PTPRT/D MUTATION OR PROMOTER METHYLATION: IMPLICATIONS FOR STAT3 INHIBITION

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Head and neck squamous cell carcinoma (HNSCC) is a morbid and frequently fatal malignancy arising from the squamous epithelium of the upper aerodigestive tract. Survival rates have remained low and stagnant in recent decades even as our understanding of this disease has led to new treatment approaches, most notably the approval in 2006 of cetuximab, a monoclonal antibody targeting the epidermal growth factor receptor. The paucity of broadly effective targeted therapies for HNSCC patients illustrates the need for new targets for pharmacologic inhibition and biomarkers for predicting exquisite response to such agents. STAT3 is a potent oncogene that is hyperactivated by constitutive tyrosine phosphorylation in nearly all HNSCCs, where STAT3 represents a rational target for inhibition. As it is increasingly clear that most targeted therapies are unlikely to be broadly effective in unselected groups of patients, we have sought to identify genetic/epigenetic alterations of phosphatases that normally downregulate STAT3 in order to assess the potential utility of these alterations as predictive biomarkers for STAT3-targeted therapeutics. Our findings reveal that somatic mutation or promoter hypermethylation of PTPRT or PTPRD leads to loss of function of these phosphatases in HNSCC, concomitant with increased activation of STAT3 in preclinical models and tumor specimens. Importantly, these events are also associated with increased sensitivity to inhibitors of the STAT3 pathway in preclinical models. Together, these studies indicate that genetic or epigenetic alterations leading to loss of function of phosphatases that target STAT3 may

ultimately serve as biomarkers for the selection of patients who will be most likely to respond to STAT3 inhibitors that are currently in preclinical and clinical development.

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List of Abbreviations:

5-aza	5-Azacytidine
AD	Adenocarcinoma
ALK	Anaplastic Lymphoma Kinase
AML	Acute Myeloid Leukemia
ATCC	American Type Culture Collection
bFGF	Basic Fibroblast Growth Factor
BLCA	Bladder Urothelial Carcinoma
BRCA	Breast Invasive Carcinoma
CCLE	Cancer Cell Line Encyclopedia
cdk	Cyclin-Dependent Kinase
CESC	Cervical Squamous Cell carcinoma and Endocervical Adenocarcinoma
COAD	Colon Adenocarcinoma
CREB	cAMP-response Element-binding Protein
CTD	Carboxy-terminal Domain
DBD	Deoxyribonucleic Acid Binding Domain
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide

DNA	Deoxyribonucleic Acid
DNMT	DNA Methyltransferase
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EGFRvIII	Epidermal Growth Factor Receptor Variant III
ESCA	Esophageal Carcinoma
FBS	Fetal Bovine Serum
FFPE	Formalin-fixed, Paraffin-embedded
FGFR	Fibroblast Growth Factor Receptor
FN3	Fibronectin Type III-like
GBM	Glioblastoma Multiforme
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
gp130	Glycoprotein 130
HBV	Hepatitis B Virus
HER2	Human Epidermal Growth Factor Receptor 2
HNSCC	Head and Neck Squamous Cell Carcinoma
IEC	Intestinal Epithelial Cell
IFN	Interferon
IgG	Immunoglobulin G
IL-6	Interleukin-6
JAK	Janus Kinase
K685	Lysine 685

KIRC	Kidney Renal Cell Carcinoma
KIRP	Kidney Renal Papillary Cell Carcinoma
LIHC	Liver Hepatocellular Carcinoma
LUAD	Lung Adenocarcinoma
LUSC	Lung Squamous Cell Carcinoma
MMP	Matrix Metalloproteinase
mRNA	Messenger Ribonucleic Acid
MSP	Methylation-specific Polymerase Chain Reaction
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear Factor Kappa-light-chain-enhancer of Activated B cells
NSCLC	Non-small Cell Lung Cancer
OCT-1	Octamer Transcription Factor 1
PBS	Phosphate-buffered Saline
PDB	Protein Data Bank
PDGFR	Platelet-derived Growth Factor Receptor
PDPN	Podoplanin
PI3K	Phophatidylinositol-4,5-bisphosphate 3-kinase
PIAS	Protein Inhibitor of Activated STAT
PPID	Protein-Protein Interaction Domain
pSTAT3	Phospho-Signal Transducer and Activator of Transcription (Tyrosine 705)
PTP	Protein Tyrosine Phosphatase
PTPN	Protein Tyrosine Phosphatase Non-Receptor-Type
PTPR	Protein Tyrosine Phosphatase Receptor-Type

PTPRT/D	Protein Tyrosine Phosphatase Receptor-Types T and D
pY	Phospho-tyrosine
RNA	Ribonucleic Acid
RPPA	Reverse-phase Protein Array
RTK	Receptor Tyrosine Kinase
S727	Serine 727
SCC	Squamous Cell Carcinoma
SH2	SRC-homology 2
shPTPRT	shRNA Targeting *PTPRT* mRNA
shRNA	Small Hairpin Ribonucleic Acid
shScr	Sequence-scrambled shRNA Control
SOCS	Suppressor of Cytokine Signaling
STAT	Signal Transducer and Activator of Transcription
SUMO	Small Ubiquitin-like Modifier
TCGA	The Cancer Genome Atlas
TCPA	The Cancer Proteome Atlas
TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factor
VEGF	Vascular Endothelial Growth Factor
WT	Wild-type
WTX	Wilms Tumor Gene on the X Chromosome Protein
Y705	Tyrosine 705

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1.0 INTRODUCTION

1.1 POTENTIAL FOR TARGETING STAT3 IN HUMAN MALIGNANCY

1.1.1 Introduction to STATs

The signal transducer and activator of transcription (STAT) family is group of ubiquitously expressed proteins involved in a wide variety of cellular processes. Canonical STAT signaling involves STAT monomers localized in the cytoplasm where they receive a wide variety of specific upstream signals. Upon activation, STATs dimerize and translocate to the nucleus where they activate transcription of specific target genes, ultimately leading to altered protein expression and cellular phenotype. To date, seven STAT family members have been identified, including STAT 1, 2, 3, 4, 5A, 5B, and 6, though multiple isoforms of each have also been found. Each STAT protein includes several conserved domains that contribute to protein function (**Figure 1**). The N-terminal protein-protein interaction domain (PPID) mediates interaction between neighboring STAT proteins (or other co-regulatory proteins) and contributes to cooperative binding of STAT dimers on DNA, leading to the formation of stabilized tetramers. [1] This function, while non-essential for transcriptional activation, may contribute to enhanced STAT3 signaling by prolonging DNA binding. The DNA-binding domain (DBD) is involved in sequence-specific DNA binding, recruitment of co-activators, and the activation of transcription of STAT3 target genes. The SRC-homology 2 (SH2) domain is the mediator of STAT dimerization via reciprocal phosphotyrosine binding, a critical step for STAT activation. It is

also involved in the recruitment of STAT to phosphotyrosine residues on other proteins, including tyrosine kinases, which then phosphorylate and activate STAT. The carboxy-terminal domains (CTD) present in STAT1 and STAT2 are involved in further protein-protein interactions that impact STAT function, including those with co-modulators of transcription such as the CREB binding protein. [2] These domains coordinately determine the varied functions of each STAT protein.

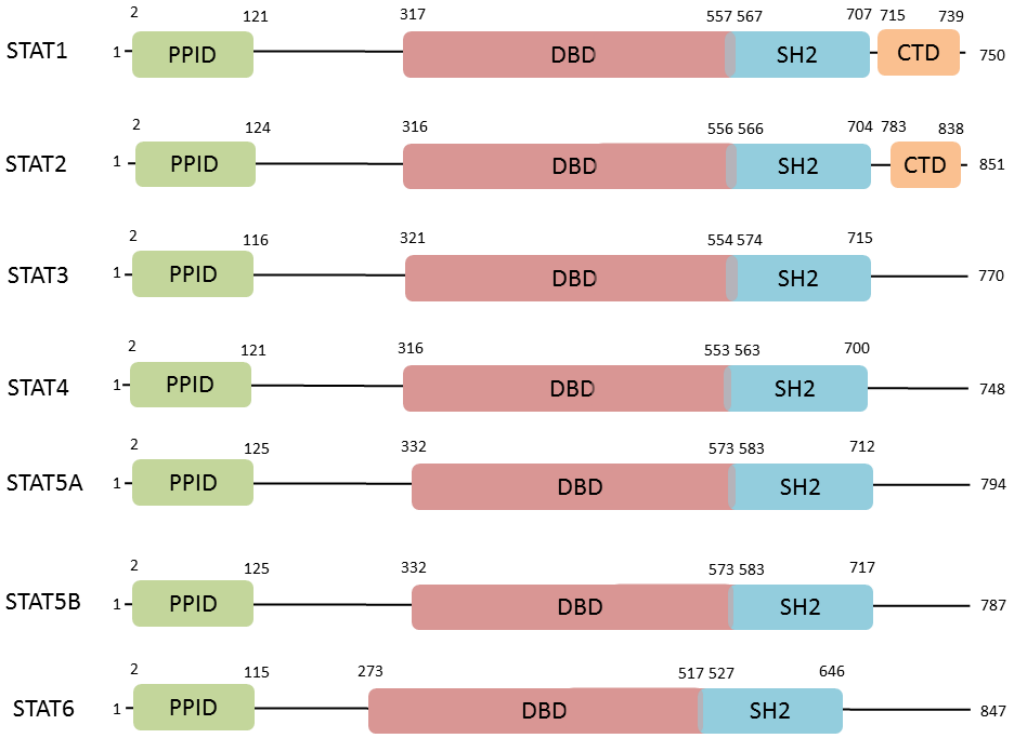


Figure 1. Domain architecture of STATs. Members of the STAT protein family share similar domain structure, with each having an N-terminal protein-protein interaction domain (PPID), a DNA-binding domain (DBD), and a SRC-homology 2 domain (SH2). STAT1 and STAT2 also have a C-terminal domain (CTD) that is involved in further protein-protein interactions. Numbers indicate amino acid positions as determined by the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov).

STAT proteins were originally identified in the context of their physiological roles as major effectors of cytokine and chemokine receptor signaling. In recent years, the discovery that

dysregulated STAT proteins are key modulators of human malignancy has driven research into the functions of these proteins. It now appears that the contribution of STAT proteins to cancer, especially overexpression and hyperactivation of STAT3, is crucial for the development and progression of many cancers. As such, STAT3 is likely a promising target for the development of inhibitors and is the focus of much ongoing research and drug development.

