibitor.

Single chemo-drug treatment induced the mRNA expression of MMP-2 which could be effectively suppressed on combination therapy with respective chemo-drug and IRAK-1 & -4 dualinhibitor. It was also observed that single drug treatment could reduced the production of IL-6 in the chemo-resistant HEp-2 which was further improved upon combining IRAK-1 & -4 dual inhibitor with the chemo-drugs.

E-cadherin expression significantly increased upon treatment with Cisplatin but the combination with IRAK-1 & -4 dual inhibitor diminished such effect by reducing the expression. All three chemo-drugs, increased expression of Vimentin. Combination treatment of IRAK-1 & -4 dual inhibitor and chemo-drugs could effectively suppress it.

Conclusion:

- TLRs are expressed in laryngeal origin HNSCC cell line HEp-2.
- TLR signaling has role in survival of laryngeal origin HNSCC cell line HEp-2.
- TLR signaling is constitutively on/activated in HEp-2 cells.
- Blocking TLR signaling pathway through downstream signaling kinases IRAK-1 and -4, has no significant anticancer effects on HEp-2.
- Few TLRs and its related downstream signaling kinases IRAK-1 and -4 were over-expressed in chemo-resistant HEp-2 as compared to the Parent HEp-2. TLR signaling was constitutively on in chemo-resistant HEp-2 as reflected by phosphorylation state of IRAK-1 and -4 which was also more as compared to parent HEp-2.
- Chemo-resistance contributed to enhanced pro-oncogenic effects evident from the increase in expression of CSCs markers, proliferation marker, metastasis and EMT markers.
- Combination therapy of chemo-drugs and TLR inhibitor (IRAK-1 & -4 dual inhibitor) was highly active against resistant head and neck cancer cell line HEp-2 as compared to standalone chemo-drugs.
- Findings need to be confirmed in more than one head and neck cancer cell lines as well as *in vivo* using pre-clinical animal model.

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