6. DISCUSSION

HNSCC remains a global health issue with a 5-year survival rate of 40-50%. India has a huge burden of oral cancer being the most prevalent one among males. Despite the advent and approval of several new anti-cancer treatment moieties in the last couple of decades, the survival rate has remained unchanged. Still, surgical resection, radiotherapy and chemotherapy are the most commonly used treatment options. Adverse events and non-responsiveness to therapy are the key reasons for a considerable number of patients ending up with an advanced stage disease ultimately leading to death (Canning *et al*., 2019).

Among the chemotherapeutic agents, Docetaxel, Cisplatin and 5-FU are FDA approved for HNSCC. Combination of all three, known as TPF is used as an induction chemotherapy and is preferred over single agent and double agents (PF therapy) for HNSCC, mainly for the treatment of advanced and metastatic tumors. Several benefits associated to TPF-treatment over other treatments are improved overall survival (OS), progression-free survival (PFS) and better larynx preservation (Ferrari *et al*., 2020). But a significant number of advanced-stage and elderly patients on redundant TPF treatment gradually become resistant to it. Also, approximately 60% advanced stage HNSCC patients develop toxicity and tolerability issues to chemo-therapy with notable side effects such as febrile neutropenia, stomatitis and diarrhea (Sher *et al.*, 2016). The resistance rates to docetaxel, cisplatin and 5-FU in HNSCC patients is 44%, 33% and 40.2%, respectively (Kanno *et al.*, 2021; Atashi *et al.*, 2021). The overall-response rate of HNSCC patients to TPF is 50-60% (Izawa *et al.*, 2015; Sher *et al.*, 2016; Ilie *et al*., 2012; Guigay *et al*., 2019). This reflects not only the requirement of alternative or new targeted therapies but also attracting our attention towards devising dose sparing strategies for the in-use chemo-drugs based treatment regimen. Dose sparing entity can produce anti-cancer efficacy equivalent to conventional chemotherapeutic drugs through combination thus allowing usage of significantly lower dose of the chemo-drugs themselves. This will not only solve the tolerability issue in a considerable cohort of the patients but also delay the process of chemo non- responsiveness. Hence, an efficacious combination therapy may benefit the patients in long term by extending their overall survival as well as progression free survival.

The aim of this research work was to understand the role of TLR signaling in the progression of HNSCC and explore it as therapeutic target using a laryngeal origin cell line HEp-2 as experimental model system. To specifically understand the role of TLR signaling during chemoresistance, a chemo-resistant lineage was derived from the parent HEp-2. The

chemo-resistant cell line was developed by exposing HEp-2 to gradually increasing doses of combination of docetaxel, cisplatin and 5-FU. Fold increase in inhibitory concentrations of the chemo-drugs on the chemo-resistant cells validated the acquisition of chemo-resistance **(Figure 5.12)**. Morphologically, chemo-resistant cells were found enlarged with high granularity than parent suggesting their increased secretory potential **(Figure 5.11)**.

Besides the morphological difference and reduced sensitivity towards tested chemo-drugs, other key attributes were also compared between the parent and resistant cell lines. Elevated levels of Bcl-2, Bcl-xL and Ki-67 observed in the chemo-resistant cell line compared to parent suggested its higher survival capacity and proliferative potential compared to the parent cell line **(Figure 5.17-Figure 5.18).**

An increase in the CSCs, as determined by CSCs markers ALDH1, CD44 and Nanog levels, in the resistant cell line compared to parent cell line also provided a reason for cells being resistant to the chemotherapies **(Figure 5.19-Figure 5.20)** (Preito-vila *et al.*, 2017; Phi *et al.*, 2018). In this study, we have also studied a novel pro-oncogenic protein, BST-2. The role of BST-2 is explored in many other cancers. Expression of BST-2 significantly correlated with expression of CD44 and Nanog in HEp-2 **(Figure 5.7)**. This suggested its association with the cancer stemness in HNSCC. The data indicated the potential of BST-2 to act as an alternative CSCs marker and a potential therapeutic target for HNSCC. For validation purpose, the TCGA data was also mined. HNSCC patients' biopsies (n=520) showed over-expression of BST-2 in primary tumors compared to tumor adjacent normal tissues. BST-2 was also over-expressed in different grades and stages of HNSCC. HNSCC was also among the different cancers in which BST-2 was highly upregulated (**Table 6.1 and Figure 6.1**) (Khan & Srivastava, 2023). BST-2 was elevated in the resistant cells compared to the parent cells, in line with the expression patterns of other CSCs markers, further strengthening its potential to act as a CSC marker of HNSCC **(Figure 5.21)**.