1.1.2 Physiological Roles of STAT3

Phosphorylation of STAT3 on tyrosine 705 (Y705) by various upstream kinases is critical for STAT3 activation. [3] A large number of protein tyrosine kinases directly phosphorylate STAT3 on Y705, including cytokine and chemokine receptors and their co-activators. Well-studied examples of such kinases include membrane integral receptor tyrosine kinases (RTKs) such as the epidermal growth factor receptor (EGFR), fibroblast growth factor receptors (FGFRs), and non-receptor tyrosine kinases that may or may not be associated with receptors such as Janus kinase (JAK), SRC, and ABL. [3-6] Upon STAT3 activation, dimers directly bind DNA at $TT(N)_{4-6}AA$ consensus sites and regulate transcription of specific target genes. [7] The binding affinity of STAT3 for this region is determined by both the nucleotide sequence and cooperative dimer-dimer interactions mediated by the amino-terminal PPID of STAT3. [7, 8] STAT3 activity can be further modulated by phosphorylation of serine 727 (S727), though the context-specific consequences of S727 phosphorylation remain incompletely understood. [9, 10] In addition, STAT3 activity can be modified by reversible acetylation of lysine 685 (K685), an event that is critical for the formation of stable STAT3 dimers and required for cytokine-induced STAT3-mediated transcription. [11, 12]

Downregulation of STAT3 occurs by several mechanisms in normal biology. The suppressor of cytokine signaling (SOCS) family of proteins, especially SOCS1 and SOCS3, inhibit STAT3 in a cytokine-inducible manner by binding to and inhibiting upstream JAKs. [13, 14] Members of the protein inhibitor of activated STAT (PIAS) family, especially PIAS3, are small ubiquitin-like modifier (SUMO)-E3 ligases that bind specifically to STAT3 and abrogate its activity. [15] The SOCS and PIAS families together constitute major mechanisms by which STAT3 activity is downregulated quickly following stimulation with specific cytokines under normal conditions. Other proteins, including GRIM-19, can also abrogate STAT3 activity via direct interactions. [16] Importantly, enzymatic removal of the phosphate group from Y705 of STAT3 by protein tyrosine phosphatases (PTPs) can also occur. Like the upstream kinases, PTPs that inactivate STAT3 can be membrane integral (PTPR family, including PTPRT and PTPRD) or cytosolic (PTPN family, including PTPN2 and PTPN11). [17-20] Additionally, removal of the acetyl group from K685 of STAT3 by deacetylases, including SirT1, can lead to STAT3 downmodulation. [21, 22] The intricacy and redundancy of the many mechanisms of STAT3 activation and deactivation illustrate the importance of maintaining tight control of the STAT3 pathway in normal biology.

Perhaps the field of normal biology in which STAT3 is most well-studied is that of inflammation and the immune response. The actions of many cytokines and chemokines that led to the discovery of the STAT family, especially IL-6 and interferon (IFN), are mediated principally by STAT3 and are critical for proper immune function. STAT3 activation is triggered in epithelial tissue and associated macrophages in response to IgG complex deposition or injury. [23] In dendritic cells, the pro-inflammatory activity of IL-6 is mediated by transient activation of STAT3 while the anti-inflammatory effects of IL-10 are due to more sustained STAT3

activation. [24] Interestingly, artificial early termination of IL-10 signaling leads to an IL-6-like cellular response. [24] This rapid termination of IL-6 signaling appears to be mediated by SOCS3, which is a STAT3 target gene upregulated by both IL-6 and IL-10 that can inhibit signaling through the IL-6 receptor, but not the IL-10 receptor. [24] These findings suggest that the divergent consequences of various signals upstream of STAT3 may in turn be determined by the contribution of STAT3 inactivators that ultimately determine the duration of STAT3 signaling.

Increased STAT3 activity is also associated with wound healing. As many of the genes involved in wound healing are also involved in oncogenesis, it is not surprising that STAT3 regulates many of the same genes in both of these processes. [25] After cutaneous wounding in mice, IL-6 is upregulated in the epidermis primarily at the leading edge of the wound. [26] Genetic knockout of *Il-6* in mice leads to deficient cutaneous wound healing, with knockout mice requiring up to three-fold longer to heal than wild-type mice. [26] Keratinocyte-specific *Stat3* knockout in mice leads to impaired skin remodeling that results from impaired epidermal cell regeneration, confirming a central role for STAT3 in normal wound healing. [27] In the gut, STAT3 activation in intestinal epithelial cells (IECs) regulates immune homeostasis. [28] Colonic $CD11c^{+}$ cells secrete IL-22 in response to Toll-like receptor activation, leading to STAT3 activation in IECs and promoting wound healing, demonstrating that STAT3 is essential for the wound healing process in a variety of tissues. [28]

STAT3 plays additional roles in several other normal cellular processes. For example, STAT3 functions as the downstream effector of important hormones such as insulin and leptin in both the brain and peripheral tissues, allowing for regulation of energy and metabolite homeostasis. [29-32] STAT3 is also involved in autophagy, embryogenesis, proper thymic

function, mammary development, and other processes. [33-36] The importance of STAT3 activity in normal biology is demonstrated in part by the ubiquity of its tissue distribution. STAT3 activation across these tissues is a transient event, and STAT3 is quickly downregulated. When aberrations occur in the strict regulation of STAT3, malignancies can develop.

1.1.3 Role of STAT3 in Cancer

1.1.3.1 Genomic and Epigenomic Deregulation of STAT3 in Cancer The STAT3 protein is overexpressed and/or hyperactivated in the majority of human cancers. [37] The prevalence of STAT3 hyperactivation in cancer cannot be explained by mutational activation of STAT3 as somatic mutation of the *STAT3* gene in cancer is rare (1.1%; 64/5626 cancers analyzed to date by whole exome sequencing by The Cancer Genome Atlas [TCGA]). [38] Instead, STAT3 is the common effector of activating events affecting oncoproteins and deactivating events affecting tumor suppressive proteins that ultimately lead to constitutive STAT3 activation. Dysregulation of diverse pathways that converge on STAT3 allows the escape from strict regulation that maintains transient STAT3 signaling in normal cell biology, leading to tumor-promoting cell proliferation, survival, motility, invasion, and angiogenesis. In addition, activation of STAT3 is associated with emergent resistance to targeted therapies and decreased patient survival. [39, 40]

Among the first observations that indicated the importance of STAT3 in cancer was the phosphorylation of STAT3 by v-SRC, a known oncoprotein, and constitutive STAT3 tyrosine phosphorylation and DNA-binding in several v-SRC-transformed cell lines. [41] Further study revealed that STAT3 activation and specific gene regulation is required for SRC-mediated transformation of NIH-3T3 cells, leading to the conclusion that activation of STAT3 signaling is a critical component of malignant transformation. [42, 43] Additional studies generated similar

findings in diverse systems, providing a strong case for the central role of STAT3 in a wide array of cancers. [39, 44-49] Years of continued research have convinced physicians and scientists of the significance of STAT3 in cancer, and have elucidated many, though certainly not all, of the mechanisms by which aberrant STAT3 signaling contributes to malignancy.

In addition to SRC, many kinases upstream of STAT3 activation are frequently altered in cancer, leading to constitutive kinase and STAT3 signaling. In neuroblastoma, frequent point mutation of the receptor tyrosine kinase ALK in the kinase domain (F1174L) leads to constitutive activation of STAT3. [50] Forced expression of this mutant, but not wild-type ALK, is sufficient to transform Ba/F3 cells, enables cytokine independent growth, and confers sensitivity to the small molecule ALK inhibitor TAE684 in neuroblastoma cell line models. [50] Further, in ALK-positive anaplastic large-cell lymphoma cells that overexpress of STAT3, inhibition of ALK leads to downregulation of total and active STAT3. [51] Similar results have been found for other kinase domain mutations, including the well-studied JAK2 mutation V617F, which is primarily found in myeloproliferative disorders. [52, 53] Activation of JAK2 caused by this mutation leads to constitutive activation of STAT3 and is associated with reduced survival in idiopathic myelofibrosis. [54, 55] Another mechanism of kinase-driven STAT3 activation in cancer is genomic amplification of kinase genes or RTK ligands with subsequent protein overexpression, leading to enhanced activation of wild-type kinases. For example, gene amplification of *PDGFRA* or *EGFR* in distinct subsets of glial tumors leads to enhanced expression of the proteins encoded by these genes and activation of downstream signaling events, including STAT3 phosphorylation. [56] Overexpression of RTK ligands, such as IL-6 or TGF-α, can also lead to persistent STAT3 activation via autocrine signaling through their receptors. [57, 58] Other genomic events and rearrangements can also lead to kinase and STAT3

activation, such as that observed for the EGFRvIII protein, a constitutively active EGFR variant that is missing a large portion of the extracellular domain and exhibits impaired EGF binding. EGFRvIII expression is sufficient to transform NR6 cells (murine fibroblasts) and is associated with STAT3 activation and target gene expression. [59, 60]

Conversely, activation of STAT3 in human cancers can result from genomic or epigenomic inactivation of proteins that normally downregulate STAT3 activity. In contrast to the frequent activation of kinases by point mutation, deactivation of tumor suppressive proteins by point mutation is relatively rare, though there are notable exceptions including deactivation of *TP53*. Other examples include recently reported mutations in GRIM-19 that ablate its STAT3 inhibitory activity and promote tumor growth. [16] Many investigators have recently begun to focus on epigenomic silencing of tumor suppressive proteins that normally downregulate STAT3, especially by promoter hypermethylation. In lung cancer for example, SOCS3 is frequently downregulated by promoter hypermethylation, and restoration of SOCS3 expression in cells where it was previously silenced leads to downregulation of active STAT3, induction of apoptosis, and suppression of cell growth. [61] As SOCS proteins have not been demonstrated to inhibit kinases other than JAKs, inactivation of the SOCS family is unlikely to contribute substantially to aberrant STAT3 signaling across cancer types. Indeed, SOCS1 is unable to inhibit STAT3-mediated transformation of NIH-3T3 cells by v-SRC and does not reduce STAT3 target gene expression in this system. [62] Abnormal epigenomic alteration of other proteins that normally cause direct inactivation of STAT3, especially protein tyrosine phosphatases, remains incompletely understood and warrants further study. For instance, frequent methylation in the promoter region of *PTPN6* is strongly correlated with decreased *PTPN6* mRNA expression and increased pSTAT3 expression in immunodeficiency-related non-Hodgkin lymphoma (but not in

human immunodeficiency virus-related Burkitt lymphoma), demonstrating that epigenetic silencing of a phosphatase targeting pSTAT3 can lead to STAT3 activation. [63] Additional STAT3 suppressive proteins, including the glutathione S-transferase family member GSTP1, which downmodulates EGF-mediated STAT3 signaling and expression of STAT3 target genes via a direct interaction with STAT3, is promoter hypermethylated in HBV-associated hepatocellular carcinoma and prostate cancer, and is subsequently downregulated. [64-66]

The diversity of genomic and epigenomic alterations in both activators and deactivators of STAT3 signaling is in part responsible for the high degree of difficulty in developing therapeutics that are applicable to a wide array of cancers and suggests that targeting STAT3 directly may prove more efficacious. The further understanding of the many mechanisms contributing to aberrant STAT3 pathway activation may lead to the identification of biomarkers that can be used to establish subsets of patients who will be most likely to respond to STAT3 inhibition.

