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IRAK-1 &-4 DUAL INHIBITOR MEDIATED BST-2 SUPPRESSION: A NOVEL THERAPEUTIC APPROACH TOWARDS HEAD AND NECK SQUAMOUS CELL CARCINOMA

KHAN H[1] AND SRIVASTAVA R[1, 2*]

1: Department of Microbiology and Biotechnology Centre, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara-390020, Gujarat, India

2: Department of Biotechnology, School of Life Sciences, Babasaheb Bhimrao Ambedkar University, Lucknow-226025, U.P., India

[*]**Corresponding Author: Dr. Ratika Srivastava: E Mail: srivastava.ratika@gmail.com**

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ABSTRACT

Head and neck squamous cell carcinomas (HNSCC) are the 7th most common cancer worldwide. Docetaxel, cisplatin and 5-fluorouracil (5-FU) are conventional chemo-drugs used for the treatment of head and neck cancer. However, treatment with these chemo-drugs is associated with severe late toxicities and acquired resistance, a major cause of mortality in HNSCC patients. Hence, identification of molecular targets associated with chemo-resistance to develop personalized and effective treatment is required. Bone marrow stromal antigen-2 (BST-2) is a Type-I interferon inducible anti-viral protein expressed on the surface, and in vesicular compartments of host cells.

Studies reveal high expression of BST-2 in a large number of cancers like breast cancer, HNSCC, lung cancer, cervical cancer, glioblastoma and myeloma. In breast cancer, over-expression of BST-2 mediates invasion, migration, progression and survival of cells. BST-2 over-expression is also reported to confer resistance to chemo-therapies such as cisplatin and gefitinib in HNSCC. In this study, we report that BST-2 is over-expressed in HNSCC through *in-vitro* and bioinformatics approaches. Treatment with conventional chemo-drugs increased the levels of BST-2 in the human laryngeal cancer cell line, HEp-2. Treatment with commercially available small molecule inhibitor of the kinase activity of Interleukin-1 receptor associated kinases (IRAK)- 1 and -4, decreased the levels of BST-2 in HEp-2. Our findings suggest BST-2 as a chemo-resistance specific druggable target in

HNSCC and report the use of IRAKs based Toll-like receptor (TLR) signaling inhibitor as a novel therapeutic approach towards HNSCC.

Keywords: BST-2, IRAK-1 &-4 dual inhibitor, Chemo-resistance, Head and Neck cancer, Cancer stem cells (CSCs)

INTRODUCTION

Head and Neck Cancer is the 7th most common cancer worldwide. Head and Neck squamous cell carcinomas (HNSCCs) are the most common histologies of head and neck cancers **[1]**. Treatment of HNSCCs requires a multidisciplinary approach comprising of surgery, radiotherapy and chemotherapy **[2, 3]**. Chemotherapy is the preferred treatment choice over others as it can destroy cancer cells that are not accessible by surgery/radiotherapy. Conventional chemo-drugs used for the treatment of HNSCC are Docetaxel, cisplatin and 5-fluorouracil (5-FU) **[4, 5]**. Combination of chemotherapies comprising of these three agents is also approved by the US FDA (Food and Drugs Administration) for the treatment of HNSCC **[6]**. Cancer stem cells (CSCs), a group of cancer initiating cells, play a role in cancer relapse and confer chemo-resistance. In presence of chemotherapies, they have the potential of self-renewal and differentiation into various cancer cell lineages **[7]**. Acquired chemo-resistance is a major cause of treatment failure resulting in high mortality rate in HNSCC. Hence, it is essential to identify molecular targets associated with chemo-resistance in order to develop personalized and effective treatment for patients.