Table 6.1: GEO data-based analysis of BST-2 expression in tumor vs normal samples of HNSCC (Source: Khan & Srivastava, 2023)

GEO accession number	Fold change in BST-2 expression (Tumor vs normal)	Number of cases (Cancer, control)
GSE23558	2.67 fold increase ($p<0.0001$)	27, 5
GSE31056	2.37 fold increase ($p<0.0001$)	23, 24
GSE74530	2.71 fold increase ($p<0.001$)	6, 6
GSE78060	2.66 fold increase ($p<0.001$)	26, 4
GSE13601	2.05 fold increase ($p<0.0001$)	31, 26
GSE30784	3.25 fold increase ($p<0.0001$)	167, 45

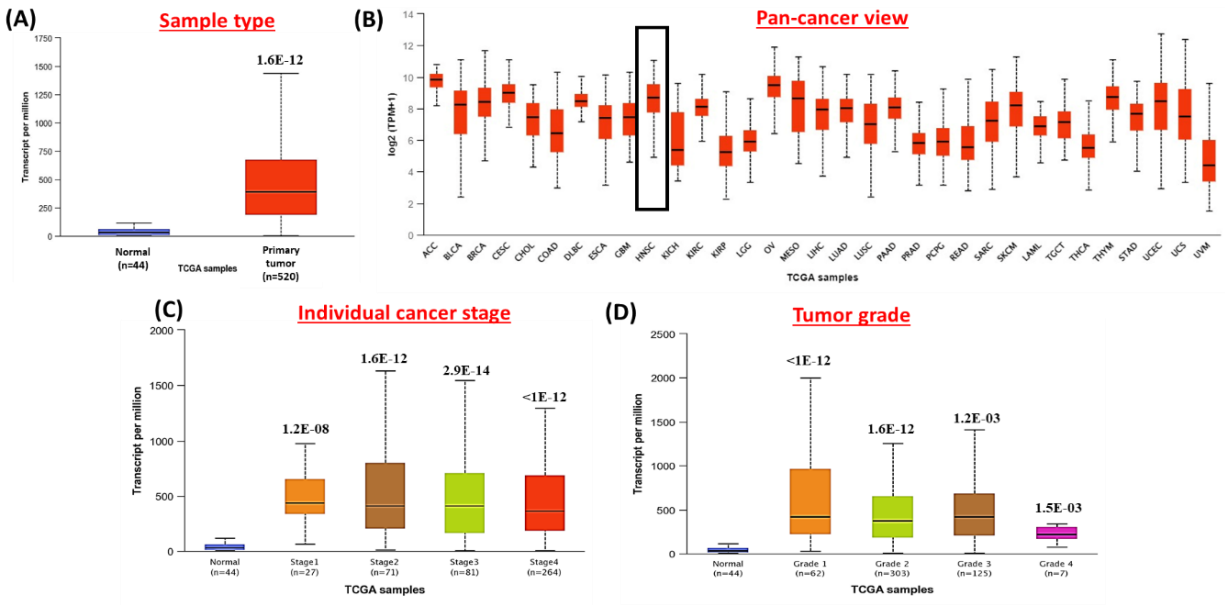

Figure 6.1: **TCGA data-based analysis of BST-2 expression in HNSCC.** BST-2 expression in (A) normal vs primary HNSCC tumor, (B) various cancers, various HNSCC (C) stages and (D) grades. (Source: Khan & Srivastava, 2023)

Alteration in the EMT profile upon chemo-resistance, observed by an increase in Vimentin and a decrease in E-cadherin in the resistant cells indicated an acquired invasive feature and metastatic potential of chemo-resistant cells. Elevated levels of MMP-2 and IL-6 in chemo-resistant cells also suggested that chemo-resistant state attained an increased metastatic potential **(Figure 5.22-Figure 5.23)**. Our findings suggests that chemo-resistant line developed in our laboratory, had better survival, aggressive proliferative potential with higher stemness and metastatic potential than parent experimental system. It well reflected the TPF-non responsive, advanced stage HNSCC patients' clinical profile and provides a valuable tool for *in-vitro* studies such as specific pathways analysis, newer drug target identification and screening etc (Amaral *et al.*, 2019; Gregoreva *et al.*, 2021).