1.1.3.2 STAT3 in Cell Growth and Proliferation STAT3 is a critical driver of dysregulated cell growth in cancer, but not in normal cells. [67] Constitutive STAT3 signaling has been implicated in aberrant cell growth and proliferation in many cancers, including head and neck squamous cell carcinoma, colorectal carcinoma, melanoma, glioblastoma multiforme, multiple myeloma, non-small cell lung cancer, and others. [58, 68-72] A critical mediator of cell growth downstream of STAT3 is its target gene *CCND1*, encoding cyclin D1, which is upregulated transcriptionally by active STAT3 and is required for STAT3-mediated transformation. [73] Cyclin D1 in turn acts through cyclin-dependent kinase (cdk)-dependent and cdk-independent mechanisms to allow passage through the G1 checkpoint of the cell cycle, ultimately leading to continuous and unregulated cell growth and proliferation. [74] In addition, the STAT3 target

gene *MYC*, which itself encodes a transcription factor, also potently promotes cell growth and is required for SRC-mediated cellular transformation via STAT3. [45]

Other target genes of STAT3 that contribute to cell growth and proliferation include cytokines and growth factors that often act in an autocrine manner to further increase STAT3 signaling and/or other mitogenic pathways. It has recently been appreciated that non-traditional gene products, including microRNA molecules that downregulate specific genes by binding to specific mRNA transcripts, are also mediators of STAT3 mitogenic function. For example, STAT3 is persistently active in Wilms tumor, a genetically heterogeneous childhood kidney cancer, where it transcriptionally upregulates the microRNA *miR-370*, which in turn regulates cell proliferation and tumorigenicity in mice. [75] Cells transfected with *miR-370* exhibit downregulation of the tumor suppressor WTX (Wilms tumor gene on the X chromosome protein) via direct binding to the 3'-untranslated region of *WTX* mRNA, leading to its degradation. [75] These cells also exhibit downregulation of the proteins $p21^{Cip1}$ and $p27^{Cip1}$ (which inhibit progress through the cell cycle) and upregulation of cyclin D1, illustrating additional mechanisms downstream of STAT3 that contribute to its proliferative capacity. [75]

1.1.3.3 STAT3 in Apoptosis and Cell Survival Constitutive STAT3 activation leads to evasion of apoptosis and a subsequent increase in cell survival. STAT3 transcriptionally regulates several BCL-2 family members, including the anti-apoptotic proteins BCL-xL, BCL-2, and MCL-1. [76] The BCL-2 family regulates apoptosis via homo/heterodimerization (the dynamics of which are determined stoichiometrically) and translocation to the mitochondrial membrane, where they ultimately regulate cytochrome c release and the initiation of apoptosis. STAT3-mediated upregulation of BCL-xL, BCL-2, and MCL-1 contributes to apoptosis evasion in several cancers. [76-78] STAT3-mediated BCL-2 expression in metastatic subclones of the parental cell line

MDA-MB435 (estrogen receptor-negative breast cancer) correlates with increased pSTAT3, but not with other transcription factors that regulate BCL-2, and contributes to chemoresistance in this cell line, suggesting that the anti-apoptotic effects of STAT3 contribute to treatment resistance. [79] Furthermore, a small peptide, ST3-H2A2, which inhibits the function of the N-terminal PPID of STAT3 induces the expression of multiple pro-apoptotic genes (and others) in prostate cancer cells, suggesting that STAT3 inhibition may restore normal apoptosis. [80]

STAT3 target genes that are not themselves in the BCL-2 family can also contribute to evasion of apoptosis. Octamer transcription factor-1 (*Oct-1*) has been reported to be a target gene of STAT3 in esophageal squamous carcinoma cells (Eca-109), where STAT3 and OCT-1 coordinately regulate apoptosis. [39] In these cells, activation of STAT3 by IL-6 treatment suppresses apoptosis as assessed by TUNEL staining, and knockdown of either *STAT3* or *Oct-1* by RNA interference enhances apoptosis. [39] Conversely, forced overexpression of OCT-1 (even in the presence of *STAT3* knockdown) is sufficient to reduce apoptosis to similar levels as IL-6 treatment, suggesting that STAT3-driven OCT-1 expression may be sufficient to reduce apoptosis to minimal levels in these cells. [39] *STAT3* and *Oct-1* knockdown leads to increased expression of pro-apoptotic BAX and BAD proteins, cytochrome c release from mitochondria, subsequent cleavage of caspase-3 and -9, and decreased expression of anti-apoptotic BCL-2 and BCL-xL proteins. [39] These findings provide a mechanism by which STAT3 hyperactivation leads to positive feedback in the suppression of apoptosis in conjunction with its target gene *Oct-1*.

1.1.3.4 STAT3 in Migration and Invasion Constitutively active STAT3 further contributes to the cancer phenotype by promoting motility and invasion, including in human melanoma where increased activation of STAT3 promotes metastasis to the brain. [81] The metastatic action of

STAT3 is in part mediated by matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases that are secreted into the extracellular matrix (ECM). There the MMPs degrade ECM proteins, leading to facilitated cell migration, invasion through the basement membrane, and ultimately the establishment of metastatic secondary tumors. The STAT3 target genes *MMP-2* and *MMP-9* are upregulated in esophageal squamous carcinoma cells (Eca-109) that express high pSTAT3, and *STAT3* knockdown by RNA interference in these cells leads to downregulation of MMP-2 and MMP-9, dysregulation of cell migration directionality, decreased migration speed, and disorganization of F-actin formation, demonstrating a central role for STAT3 in MMP-2/9-mediated cell motility. [39] In addition, activation of STAT3 is required for maximal MMP-1 and MMP-10 induction in response to EGF in T24 bladder cancer cells, where STAT3 is a critical mediator of malignant characteristics. [82]

Other mechanisms that contribute to STAT3-mediated cell migration have been elucidated. For instance, EGFR activation via autocrine signaling in near-confluent, but not sparse, squamous cell carcinoma cells leads to activation of STAT3 and subsequent overexpression of the transmembrane glycoprotein podoplanin (PDPN). [83] This cell density-regulated PDPN expression leads to increased cell migration and invasion, and these effects are reversed by shRNA knockdown of *PDPN*. [83] Importantly, the observation of increased PDPN extends to clinical samples, in which PDPN is overexpressed in basal cell layers at the invading front of *in situ* SCC lesions, providing an additional clinically relevant mechanism by which STAT3 contributes to motility and invasion. [83] Similarly, STAT3 is necessary for EGFR-mediated migration and invasion in prostate carcinoma cells. [84] shRNA knockdown of *STAT3* in Tu-2449 glioma cells additionally leads to decreased PDPN expression and microvilli

formation relative to vector infected cells. [85] Thus, inhibition of STAT3 may be an effective strategy for preventing malignant transformation and metastasis in several human cancers.

1.1.3.5 STAT3 in the Tumor Microenvironment STAT3 is a critical regulator of the tumor microenvironment. For example, STAT3 is the downstream effector of several cytokine receptors that are involved in promoting angiogenesis, including those for vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), leptin, IL-6, and granulocyte-macrophage colony-stimulating factor (GM-CSF). [86-89] In addition, STAT3 can promote transcription of pro-angiogenic factors, including VEGF and IL-6, leading to paracrine and/or autocrine feedback. [90-92] Cytokine excretion from tumor cells also act upon neighboring endothelial cells to promote proliferation, migration, and microvascular tube formation, leading to the development of mature blood vessels. The contribution of STAT3 activation to tumor angiogenesis both in tumor cells and in endothelial cells suggests that inhibition of STAT3 may be an efficient method for blocking angiogenesis and tumor progression.

STAT3 is also involved in inflammation-associated carcinogenesis, suppression of the anti-tumor immune response, and maintenance of cancer stem cells. For example, NF-κB-mediated expression of IL-6 and subsequent activation of STAT3 is required for survival and evasion of apoptosis in intestinal epithelial cells during the development of colitis-associated cancer, a serious complication of irritable bowel disease. [93, 94] Furthermore, STAT3 activity is associated with reduced T cell infiltration in isogenic murine melanomas, suggesting a role for STAT3 in suppressing anti-tumor immunity. [95] Inhibition of STAT3 in these tumor cells and also in glioblastoma multiforme (GBM) cell models stimulates secretion of soluble factors, including TNF-α and IFN-β, that ultimately lead to increased infiltration of lymphocytes, natural killer cells, neutrophils, and macrophages, and also activate nitric oxide production from

macrophages *in vivo* and *in vitro*. [95, 96] In addition, genetic or pharmacologic inhibition of STAT3 in GBM stem cells, even transiently, leads to a loss of multipotency and irreversible growth arrest, suggesting that STAT3 is required for maintenance and proliferation of cancer stem cells in this system. [97] Thus, several mechanisms exist by which STAT3 inhibition may lead to tumor microenvironment disruption and subsequent regression.