Bone marrow stromal antigen-2 (BST-2) also called tetherin or CD317, is a type-I interferon inducible anti-viral protein located on the cell surface, inside the trans-golgi network and in vesicular compartments of the cells **[8]**. Structurally, BST-2 is a 180 amino acid long type-II transmembrane glycoprotein tethering various kind of viruses like HIV **[9]**, herpesvirus **[10]**, and retrovirus **[11]**. It comprises of a coiled-coil ectodomain with a C-terminal glycosylphosphatidylinositol (GPI)-anchor and transmembrane domain attached to N-terminal cytoplasmic tail. BST-2 upon crosslinking leads to NF-κB activation. NF-κB signaling leads to the production of cytokines CXCL10 and IL-6 **[8]**.

Studies reveal high expression of BST-2 in a large number of cancers like breast cancer **[12]**, HNSCC **[13]**, lung cancer **[14]**, cervical cancer **[15]**, glioblastoma **[16]** and myeloma **[17]**. Despites its anti-viral activity, high expression of BST-2 is associated with poor survival in breast cancer **[18]** and HNSCC **[13]**. In breast cancer, over-expression of BST-2 mediates

invasion, migration, progression and chemo-resistance in cells **[19, 20]**. BST-2 over-expression is also reported to confer resistance to chemo-therapies such as cisplatin and gefitinib in HNSCC **[21, 22]**. Hence, therapeutic approaches to target BST-2 are required for the treatment of the cancer.

In the present study, using *in-vitro* and bioinformatics-based approaches, we investigated the association of BST-2 with cancer stem cells and clinicopathological parameters of HNSCC. We also explored the therapeutic advantage of using a commercially available small molecule based Toll like receptor signaling inhibitor through blocking of the kinase activity of Interleukin-1 receptor associated kinases-1 and 4 (IRAK-1 and IRAK-4) in suppressing the BST-2 expression.

MATERIALS AND METHODS

Cell culture

HEp-2, a human laryngeal cancer cell line was purchased from National Centre for Cell Science (NCCS), Pune, India. The cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM) (Hyclone, GE) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco) and 1% antibiotic/antimycotic (Gibco)in a humidified incubator with 5 % CO_2 at 37°C.

Reagents

Docetaxel (Zydus Cadila Pharmaceuticals, India), cisplatin (Celplat) and 5-fluorouracil (5-FU) (Zydus Cadila Pharmaceuticals, India) were commercially procured. IRAK-1 &-4 dual inhibitor solution (Cat# 407602) was purchased from Sigma-Aldrich, St. Louis, MO, USA and was used as a pharmacological small molecule inhibitor. Fluorochrome conjugated anti-BST-2, anti-CD44 and anti-Nanog were purchased from Invitrogen, CA, USA.

UALCAN database analysis

UALCAN browser (http://ualcan.path.uab.edu/index.html) is an online user-friendly and interactive resource for genomic and proteomic data analysis in TCGA tumors. BST-2 expression across various cancers and its association with HNSCC tumor stages and grades for 520 patient samples was analyzed using UALCAN database.

Gene expression omnibus database analysis

Gene expression omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo), a public genomic database by NCBI was used to analyze BST-2 gene expression in HNSCC. The database was searched using the keywords "oral cancer", "head and neck cancer" and "HNSCC". Fold change in BST-2 expression in tumor vs. normal samples was analyzed using GEO2R. List of data sets analyzed along with the

corresponding number of samples are mentioned in **Table 1**.

Treatment of cells

10,000 cells per well were seeded in a 12 well plate and incubated overnight at 37℃ with 5 % CO_2. Cells were further treated with 1 nM docetaxel, 11 uM cisplatin and 0.3 mM 5-FU alone and in combination with 5.1 uM IRAK-1 &-4 dual inhibitor for 72 hours and incubated at 37℃ with 5 % CO_2.

Flow cytometry

Cells were harvested and stained with fluorochrome conjugated surface antibodies CD44 and BST-2 for 30 mins at room temperature (RT). Intracellular staining of Nanog was performed by fixing the cells with 2% formaldehyde for 10 mins followed by permeabilizing with 100% cold methanol for 30 mins and incubating with fluorochrome conjugated anti-Nanog for 30 mins at RT. Samples were washed and analyzed on FACS Calibur flow cytometer (BD Biosciences, USA). FACS data was analyzed using flowing software (version 2.5.1, Turku. Cent. Biotechnology).