Almost all the TLRs were found equivalently expressed on both the parent and chemo-resistant line with lack of TLR 2 and TLR 10 expression **(Figure 5.1, Figure 5.13 and Figure 5.14).** TLRs expression have been reported in head and neck cancers of different origins (Pisani *et al.*, 2017). The presence of the TLRs on both cell lines suggested a probable role of TLR signaling in HNSCC carcinogenesis. To know whether these TLRs are undergoing signaling or not, expression and phosphorylation state of IRAK-1 and -4 were determined. Both IRAK-1 and IRAK-4 were found expressed and significantly phosphorylated in parent (60-90%) and chemo-resistant form (80-90%) **(Figure 5.2 and Figure 5.15)** indicating that TLR signaling was ON in these cells. As we have not added any synthetic TLR ligands externally into our

culture system, the endogenous ligands, mainly DAMPs generated during cell culture progression could be triggering the TLR signaling. Although, the study has not quantitated any endogenous TLR ligands levels in experimental system.

While the TLRs expression levels were found equivalent, the IRAKs expression were around 3-6 folds higher in chemo-resistant state as compared to the parent line, with most of it remaining in phosphorylated state **(Figure 5.15)**. Chemo-resistance linkage with over-expression and phosphorylation of IRAK-1 and IRAK-4 is previously demonstrated in paclitaxel resistant TNBC (Wee *et al*., 2015), NPC (Liu *et al*., 2021), various chemo therapies resistant cancers including PDAC (Zhang *et al*., 2017), CRC (Li *et al*., 2019) and HCC (Cheng *et al*., 2018). In 2015, Adams *et al.,* reported over-expression of IRAK-1 in HNSCC patients based on TCGA data analysis. This study demonstrated the role of IRAK-1 and IRAK-4 expression in promoting the proliferation using laryngeal cancer cell line.

IRAK upregulation observed in derived chemo-resistant line could be an end result of the feedback loop of constitutively ON TLR signaling (Rhyasen and Starczynowski, 2015, El-Zayat *et al.,* 2019). The data suggest that even with the similar levels of expression of TLRs, the TLR signaling can be activated at higher amplitude in chemo-resistant line than the parent. This could be possible either due to generation of higher amount of endogenous TLR ligands being produced in chemo-resistant state or due to amplified downstream signaling through higher expression of downstream adaptor involved in TLR signaling like IRAKs. High mobility group box-1 (HMGB-1) is one of the most abundant DAMP binding to TLR 4 is generated during cellular stress. Overexpression of HMGB-1 in the blood and tumor tissues of patients with head and neck squamous cell carcinoma is already reported (Wild *et al.,* 2012; Mohajertehran *et al.,* 2018). In fact, its levels have been shown to be of prognostic importance in association with clinical and pathological characteristics of head and neck squamous cell carcinomas by multiple studies (Qiu *et al.,* 2014; Nguyen *et al.,* 2016). HMGB-1 is a key mediator of chemotherapy-induced peripheral neuropathy (CIPN) providing evidence that its level being considerably high post chemo exposure in host (Sekiguchi and Kawabata, 2020). Although, present study has not estimated the DAMPs level in cell culture supernatants. Many other DAMPs binding to other TLRs than TLR 4 are also known to be well present in HNSCC patients such as S100A4 (Fei *et al.,* 2017), S100A8 (Funk *et al*., 2014), S100A12 (Funk *et al*., 2014), HMGB1 (Mohajertehran *et al*., 2018), HSP60 (Tsai *et al*., 2009) capable of triggering multiple TLR signaling.

To know whether TLR signaling has any role in the HNSCC progression in native and chemo-resistant state, we have used the approach of blocking the TLR signaling pathway to study its impact on these pro-oncogenic attributes in both parent and chemo-resistant line. Various pro-oncogenic properties such as proliferative potential, survival, expression of CSCs, metastasis, and EMT were evaluated with and without TLR inhibitor. Also, the comparative analysis was performed between parent and chemo-resistant state. In this study, we used a commercially available benzimidazole based small molecule IRAK-1 &-4 dual inhibitor, to disrupt the TLR signaling.