1.1.4 Overview of the Current STAT3 Inhibitors in Clinical Development

Table 1-1 summarizes the STAT3 inhibitors that are currently in clinical development according to www.clinicaltrials.gov. Both the Isis and AstraZeneca compounds are antisense oligonucleotide inhibitors of *STAT3* mRNA, while the Otsuka compound is a small molecule that binds to a distinct pocket in the SH2 domain. A Phase I study of OPB-51602 in refractory solid tumors has recently established a maximum tolerated dose and recommended Phase II dose, with common toxicities including fatigue, nausea, anorexia, and early-onset peripheral neuropathy. [98] Preliminary data from a Phase I trial of AZD9150 presented at the 2014 EORTC-NCI-AACR Symposium on Molecular Targets and Cancer Therapeutics indicate that a tolerable dose with pharmacodynamic activity has been identified in patients, and further investigation is ongoing. [99]

Table 1. STAT3 inhibitors currently in clinical development according to clinicaltrials.gov

Drug Name	Phase	Condition	STAT3 Targeting Strategy	Sponsor	Status	PubMed Identifier
STAT3 Decoy	0	HNSCC	DBD oligonucleotide decoy	University of Pittsburgh	Complete	22719020
AZD9150	1	Advanced adult hepatocellular carcinoma, heptaocellular carcinoma metastatic	Antisense oligonucleotide	AstraZeneca	Complete	Not yet published
ISIS-STAT3$_{Rx}$	1/2	Advanced cancers, DLBCL, lymphoma	Antisense oligonucleotide	Isis Pharmaceuticals	Active, not recruiting	Not yet published
OPB-51602	1	Advanced cancer	Small molecule, target unknown	Otsuka Pharmaceutical Development & Commercialization, Inc.	Complete	25609248

For a full report of the completed STAT3 decoy Phase 0 trial and further development, see [100]. The decoy, which was designed to bind to the STAT3 DBD and prevent STAT3 binding to STAT3 response elements in genomic DNA, consists of a 15-mer duplex oligonucleotide with phosphorothioate caps at the 5' and 3' ends to enhance stability *in vivo*. Intratumoral injection of this molecule immediately prior to surgical resection in HNSCC patients led to decreased expression of STAT3 target genes relative to saline-injected tumors in a Phase 0 trial, confirming the ability of the decoy to downregulate STAT3 signaling in human tumors. Systemic administration of the decoy in a murine xenograft model failed to demonstrate any effect on tumor growth or STAT3 signaling. One challenge presented by this approach was the low stability of the decoy molecule in serum. To overcome this difficulty, modified decoys were designed and tested. A circularized decoy consisting of the original decoy with two hexaethylene glycol linkages demonstrated enhanced stability in serum, with detectable levels up to 12 hours. Importantly, systemic administration of the cyclic decoy in murine xenograft models by intravenous injection inhibited tumor growth and expression of STAT3 target genes, demonstrating a successful strategy for inhibiting intratumoral STAT3 signaling via systemic, rather than intratumoral, administration. The cyclic decoy has not yet been tested in humans, as efforts are underway to further improve its preclinical pharmacodynamic and pharmacokinetic parameters.

In addition to targeting STAT3 via its DNA-binding domain with an oligonucleotide decoy, STAT3 may be targeted via its SH2 domain by small molecules, peptides, or peptidomimetic compounds. [101-104] Such molecules are designed to disrupt STAT3 dimerization, thus preventing its translocation to the nucleus and transcription. Other inhibition strategies include the introduction of antisense oligonucleotides (as in the case of the

Isis/AstraZeneca drug in clinical development) designed to cause degradation of *STAT3* mRNA or prevent its translation via complementary base paring, thereby reducing total STAT3 protein levels. [105] Antisense strategies in particular will require exquisite tissue specificity, as they may lead to reduced expression of STAT3 in normal tissues where its function is required. Recent high throughput and *in silico* screens also have the potential to identify novel strategies for targeting STAT3. [101, 106]

1.1.5 Critical Analysis of the Potential use of STAT3 Inhibitors in the Management of Human Malignancy

In order for any protein to be the optimal target of inhibition for the treatment of cancer, it must exhibit several characteristics. Inhibition of the target protein must lead to suprression of cell growth/proliferation/survival, motility/invasion, and angiogenesis, and enhancement of the anti-tumor immune response. The ideal target would also be applicable across a wide variety of cancer types. The inhibition of STAT3 in preclinical models has demonstrated all of these characteristics across a wide variety of cancers, most likely via reversal of the many mechanisms discussed above, suggesting that STAT3 inhibitors may ultimately be of especially high impact in treating human malignancy. Importantly, a Phase 0 trial has demonstrated that STAT3 can be effectively targeted in human tumors, and further preclinical studies have suggested that systemic delivery of STAT3 inhibitors is likely to be effective. [100] In addition, because STAT3 signaling is transient in normal tissues and cells, the potential for adverse events following systemic administration of a STAT3 inhibitor is minimal. Indeed, toxicology studies in non-human primates demonstrate a lack of toxicity of a STAT3 decoy oligonucleotide. [107]

Many of the recently approved therapies for cancer target tyrosine kinases that are upstream of STAT3 activation, among other pathways. It is thought that mutations in these kinases would signify constitutive activation and serve as a biomarker for patients that will most likely respond to these therapies. Unfortunately, there has been limited clinical success with currently available inhibitors of these upstream kinases, though in some cases they do prove extremely effective. Studies in preclinical models demonstrate that NSCLC cell lines with mutations in select tyrosine kinases do not exhibit decreased STAT3 activation upon treatment with the respective targeted small molecules erlotinib (EGFR), U0126 (MEK1/2), sunitinib (PDGFRA), or crizotinib (MET), though other downstream effects of these inhibitors, including downmodulation of PI3K signaling, does occur. [108] These findings support the notion that directly targeting STAT3, rather than any large number of its upstream activators, may be more efficacious in reversing the effects of constitutive STAT3 signaling.

Though some patients initially respond to targeted therapies, many develop chemoresistance and secondary cancers that are associated with increased STAT3 signaling. For example, hyperactivation of STAT3 is associated with resistance to EGFR-targeted therapies in several cancers, including HNSCC, bladder cancer, and others. [40, 79] Biopsies of recurrent HNSCC following treatment with cetuximab, an FDA-approved monoclonal antibody targeting EGFR, exhibit elevated pSTAT3 relative to pre-treatment samples, suggesting that STAT3 inhibition may be effective at overcoming acquired resistance or as adjuvant therapy to prevent recurrence. [40] Targeting STAT3 in bladder cancer cell lines that are resistant to cetuximab and exhibit elevated levels of activated STAT3 relative to cetuximab-sensitive cell lines leads to reduced cell viability and downregulation of STAT3 target genes. [40] Importantly, combination of STAT3 inhibition with EGFR blockade significantly enhances antitumor effect *in vivo* relative

to EGFR blockade alone, suggesting that the efficacy of already existing (and approved) drugs may be significantly increased by concomitant treatment with STAT3 inhibitors. [40] An additional mechanism of acquired resistance to targeted therapy is the activation of IL-6 following treatment. For example, acquired resistance to trastuzumab (a monoclonal antibody targeting the HER2/neu receptor) in HER2-positive breast cancer is associated with the activation of an IL-6 inflammatory feedback loop in which downstream STAT3 signaling contributes to cancer stem cell proliferation, providing additional rationale for co-targeting with a STAT3 inhibitor. [109] Likewise, resistance to the tyrosine kinase inhibitor imatinib, which targets the BCR-ABLBCR-ABL oncoprotein, in chronic myeloid leukemia cell models is also associated with increased STAT3 activation and target gene expression. [110] Knockdown of *STAT3* by RNA interference in this context re-sensitizes the cells to imatinib-induced cell death, suggesting that STAT3 inhibition may also be effective at overcoming targeted therapy resistance in hematological malignancies. [110]

Certain challenges in the development of STAT3 inhibitors to date have been overcome. Firstly, STAT3 and other transcription factors were widely regarded as undruggable mainly because they are not exposed to the extracellular surface and do not have clear ligand-binding domains that can be targeted for competitive inhibition. These perceived difficulties have proven surmountable in the case of STAT3 in both preclinical and clinical models, with STAT3-targeting agents effectively downmodulating the pathway and reversing its oncogenic effects. A second obstacle in targeting STAT3 was its structural homology with STAT1, a family member with tumor suppressive properties in many systems. This challenge has been overcome both with oligonucleotide inhibitors (which exploit the exquisite specificity of the DNA-binding domain) and with recent high-throughput *in silico* and *in vitro* screens of compound libraries to identify

candidates that specifically inhibit STAT3 and not STAT1. Both of these strategies may lead to novel clinical therapeutics targeting STAT3 in the near future.

1.1.6 Conclusions

Recent advances in the understanding of STAT3 signaling and its role in cancer have led to the establishment of STAT3 as a potential target for a wide variety of human malignancies. While some clinical success has been found in the treatment of cancer with non-specific chemotherapeutics and some targeted agents, there remains a large need for new classes of inhibitors of novel targets that will be widely applicable, well-tolerated, and highly effective. There is an additional need for the identification of predictive biomarkers of response to these emerging agents. The sum of preclinical and clinical data to date supports a unique role for STAT3 as one such target. Indeed, many therapeutic clinical successes thus far have been associated with decreased STAT3 signaling, but because of the diversity of signaling components upstream of STAT3, the high cost of developing inhibitors for each one, the potential for emergent compensatory mechanisms, and the development of resistance to a given therapy, a more effective strategy will likely be to directly target STAT3.

2.0 FREQUENT MUTATION OF RECEPTOR PROTEIN TYROSINE PHOSPHATASES PROVIDES A MECHANISM FOR STAT3 HYPERACTIVATION IN HEAD AND NECK CANCER

2.1 INTRODUCTION

Tyrosine phosphorylation regulates a multitude of cellular processes by coordinately activating and inactivating signaling proteins. Aberrations of protein tyrosine phosphorylation and signaling are a hallmark of oncogenic events found in most human cancers. The phosphorylation/dephosphorylation of tyrosine residues on signaling proteins is directly mediated by protein tyrosine kinases and phosphatases. Although many cellular factors are known to dynamically control the activity of these enzymes, genetic alterations of kinases and phosphatases in human cancers lead to perturbations in the levels of tyrosine phosphorylated proteins, uncontrolled cell growth, and tumor formation. While activating mutations of tyrosine kinases have been extensively studied [111, 112], cancer-associated mutations of tyrosine phosphatases remain incompletely understood, partly due to the lack of comprehensive genomic analysis of these large arrays of phosphatases, as well as their largely unknown and often ambiguous actions in normal physiology and cancer biology.