Statistical analysis

Statistical analysis was performed using GraphPad Prism, California, USA (version 8.0.1). Data are represented as mean ± S.D. of more than three independent experiments. Comparison of two data sets was performed using unpaired student's *t*-test and comparison of multiple sets was performed using Analysis of Variance (ANOVA). Correlation between CSCs expression and BST-2 expression were assessed using Pearson's correlation and Pearson correlation coefficient (r) are mentioned in figures. $P < 0.05$ was considered for statistical significance.

RESULTS

Association between BST-2 and Cancer Stem Cells (CSCs)

We analyzed the correlation between expression of CSCs marker CD44 and Nanog with BST-2 in HEp-2 by flow cytometry. The results showed a significant positive correlation between CD44 Mean Fluorescence Intensity (MFI) and BST-2 MFI ($r=0.8120$, $p=0.0497$) **(Figure 1A)**. A strong significant positive correlation between Nanog MFI and BST-2 MFI ($r=0.9393$, $p=0.0054$) was also observed **(Figure 1B)**.

BST-2 is overexpressed in HNSCC

We evaluated the expression of BST-2 in HNSCC tissue samples data available in TCGA using UALCAN browser. We observed that BST-2 was upregulated in primary HNSCC compared to the adjacent healthy tissue **(Figure 2A)**. On analyzing the pan-cancer view, BST-2 was up regulated in HNSCC besides Adrenocortical carcinoma (ACC), Cervical and endocervical squamous cell carcinoma (CESC) and Ovarian cancer (OV) **(Figure**

2B). On analyzing GEO datasets, BST-2 was found significantly over-expressed in HNSCC patient samples compared to non-cancer patient samples in GSE23558, GSE31056, GSE74530, GSE78060, GSE13601 and GSE30784 datasets **(Table 1)**. Analysis of association of BST-2 with clinicopathological parameters of HNSCC using UALCAN browser also showed significantly elevated BST-2 expression in individual tumor stages **(Figure 2C)** and grades of HNSCC tissue samples in comparison to normal tissues **(Figure 2D)**.

Cisplatin and 5-FU treatment induces BST-2 expression in HEp-2

We evaluated BST-2 protein levels in HEp-2 by flow cytometry and observed 20% of HEp-2 cells were positive for BST-2. BST-2 expression remained unchanged on treatment with docetaxel in HEp-2 cells. While, treatment with other chemo drugs, cisplatin and 5-FU increased the percentage of BST-2+ cells by 15% and 30%, respectively **(Table 2)**. A significant increase in the mean fluorescence intensity (MFI) of the BST-2 upon cisplatin and 5-FU treatment was also observed **(Table 2)**.

IRAK-1 &-4 dual inhibitor treatment alone and in combination with chemo-drugs reduces BST-2 expression in HEp-2

Treatment with IRAK-1 &-4 dual inhibitor significantly reduced the percentage of BST-2+ cells in HEp-2 by 10% **(Figure 3A)**. A significant reduction in BST-2 MFI in HEp-2 was also observed upon treatment with IRAK-1 &-4 dual inhibitor **(Figure 3B)**. Combining IRAK-1 &-4 dual inhibitor with docetaxel, cisplatin and 5-FU reduced the percentage of BST-2+ cells by 6%, 18% and 15% respectively **(Figure 3A)**. A significant reduction in BST-2 MFI was also observed on treatment of IRAK-1&-4 dual inhibitor when combined with cisplatin or 5-FU. No significant difference in BST-2 MFI was observed on combining IRAK-1 &-4 dual inhibitor with docetaxel **(Figure 3B)**.