A concentration dependent inhibition in the viable cell count of HEp-2 cells upon IRAK-1 &-4 dual inhibitor treatment was observed suggesting the cytostatic effect of the inhibitor on the cells**.** Interestingly, a 5-fold shift in the IC_{50} of IRAK-1 &-4 dual inhibitor on the chemo-resistant cells compared to the parent cells was also noted **(Figure 5.3 and Figure 5.16)**. This could be due to higher target expression in chemo-resistant state [3-6 fold higher levels of IRAKs] and better proliferative potential as suggested by Ki-67 data. Data well suggested that TLR signaling contributes to the proliferation of parent cells and chemo-resistant cells although the impact is more on chemo-resistant state than parent. Toll-like receptor signaling stimulates cell cycle entry and progression in fibroblasts (Hasan *et al.*, 2005). This particular study provide evidence of a non-immune and cell autonomous role of TLR signaling, for inducing cell proliferation. The resazurin based assay performed in this study reflects the total number of metabolically active live cells which can be affected by many attributes including cell survival, proliferation, presence of cancer stem cells and other promoting factors. In the parent line, blocking the TLR signaling showed limited effect only on proliferative potential while it impacted chemo-resistant line quite significantly affecting almost all attributes including survival, proliferation, CSCs formation, EMT and metastasis.

BST-2, used as an additional pro-oncogenic marker was downregulated by blocking TLR signaling in parent line **(Figure 5.7)**. BST-2 takes part in cell invasion, migration, progression and chemo-resistance in cancers (Cai *et al.*, 2009; Mahauad-Fernandez *et al.*, 2018; Woodman *et al.*, 2016; Yi *et al.*, 2013; Kuang *et al.*, 2017; Jin *et al.*, 2019). Its role in HNSCC is less explored. Pre-clinical reports indicate the use of anti-BST-2 antibody for the treatment of myeloma (Tai *et al.*, 2012), lung cancer (Wang *et al.*, 2009) and endometrial cancer (Yokoyama *et al.*, 2013). B49, a BST-2 based peptide, demonstrated inhibition in invasion and migration of breast cancer cells (Mahauad-Fernandez *et al.*, 2018). This indicates the limited

therapeutic options available for targeting BST-2. The present study also opens up the avenues to test BST-2 inhibitors as potential therapeutic drug candidates in HNSCC.

Similarly, negligible impact of TLR signaling was observed on metastasis as reflected by EMT signatures along with IL-6 and MMP-2 **(Figure 5.8-Figure 5.9)** in parent while in chemo-resistant cell line, these processes were TLR signaling dependent.

Overall, the data indicates minimal role of TLR signaling in parent HEp-2 progression while dependency of chemo-resistant HEp-2 cells on TLR signaling was considerably high, influencing almost all the cancer promoting attributes studied **(Table 6.2).**

Table 6.2: Summary of pro-oncogenic features of parent and chemo-resistant HNSCC cells dependent on TLR signaling

Pro-oncogenic effects associated markers		Parent HEp-2	Chemo-resistant HEp-2
Survival	Bcl-2	—	↓
	Bcl-xL	—	↓
Proliferation	Ki-67	↓	↓
CSCs formation	CD44	—	—
	Nanog	—	↓
	ALDH1	—	↓
	BST-2	↓	—
EMT	E-cadherin	—	↓
	Vimentin	—	↓
Metastasis	MMP-2	—	↓
	IL-6	—	↓

↓ - Suppressive effect of IRAKs-based TLR inhibitor
— - No significant impact of IRAKs-based TLR inhibitor

These results supported the rationale to test the TLR inhibitors as a therapeutic entity in HNSCC as stand-alone as well as combination therapy along with ongoing chemotherapy. Although in the parent line, TLR signaling blocker i.e., IRAK inhibitor, have shown limited impact, it was tested as chemo-drug sparing regimen in combination with chemo-drugs. Each chemo-drug, had different efficacy for the tested oncogenic properties. Overall, the TLR inhibitor-based chemo-drug combination therapy showed moderate effect on the parent line. The combination therapy with cisplatin downregulated Ki-67 levels, whereas the combination with docetaxel downregulated Nanog levels in the parent cells **(Figure 5.29 A and Figure 5.33 A)**. Strikingly, the combination therapy clearly downregulated the BST-2 levels with all