Among the 107 known protein tyrosine phosphatases, the Receptor-like Protein Tyrosine Phosphatases (PTPRs) represent the largest family of the human tyrosine phosphatome,

comprising 21 family members [113]. These PTPRs are believed to be crucial for the regulation of inter- as well as intracellular signaling due to the cell surface localization of PTPRs. Selected members of the PTPR family have been reported to function as tumor suppressors, where gene mutation, deletion, or methylation may contribute to the cancer phenotype [113].

STAT3 is an oncogene and constitutive STAT3 activation is a hallmark of human cancers. Activating STAT3 mutations are rare in all cancers studied to date, including HNSCC [114]. While activating mutations of upstream receptor tyrosine kinases leading to increased STAT3 phosphorylation characterize some malignancies (e.g. EGFR mutations in NSCLC [115]), most cancers lack these alterations yet harbor elevated pSTAT3 levels. Importantly, it has been reported that pSTAT3 serves as a substrate for wild-type PTPRT in colorectal cancer cells (SW480 and HT29) and HEK293T cells [17]. pSTAT3 has additionally been reported to be a substrate of PTPRD in glioblastoma models, suggesting that several PTPR family members may exhibit tumor suppressive function by dephosphorylating STAT3 [20].

In the present study we hypothesized that mutation of PTPR family members, including *PTPRT* and *PTPRD,* results in elevated expression levels of tyrosine phosphorylated STAT3 in human HNSCC, and that these mutations may predict sensitivity to STAT3 pathway inhibitors in preclinical and clinical development. *PTPRT/D* are two distinct genes that each code for distinct phosphatases that directly target p-STAT3. Analysis of reverse phase protein array (RPPA) and whole-exome sequencing data demonstrated significant association between *PTPR* mutation and increased p-STAT3 expression levels in HNSCC. Studies in HNSCC models demonstrate that *PTPRT/D* mutations induce pSTAT3 and HNSCC survival, consistent with a “driver” phenotype, while computational modeling revealed functional implications of *PTPR* mutations on p-Tyr-substrate interactions. Collectively, these studies establish first-time evidence of the *de novo*

signaling consequences of *PTPR* mutations on major oncogenic pathway that drives human carcinogenesis. Analysis of whole-exome sequencing results of 374 primary (HNSCCs) revealed that *PTPR* genes are mutated in nearly one-third (30.7%) of HNSCC tumors, compared to a 15.2% mutation rate in the *PTPN* family. This pattern is strikingly consistent across an additional 14 types of solid tumors, implicating a potentially important pathologic contribution of *PTPR* mutations to human carcinogenesis. These cumulative findings suggest that genetic alterations of selected *PTPRs*, including *PTPRT/D*, may induce STAT3 activation and serve as predictive biomarkers for treatment with emerging STAT3 pathway inhibitors.

2.2 MATERIALS AND METHODS

2.2.1 Data Download and Analysis

Mutation, copy number alteration, and RNA-Seq data were aggregated from the cBio Portal and published reports. [38, 116-118] DNA and protein sequences and domain annotations were obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Reverse phase protein array data were obtained from The Cancer Proteome Atlas (http://app1.bioinformatics.mdanderson.org/tcpa/_design/basic/index.html). Statistical tests were performed using GraphPad Prism 5 (GraphPad, La Jolla, CA) or StatXact (Cytel, Cambridge, MA) where noted.

2.2.2 Cell Culture

All HNSCC cell lines were genotypically verified by short tandem repeat DNA profiling. Cal27 and FaDu cells were obtained from ATCC (Manassas, VA). PE/CA-PJ34.12 and PE/CA-PJ49 cells were obtained from Sigma-Aldrich (St. Louis, MO). 686LN cells were obtained from Georgia Chen at MD Anderson Cancer Center (Houston, TX). Cal33 cells were obtained from Gerard Milano (Centre Antoine Lacassagne, Nice, France), and the PCI-52-SD1 cell line was obtained by clonal selection of the parental PCI-52 cell line (University of Pittsburgh Cancer Institute) by rounds of graded serum selection as described. [119] Cal27, Cal33, FaDu, and PCI-52 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Inc., Manassas, VA) supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-Products, West Sacramento, CA). 686LN cells were cultured in DMEM/F12 (Life Technologies, Grand Island, NY) supplemented with 10% FBS. PE/CA-PJ34.12 and PE/CA-PJ49 were cultured in Iscove's Modification of DMEM (Mediatech Inc., Manassas, VA) supplemented with 10% FBS and 2 mM L-glutamine (Life Technologies, Grand Island, NY). All cells were maintained in an incubator at 37°C and 5% CO_2.

2.2.3 Plasmid Constructs and Site-Directed Mutagenesis

pCl-Neo-*PTPRT* was obtained from Addgene (plasmid 16630, Cambridge, MA). pMXs-puro-EGFP vector was obtained from Cell Biolabs (San Diego, CA). The *PTPRT* WT gene was subcloned into the retroviral vector pMXs-puro, and the pMXs-puro-*PTPRT* WT was used as a template for site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) or Phusion site-directed mutagenesis kit (Thermo

Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. pcDNA3.1-*PTPRD* and empty vector were obtained from Timothy Chan (MD Anderson Cancer Center, Houston, TX) and used as a template for site-directed mutagenesis as above. *E. coli* clones were grown on LB/agar plates containing the appropriate selection antibiotic. Three to five clones were selected and grown in 5 mL of LB containing the appropriate antibiotic overnight, and plasmid DNA was isolated using the QIAprep Spin Miniprep Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Mutation sites were then verified by Sanger sequencing. After identification of clones expressing the intended plasmids, 250 mL cultures of the clones were established overnight prior to plasmid DNA isolation with the Hurricane Maxi Prep Kit according to the manufacturer's instructions (GerardBIOTECH, Oxford, OH). Glyerol stocks of each culture were stored at -80°C for subsequent rounds of plasmid amplification and isolation.

2.2.4 Transient Transfection

Cells were transfected using Lipofectamine 2000 (Life Technologies, Carlsbad, CA) or FuGENE HD (Promega Corporation, Madison, WI) according to the manufacturer's instructions. Cells were plated at 250,000 cells per well in 6-well plates one day before transfection with 4 μg of DNA diluted in 200 μL of Opti-MEM (Life Technologies, Carlsbad, CA) containing the appropriate quantity oftransfection reagent. For Lipofectamine 2000 transfections, cells in transfection medium were incubated at 37°C and 5% CO_2 for 4 hrs before the medium was replaced with complete growth medium containing 10% or 0% fetal bovine serum as experimentally appropriate. For FuGENE HD transfections, the transfection mixture was added

directly to growth medium and left unchanged for the duration of the experiment. Cells were then incubated as above for 48-72 hrs before analysis.

2.2.5 Retroviral Infection of HNSCC Cells

Retroviruses were generated using the Platinum Retrovirus Expression Systems (Cell Biolabs, San Diego, CA) according to manufacturer's instructions. Plat-A cells were transfected with 3 µg of retroviral vector carrying the gene of interest (pMXs-puro-*EGFP* as control, pMXs-puro-*PTPRT* WT, pMXs-puro-*PTPRT* mutants). Three days after transfection, fresh retroviruses (in the supernatant of the Plat-A cells) were collected by centrifugation at 1,500 rpm for 5 mins at 4°C to pellet cell debris. Any cell debris left in the supernatant was removed by filtration through a 0.45 µm syringe filter. Fresh retroviruses were immediately used for infection of HNSCC cells. HNSCC cells were plated at ~20% confluency in a T75 flask one day before infection. Infection of HNSCC cells was performed by adding 4.5 mL of fresh retrovirus to the culture flask containing 7.5 mL of complete culture media. Then, 38 µL of polybrene (4 µg/µL stock solution, Sigma-Aldrich, St. Louis, MO) was added to the cells with gentle mixing to improve infection efficiency. Cells were then incubated at 37°C and 5% CO_2 for an additional 48-72 hrs, after which the infection medium was replaced with fresh complete medium. Infection efficiency was estimated by visualization of GFP-expressing cells under a fluorescence microscope. Expression of the gene of interest and alteration of the signaling pathway was assessed within 7-10 days of infection by Western blot.

2.2.6 Western Blotting

Whole cell lysates were prepared in lysis buffer containing phosphatase and protease inhibitors using cell scrapers and sonication followed by centrifugation to remove cell debris. Lysate concentrations were determined with the Bio-Rad Protein Assay reagent (Bio-Rad Laboratories, Inc., Hercules, CA). 40 μg of protein was mixed with the appropriate volume of 4X loading dye containing β-mercaptoethanol and incubated in boiling water for 5 minutes. Samples were loaded into 8-10% polyacrylamide gel containing sodium dodecyl sulfate and allowed to separate at ~125 V. Gels were blotted onto nitrocellulose membranes by semi-dry transfer at 21 V for 50 min. Blots were blocked in 5% milk for 1 hr at room temperature, then incubated with primary antibody in 1% milk overnight at 4°C with agitation. The next day, primary antibody was rinsed off, followed by incubation with horse radish peroxidase-conjugated secondary antibody in 5% milk for 1 hr at room temperature. Blots were then imaged using Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Dallas, TX) and the ChemiDoc™ XRS+ System and Image Lab™ software (Bio-Rad Laboratories, Inc., Hercules, CA). Primary antibodies for pSTAT3 and STAT3 were purchased from Cell Signaling Technology, Inc. (Boston, MA; typical dilution 1:1000-1:3000). PTPRT antibody was produced by and obtained from Dr. Zhenghe Wang (Case Western Reserve University, Cleveland, OH; typical dilution 1:500). β-tubulin primary antibody was purchased from Abcam (Cambridge, MA; typical dilution 1:50,000). Secondary antibodies were purchased from BioRad (Hercules, CA; typical dilution 1:1000-1:3000). All milk and antibody solutions were made in tris-buffered saline containing Tween-20 at pH 7.6. Blots were quantitated by densitometry using ImageJ software.