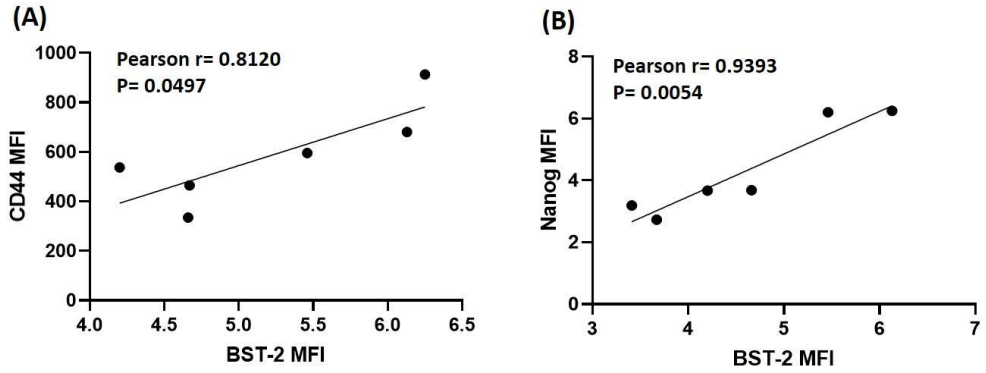

Figure 1: Association of BST-2 expression with CSCs markers (A) CD44 and (B) Nanog expression in HEp-2

Table 1: Fold change in BST-2 expression in tumor vs normal samples of HNSCC in GEO datasets

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

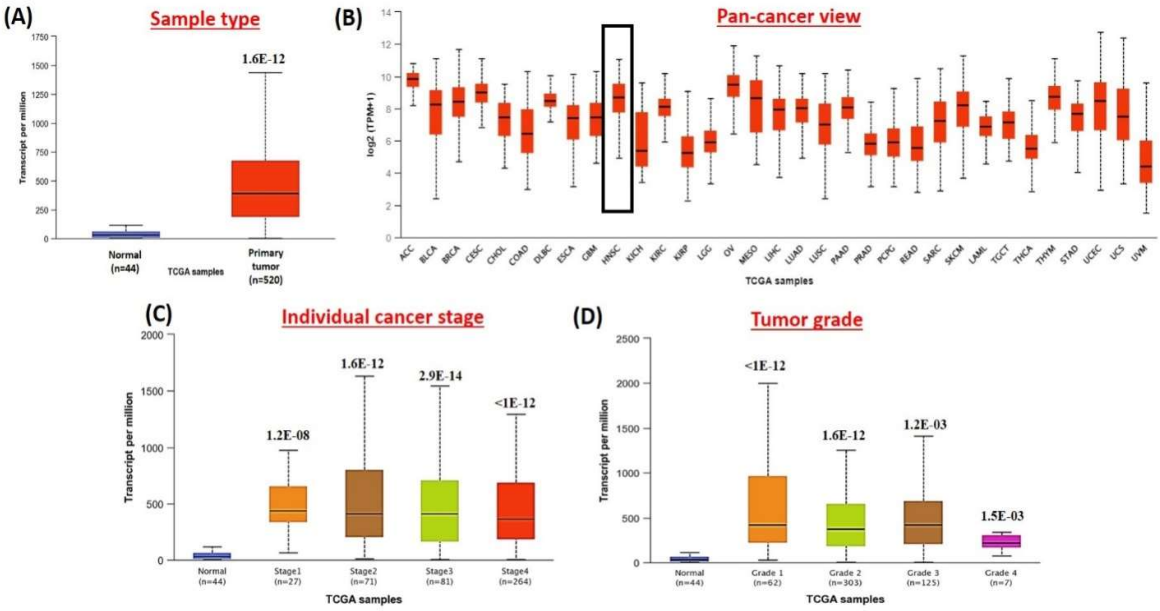

Figure 2: Bioinformatics based analysis of BST-2 expression in HNSCC: (A) Expression of BST-2 in normal vs primary tumor in HNSCC. (B) Expression of BST-2 across TCGA cancers. (C) Expression of BST-2 in HNSCC based on individual stages of tumor. (D) Expression of BST-2 in HNSCC based on grades of tumor. *P*-values are indicated on individual box plots. (Source: UALCAN browser)