chemo-drugs in parent cells suggesting it as a promising approach for HNSCC treatment through reducing BST-2 levels **(Figure 5.39 A)**. In chemo-resistant cells, the effect of combination therapy was better than parent line with more cancer promoting attributes being suppressed significantly by TLR inhibitor alone and even better in combination with chemo-drugs. Among the tested drugs the efficacy of combination therapy was best with cisplatin chemo-drug than docetaxel and 5-FU **(Figure 5.27–Figure 5.39)**. In parent line, blocking the pathway in presence of chemo-drugs can extensively delay the development of chemo-drug resistance in early-stage chemo responsive cancers if treated with lower doses of chemo-drug with TLR inhibitor. While in chemo-resistant state, TLR blockers can covert a chemo non-responsive state to responsive one by adding TLR inhibitors with individual chemo-drug, thus may influencing the OS.

Another interesting finding of the study was upregulation of various pro-oncogenic proteins by treating the cells by chemo-drugs itself, in both parent and chemo-resistant state **(Table 6.3)**. Such data alarms for chronic usage to chemotherapy as standalone therapy. Interestingly, when the chemo-drugs were combined with TLR signaling blockers, such effects could be diminished very well. Data also advocates the usage of the TLR inhibitors along with chemo-drugs at early and late stages cancers both. Our data not only highlights the role of TLR signaling in HNSCC progression and chemoresistance development but also showed its therapeutic utility as standalone and combination therapy. Current data sets the ground to test IRAK inhibitors based TLR signaling blockers in preclinical animal models of HNSCC followed by clinical trial/s. Till date no TLR inhibitors has been approved by any national and international drug regulatory authorities for HNSCC. Developing such therapeutic modality can change the treatment landscape for HNSCC patients. India has considerably high burden of HNSCC, with significant number of the patients belonging to lower economic background. A large cohort of them can't afford the recently approved targeted therapies like checkpoint inhibitors or EGFR blockers. Developing an affordable small molecule inhibitor of TLR signaling such as IRAK inhibitor can be of great benefit towards reducing huge and escalating HNSCC burden in country like India.

Table 6.3: Summary of pro-oncogenic features upon exposure to combination of chemotherapy and IRAKs based-TLR inhibitor in parent and chemo-resistant HNSCC cells

Pro-oncogenic effects associated markers	Survival		Proliferation		CSCs formation							EMT		Metastasis	
	Bcl-2	Bcl-xL	Ki-67		CD44		Nanog		ALDH1	BST-2		E-cadherin	Vimentin	MMP-2	IL-6
Cell line / Chemo-drug	Chemo-resistant HEp-2	Chemo-resistant HEp-2	Parent HEp-2	Chemo-resistant HEp-2	Parent HEp-2	Chemo-resistant HEp-2	Parent HEp-2	Chemo-resistant HEp-2	Chemo-resistant HEp-2	Parent HEp-2	Chemo-resistant HEp-2	Chemo-resistant HEp-2	Chemo-resistant HEp-2	Chemo-resistant HEp-2	Chemo-resistant HEp-2
Docetaxel IC_{25}	↑↓	↑↓	↑↑↓	–	↑	↑↓	↑↓	↑↓	↓↓	↓	–	–	↑↑↓	–	↓↓
Docetaxel $IC_{12.5}$	↑↑↓	↑↓	↑	↑↓	↑	↑↓	↑	↑↑↓	↓	↓	↓	–	↓	↑↓	↓↓↓
Cisplatin IC_{25}	↑↓	↑↑↓	↑↑↓	↑↑↓	↑	–	↑	↑↓	–	↑↓	–	↑↓	↑↓	↑↑↓	↓↓
Cisplatin $IC_{12.5}$	↓	↑↓	–	↓	–	–	–	↑↓	–	↓	↓	–	↓	↑↓	↓↓
5-FU IC_{25}	↑↓	↑↓	–	↓	–	–	↑	–	↓↓	↑↑↓	–	–	–	↑↓	↓↓
5-FU $IC_{12.5}$	↑↑↓	↑↑↓	–	↓	–	–	–	↑↓	↓	↓	–	↓	↑↓	–	↓

↑ - Upregulation of expression of markers upon chemotherapy
↓ - Downregulation of expression of markers upon chemotherapy
↓ - Downregulation of expression of markers upon combining TLR inhibitor with chemotherapy
— - Unaltered expression of markers upon chemotherapy/combination therapy