2.3 RESULTS

2.3.1 *PTPR* Genes are Frequently Mutated Across Cancers and *PTPRT* is the Most Commonly Mutated Family Member

In order to understand the potential genetic contributions of the *PTPR* family to tyrosine phosphorylation-mediated signaling and dysregulation in HNSCC, we comprehensively analyzed *PTPR* family mutations in large cohorts of primary HNSCC tumors. Whole-exome sequencing data of 374 primary HNSCCs were included. Strikingly, 30.7% (115/374) of HNSCC tumors harbored non-synonymous somatic mutations of at least one *PTPR* family member, compared to only 15.2% (57/374) of tumors with mutations of *PTPN* genes, which code for the cytoplasmic PTPs that comprise the second major family of PTPs in the human genome (**Figure 2A**). Further, 7.8% (29/374) of HNSCC tumors contained multiple mutations of *PTPR* family members (from 2-6 *PTPR* mutations per tumor; **Figure 2B**), suggesting a potentially significant contribution of *PTPR* family mutations in this context. Further investigation demonstrated that this high rate of somatic mutation of the *PTPR* family (relative to the *PTPN* family) found in HNSCC is also detected in all 14 types of human solid tumors analyzed (4039 total solid tumors sequenced by TCGA at time of analysis), but not in a hematopoietic malignancy (6/196 AML cases; 3.1%) (**Figure 2C**), suggesting that *PTPR* mutation may substantially contribute to many types of solid tumors.

The availability of comprehensive whole-exome sequencing data (from TCGA) on all *PTPR* genes allowed us to identify the most commonly mutated *PTPR* family members in all sequenced human cancers, including HNSCC. Among the 374 sequenced HNSCC tumors, *PTPRT* is the most frequently mutated *PTPR* (5.6% of cases; 22 mutations total, with one tumor

harboring 2 *PTPRT* mutations), followed by *PTPRD*, *PTPRC*, and *PTPRM*, each mutated in 3.7% of cases. Missense mutation sites in each of the corresponding proteins are depicted in **Figure 2D**. Although *PTPR* mutation rates vary among different cancer types, cumulative results reveal that *PTPRT* is the single most commonly mutated *PTPR* in human cancers (6.2%, 285 mutations in 4609 solid and hematopoietic cancers sequenced), with the highest mutation frequency of *PTPRT* in cutaneous melanoma (a total of 99 mutations in 253 tumors sequenced; 39.1%).

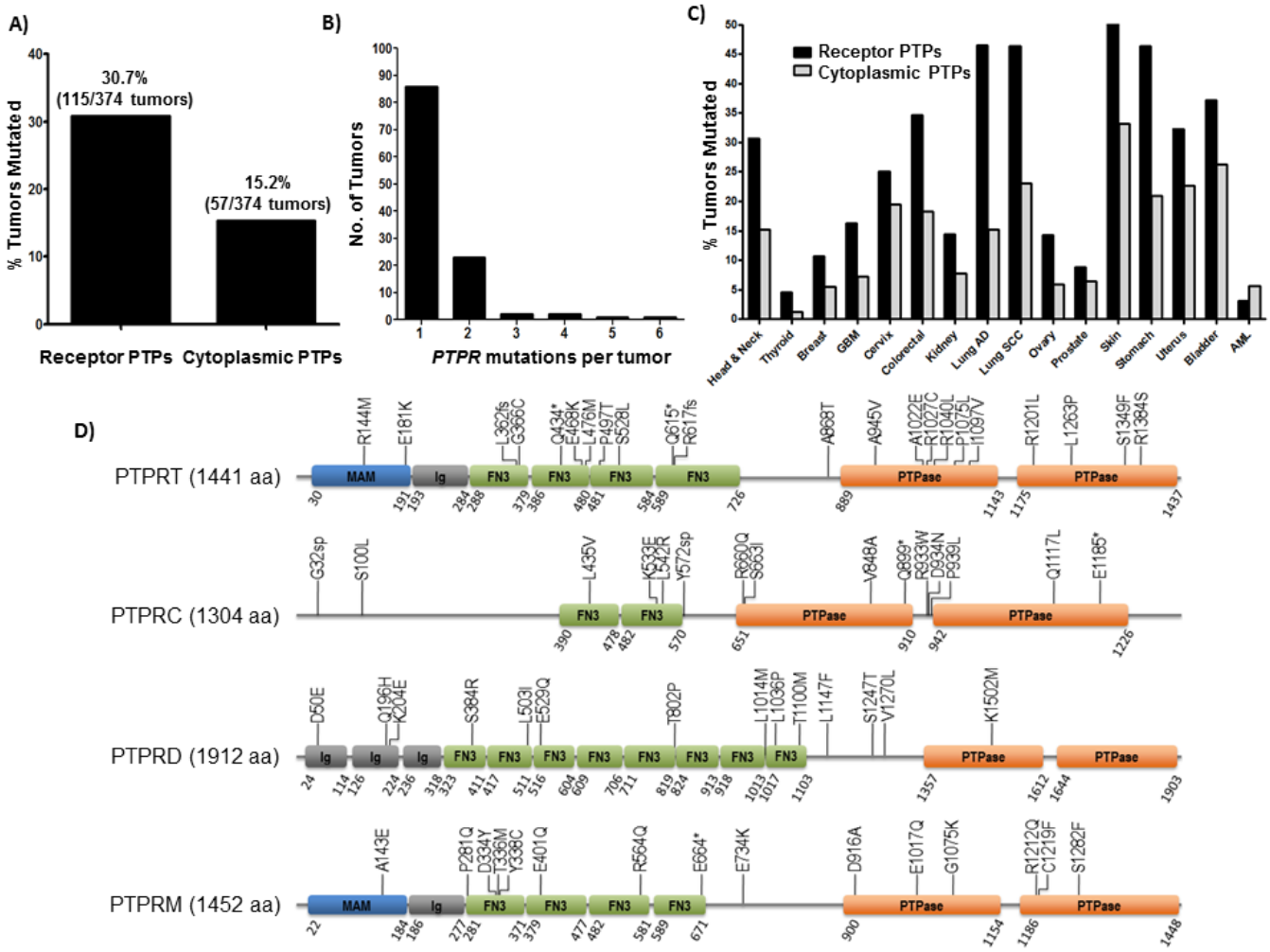


Figure 2. Whole-exome sequencing of human cancers demonstrates high rates of *PTPR* somatic mutations. A) Frequencies of *PTPR* and PTPN somatic mutations in 374 HNSCC tumors. B) A subset (29/374, 7.8%) of HNSCC tumors harbor mutations of multiple *PTPR* genes. C) The *PTPR* gene family is mutated at a

higher rate than the cytoplasmic PTP gene family across 15 cancers). Glioblastoma Multiforme (GBM); Adenocarcinoma (AD); Squamous Cell Carcinoma (SCC), Acute Myeloid Leukemia (AML). D) Mutation and domain-mapping of the 4 most frequently mutated PTPRs in HNSCC. Domains defined as in the Swiss-Prot entries, Protein Knowledgebase, UniProtKB.

Cumulative mutation data for *PTPRT* in 16 types of sequenced tumors indicates that 37.9% (108/285) of *PTPRT* mutations are found in the catalytic phosphatase domain, while 33.0% (94/285) occur in the extracellular fibronectin type III-like (FN3) domain. In HNSCC, 45.5% (10/22) of *PTPRT* mutations are located in the catalytic domain, indicating the potential pathologic relevance of these genetic alterations leading to loss of phosphatase activity or substrate recognition.

2.3.2 *PTPR* Mutations are Associated with Increased pSTAT3 Expression in HNSCC Tumors and Cell Lines

Using whole-exome sequencing and RPPA data available from TCGA and The Cancer Proteome Atlas (TCPA, a resource for accessing proteomic data from tumors previously analyzed by TCGA), we sought to determine if mutation of *PTPR* family members with putative tumor suppressive functions is associated with elevated pSTAT3 expression in HNSCC tumors. A literature review identified 7 *PTPR* family members (types T, D, J, K, M, O, and S) that have consistently been reported as tumor suppressors without also having been reported as oncogenic in alternate systems. Examination of 212 primary HNSCC tumors showed that tumors harboring mutations in *PTPR* tumor suppressor genes expressed significantly higher levels of pSTAT3 compared to tumors with wild-type *PTPR* family members ($P = 0.02$; **Figure 3A**). When each family member is instead analyzed individually, only *PTPRD* mutation is significantly associated

with increased pSTAT3 in HNSCC tumors (P = 0.01; **Figure 3B**), suggesting that these mutations may have a particularly profound signaling effect.

We next sought to determine if this trend held true in HNSCC cell lines that harbor endogenous *PTPRT/D* mutations as determined by the Cancer Cell Line Encyclopedia (CCLE). [120] We identified three HNSCC cell lines with such mutation: FaDu harboring PTPRT p.E985K, Cal27 harboring PTPRD p.S387L, and PE/CA-PJ49 harboring PTPRD p.I1821V. Western blot analysis revealed that these *PTPRT/D* mutant cell lines all express significantly greater pSTAT3 than does a cell line with no *PTPR* gene family mutations (PE/CA-PJ34.12; **Figure 3C and 3D**). These findings suggest that these cell lines may represent sufficient models for further investigation of the contribution of *PTPRT/D* mutation to STAT3 signaling and sensitivity to STAT3 inhibition.