Table 2: Expression of BST-2 upon chemo-drug treatment in HEp-2

Levels of BST-2 in HEp-2		
	% Positive cells (n=3)	MFI (n=3)
Untreated	20.91 ± 0.611	4.412 ± 0.431
Docetaxel 1nM	18.96 ± 1.21	4.181 ± 0.38
Cisplatin 11uM	34.99 ± 0.92	5.810 ± 0.46
5-FU 0.3mM	42.91 ± 2.92	6.720 ± 0.15

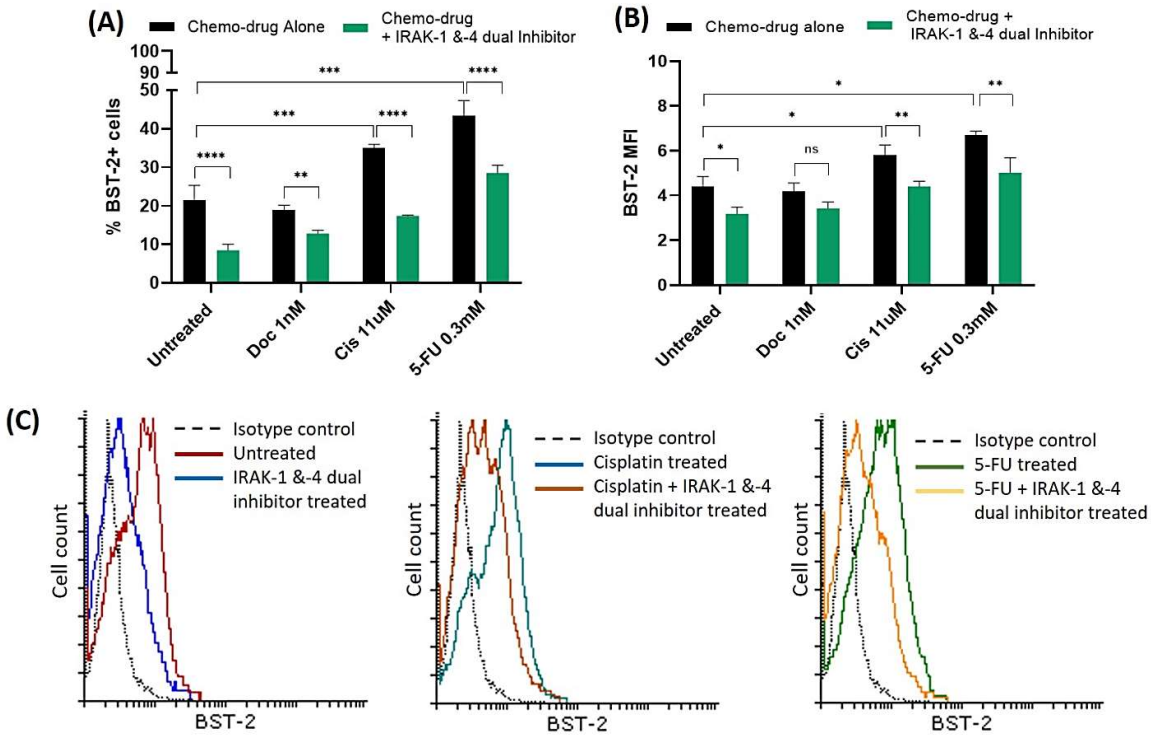

Figure 3: Treatment with IRAK-1 &-4 dual inhibitor suppresses BST-2 expression alone and in combination with chemo-drugs. Statistical analysis of (A) percentage of BST-2+ cells and (B) MFI of BST-2 in HEp-2 upon combination treatment. (C) Representative flow cytometric histogram images demonstrating the effect of treatments on BST-2 expression in HEp-2. (ns: not-significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.)