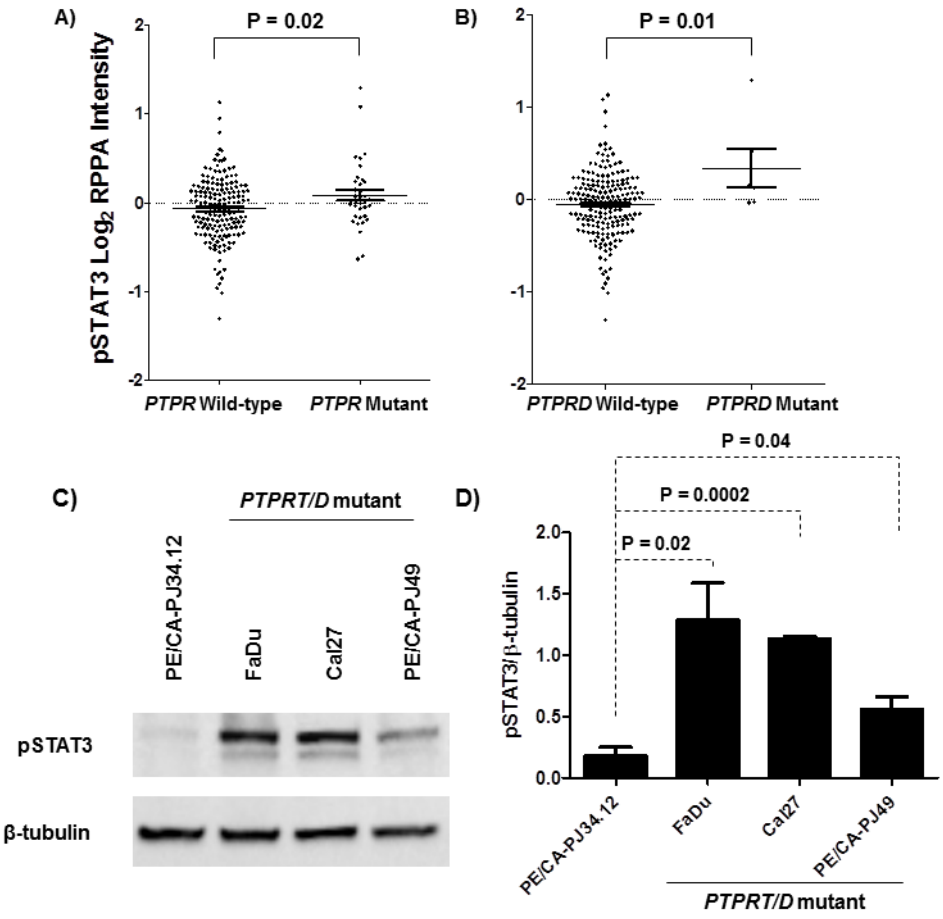


Figure 3. *PTPR* mutation is associated with increased pSTAT3 expression in HNSCC tumors and cell lines. A) Significant increase in pSTAT3 expression in HNSCC patient tumors harboring *PTPRT/D/J/K/M/O/S* mutation (n=37) versus tumors without *PTPR* mutation (n=171). Two-tailed t test. B) Significant increase in pSTAT3 expression in HNSCC patient tumors harboring *PTPRD* mutation (n=6) versus tumors without *PTPRD* mutation (n=194). Two-tailed t test. C) Western blot of HNSCC cell lines harboring *PTPR* WT (PE/CA-PJ34.12) or *PTPRT/D* mutant (FaDu, Cal27, PE/CA-PJ49). (B) Graphical representation of pSTAT3 levels normalized to β-tubulin loading control. Two-tailed t tests, n = 3.

2.3.3 *PTPRT/D* Mutations Dysregulate pSTAT3 Expression and Growth/Proliferation in HNSCC Cells

We next sought to directly test the effects of overexpression of wild-type (WT) *PTPRT/D* or mutant constructs in HNSCC cells. First, PCI-52-SD1 cells (with unknown *PTPR* mutation status) were stably infected with WT *PTPRT* and analyzed by Western blot (**Figure 4A**). A significant downregulation of pSTAT3 was observed in *PTPRT*-infected cells indicating that PTPRT is capable of regulating pSTAT3 activation in HNSCC cells. Conversely, transient overexpression of an HNSCC-derived *PTPRT* mutant (A1041E, localized in the catalytic domain) in Cal33 cells (with no *PTPR* family mutations) results in increased pSTAT3 expression relative to WT-expressing cells, suggesting that this mutation results in loss of catalytic function (**Figure 4B**). Interestingly, overexpression of another HNSCC-derived mutant (P497T, localized in the extracellular domain) does not result in increased pSTAT3 expression relative to WT in the same context. This may suggest that mutations located in the extracellular region of the PTPRT may not manifest through altered catalytic activity, but rather through alternative mechanisms such as altered cell-cell interaction, cell adhesion, motility and/or invasion.

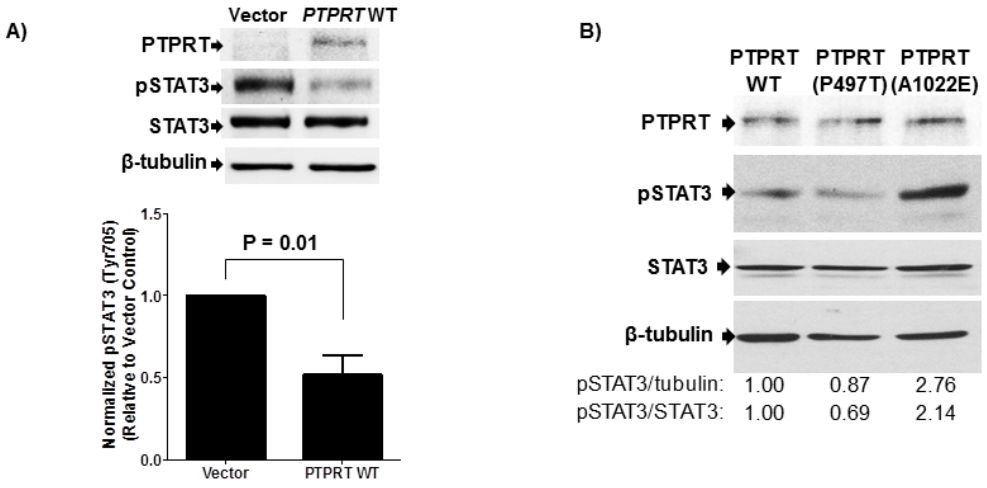


Figure 4. *PTPRT* wild-type, but not a catalytic domain mutant, leads to decreased pSTAT3 expression. A) Stable expression of WT PTPRT reduced basal pSTAT3 expression in PCI-52-SD1 cells. Graph showing cumulative results of from 5 independent experiments. Two-tailed t test. D) Cal33 cells were transiently transfected with a representative catalytic domain mutation (A1022E) or FN3-domain mutation (P497T).

Similar experiments were then performed to test if *PTPRD* and its HNSCC-derived mutants are similarly able to regulate pSTAT3 expression. Upon transient overexpression of WT PTPRD in Cal27 cells (with endogenous *PTPRD* mutation), a significant reduction in pSTAT3 expression is observed by Western blot (**Figure 5A**), indicating that PTPRD also has the ability to regulate pSTAT3 in HNSCC cells. A similar reduction in pSTAT3 expression is also observed after transient overexpression of WT PTPRD in PE/CA-PJ34.12 cells (with no *PTPR* family mutations) (**Figure 5B**), demonstrating a consistent effect in an additional HNSCC cell line. Transient overexpression of five representative HNSCC-derived PTPRD mutants in this cell line conversely leads to no significant reduction in pSTAT3 expression relative to vector control, with the exception of one mutant (L1147F). Interestingly, only one of these mutants (K1502M) is located in the catalytic domain, suggesting that even those mutations that are located in the

extracellular region can affect the catalytic function of PTPRD. While these mutations likely manifest through allosteric mechanisms, the current lack of a solved crystal structure of the PTPRD extracellular domain precludes a more thorough mechanistic analysis.

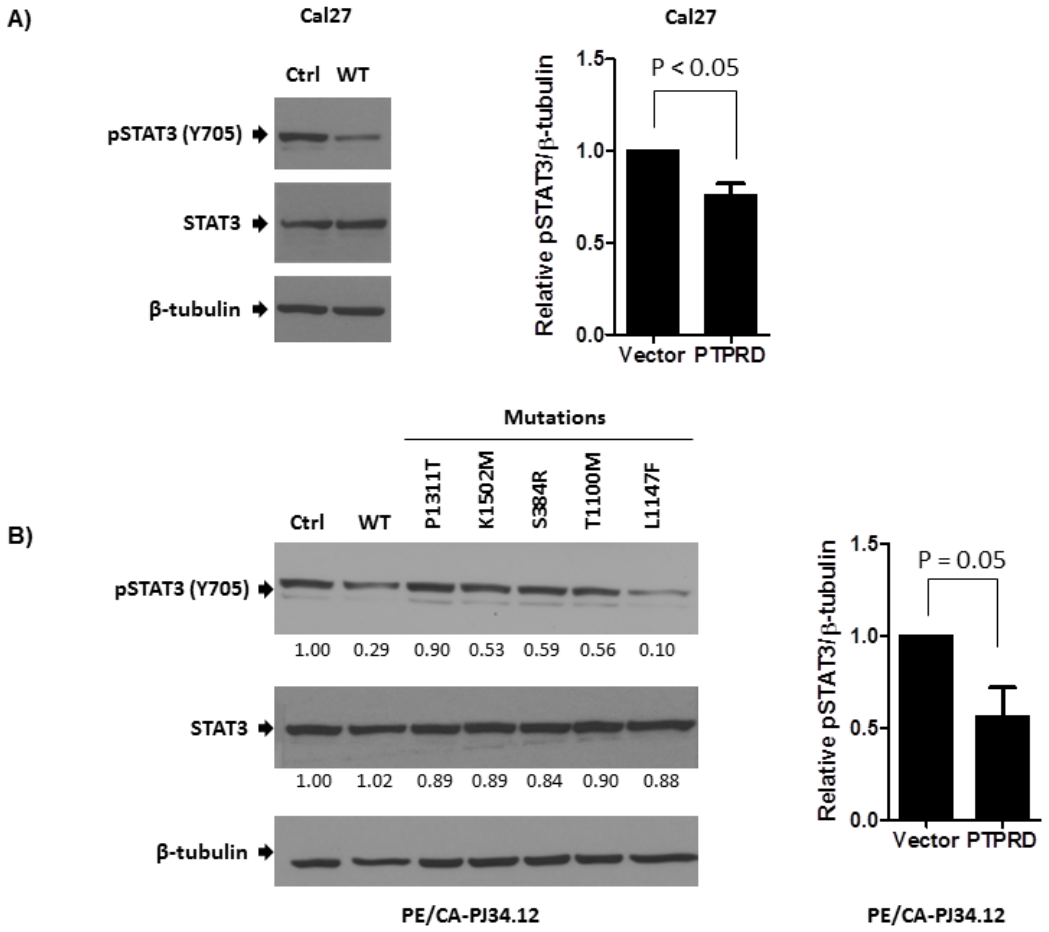


Figure 5. *PTPRD* wild-type, but not mutants, leads to decreased pSTAT3 expression. (A) Overexpression of WT PTPRD in a *PTPRD*-mutant cell line (Cal27) leads to decreased pSTAT3 expression. Two-tailed t test. (B) *PTPRD*-wild-type HNSCC cells (PE/CA-PJ34.12) transiently overexpressing mutant PTPRD exhibit increased pSTAT3 expression relative to wild-type-expressing cells. Two-tailed t test.

Having now established that *PTPRT/D* mutations lead to increased pSTAT3 activation in HNSCC cells, we next tested whether this activity leads to phenotypic changes associated with cancer progression. First, we tested whether PCI-52-SD1 cells, which are exquisitely sensitive to

serum deprivation and die rapidly upon serum removal [119], were able to overcome their serum dependence upon expression of HNSCC-derived PTPRT mutants. Using this model, stable expression of two representative mutants resulted in increased survival/growth relative to GFP-vector control, suggesting a "driver" phenotype for these mutants in HNSCC cells (**Figure 6**). These results suggest a dominant negative mechanism of *PTPRT* mutation that may be due to exogenous mutants inhibiting endogenous wild-type proteins by dimerization or other mechanisms.