DISCUSSION AND CONCLUSION

HNSCC is a highly malignant cancer and is associated with poor prognosis. Due to the lack of effective molecular predictors, most patients present with advanced HNSCC. Treatment options of HNSCC are surgery, radiotherapy and chemotherapy but are associated with poor outcomes. Combination of Docetaxel (T), Cisplatin (P) and 5-FU (F) is the preferred chemotherapeutic treatment approved by the US FDA but the overall response rate in patients is only 50% **[23]**. A major cause of chemo-resistance is the presence of cancer stem cells in the micro-environment which eventually leads to cancer relapse **[7]**. Hence, Identification of cancer associated molecular targets is required to facilitate the treatment of patients. In this study, we utilized bioinformatics and *in-vitro* approaches to explore the diagnostic and prognostic potential of an anti-viral protein bone-marrow stromal antigen-2 (BST-2) in HNSCC. Previously, over-expression of BST-2 has been reported to confer pro-oncogenic features such as invasion, migration and therapy resistance in breast cancer **[19, 20]**. Expression of BST-2 also conferred resistance to chemo-therapies such as cisplatin and gefitinib in HNSCC **[21, 22]**.

Data analysis from GEO datasets (n=6) and TCGA samples (n=520) revealed that in comparison to normal tissues, BST-2 was highly over-expressed in HNSCC samples. Further exploration also demonstrated that HNSCC is one of those cancer in which BST-2 expression in high, next to ACC, CESC and OV. BST-2expression also correlated with the tumor stages and grades demonstrating a prognostic value and its significant role in progression of HNSCC. BST-2 expression also correlated with CSCs expression in the human laryngeal cancer cell line HEp-2 indicating its association with stemness in HNSCC. These results largely indicate that BST-2 is a robust prognostic biomarker of HNSCC. It can act as a potential oncogene of HNSCC and a druggable target for the treatment of the cancer.

Moreover, high expression of BST-2 was also observed in cisplatin and 5-FU treated HEp-2 cells. This suggested that chronic chemo-drugs exposure can further aggravate the disease condition and elevating CSCs as evident from BST-2 over-expression. This also suggested that BST-2 could act as a marker of chemo-resistance.

Till date, the use of anti-BST-2 monoclonal antibody for the treatment of myeloma **[17]**, lung cancer **[24]** and endometrial cancer **[25]**, and B49, a BST-2 based peptide for inhibition of invasion and migration of breast cancer **[26]** have been reported in pre-clinical studies. This indicates the limited therapeutic options available for targeting BST-2 and a requirement for investigating and devising new targeted therapies. IRAK-1 &-4 dual inhibitor is a commercially available small molecule inhibitor of the kinase activity of Interleukin-1 receptor associated kinases-1 and -4, downstream kinases in the Toll-like receptor signaling pathway in immune and non-immune cells. This inhibitor has been used to target IRAK-1 & IRAK-4, that are generally found over-expressed in diseased conditions **[27, 28]**. Use of small molecule inhibitors is advantageous as they can be orally administered and can easily bind to intracellular targets. In this study, we observed that treatment with IRAK-1 &-4 dual inhibitor reduced the expression of BST-2 in HEp-2. IRAK-1 &-4 dual inhibitor also suppressed the chemo-therapy mediated BST-2 expression in HEp-2. Through this study, we report the use of IRAK-1 &-4 dual inhibitor as a prospective treatment for HNSCC and to overcome chemo-resistance in HNSCC. This holds promising effect for future HNSCC immunotherapy. However, findings need to be validated in other HNSCC cell lines and pre-clinical models. Our results also support the approach to explore IRAKs based TLR signaling

inhibition effect on BST-2 expression in other cancer types.

CONFLICT OF INTERESTS

None declared.

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