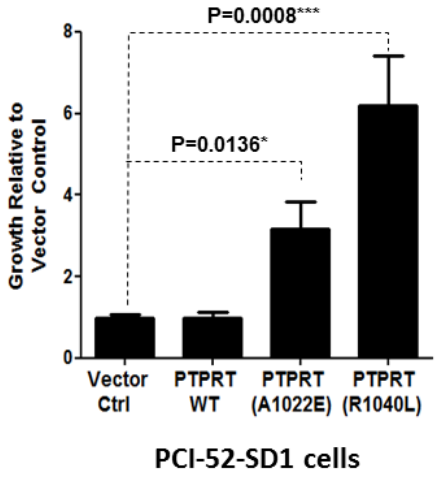


Figure 6. HNSCC-derived *PTPRT* mutants lead to increased growth in serum dependent HNSCC cells. Serum-dependent PCI-52-SD1 cells stably expressing EGFP-vector control, wild-type PTPRT, or representative mutants were assessed by MTT assay for growth in the absence of serum. Cumulative growth relative to vector control (n≥9) is shown. Two-tailed t test.

As we have not yet been successful in stably expressing PTPRD efficiently in HNSCC cells, likely due to its size (1912 amino acids), a different approach was undertaken to establish a phenotypic effect of mutant overexpression. Equal numbers of PE/CA-PJ34.12 cells were plated on 6-well plates followed by transient transfection in triplicate. 48-72 hrs after transfection, cells were assessed by MTT or trypan blue exclusion assays. As assessed by MTT assay, transient

overexpression of WT PTPRD leads to significantly decreased cell growth relative to vector control (**Figure 7A**). For all PTPRD mutants tested, this growth suppression was not observed, again suggesting these mutations are loss of function events. This result was confirmed with two representative mutants by trypan blue exclusion assay (**Figure 7B**). These results are consistent with the observed effects on pSTAT3 expression following transient overexpression (**Figure 5B**), with the exception of the L1147F mutation. While expression of this mutation leads to decreased pSTAT3 expression, it does not also lead to decreased growth. This is likely an example of a mutation that manifests through alternative mechanisms that are independent of STAT3.

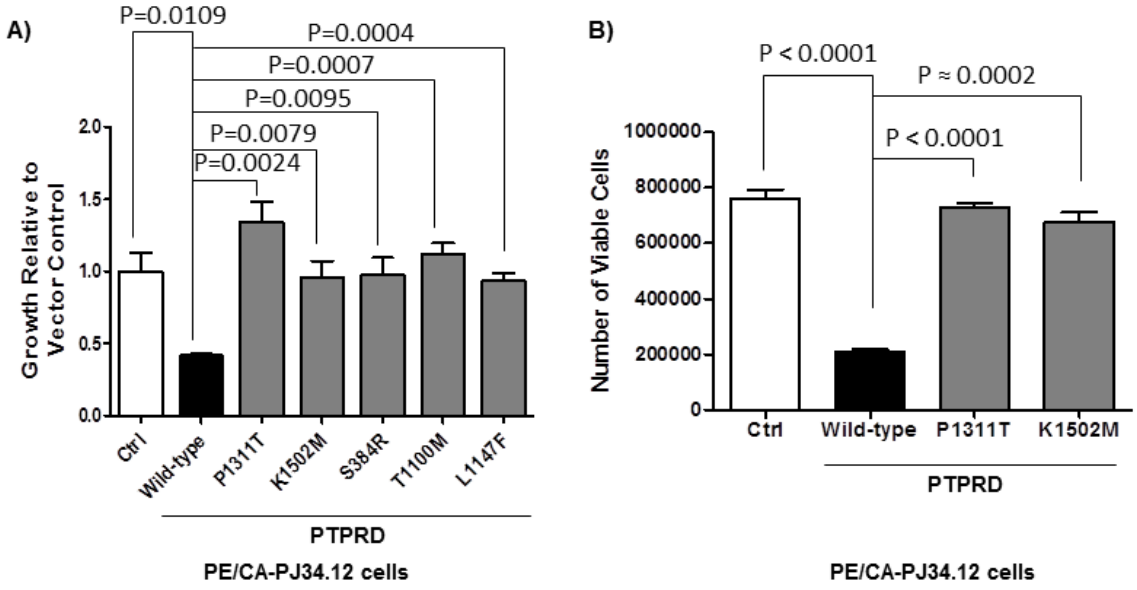


Figure 7. HNSCC-derived *PTPRD* mutations lead to increased cell growth/proliferation in HNSCC cells. (A) Cell growth was assessed by MTT assay 48 hrs after transfection and normalized to vector-transfected controls. One-way ANOVA P = 0.0007. Depicted P values represent the results of pairwise two-tailed unpaired t tests. The experiment was performed three times with similar results. (B) Cell proliferation was assessed by trypan blue exclusion assay 72 hrs after transfection. One-way ANOVA P < 0.0001. Depicted P values represent the results of pairwise two-tailed unpaired t tests.

Together, these experiments demonstrate that PTPRT/D are capable of regulating STAT3 activity in a variety of HNSCC cell models and that most HNSCC-derived *PTPRT/D* mutations are likely to be loss-of-catalytic function events. In addition to regulation of pSTAT3 expression, these mutants are also capable of affecting cell growth/proliferation in HNSCC cell lines, confirming an oncogenic role for mutant *PTPRT/D*.

2.3.4 *PTPRT/D* Mutation is Associated with Enhanced Sensitivity to STAT3 Pathway Inhibition in HNSCC Cells

The proximal nature of *PTPRT/D* mutation to STAT3 hyperactivation led us to hypothesize that these mutations may signify increased dependence upon STAT3 signaling for cell growth and survival. We therefore sought to test if these mutations led to sensitivity to pharmacologic STAT3 pathway inhibition. Treatment of PCI-52-SD1 cells stably expressing a catalytic domain PTPRT mutant (R1059L) or EGFP control with JSI-124, a selective JAK/STAT3 inhibitor [121], revealed that cells overexpressing mutant PTPRT are indeed more sensitive to STAT3 inhibition than vector control cells as determined by MTT assay (**Figure 8**), suggesting that these mutations may signify enhanced sensitivity to STAT3 pathway inhibition.

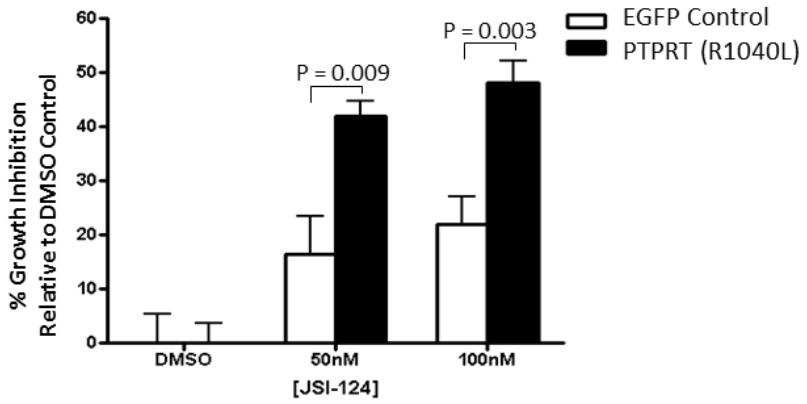


Figure 8. *PTPRT* mutation is associated with increased sensitivity to the STAT3 pathway inhibitor JSI-124. PCI-52-SD1 cells stably expressing the indicated constructs were treated with JSI-124 followed by MTT assay. Two-tailed t tests.

Our lack of success in stably expressing PTPRD efficiently in HNSCC cells again led us to employ an alternate method for determining relative sensitivity to STAT3 inhibition in *PTPRD* WT versus mutant cells. We identified one cell line that harbors an endogenous *PTPRD* mutation (PE/CA-PJ49) and another with no *PTPR* family mutations (PE/CA-PJ34.12) according to the CCLE. Treatment of these cell lines with JSI-124 reveals that cells harboring mutant *PTPRD* are more sensitive than WT cells as assessed by MTT assay (**Figure 9**), suggesting that *PTPRD* mutation may also signify enhanced sensitivity to STAT3 pathway inhibition.

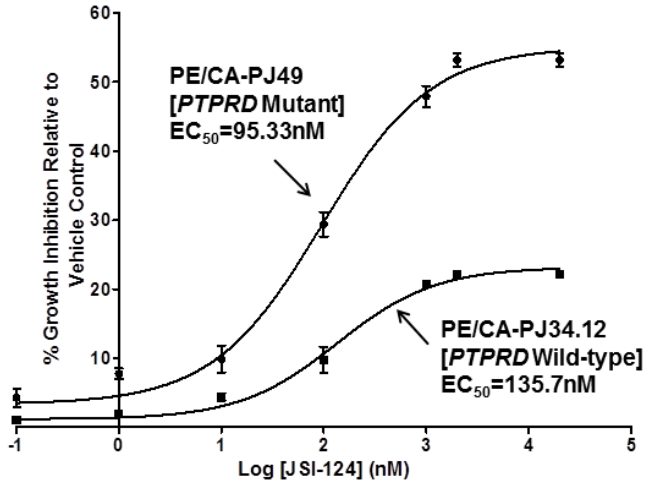


Figure 9. HNSCC cells harboring an endogenous *PTPRD* mutation (PE/CA-PJ49) are more sensitive to the STAT3 pathway inhibitor JSI-124 relative to representative *PTPRD* WT HNSCC cells (PE/CA-PJ34.12). Cells were treated with increasing concentrations of JSI-124 for 24 hours followed by MTT assay. The experiment was performed three times with consistent results.

Together, these findings indicate that *PTPRT/D* mutations may lead to increased sensitivity to STAT3 inhibition in HNSCC. These mutations may therefore ultimately serve as predictive biomarkers of exquisite response to emerging STAT3-targeted therapeutics in HNSCC patients.

2.4 DISCUSSION

These cumulative results indicate that tumor-associated *PTPRT/D* mutations can alter STAT3 phosphorylation/activation in HNSCC, and likely across many tumor types. This suggests a novel and common mechanism for dysregulated cell survival and growth involving *PTPR* mutation and STAT3 hyperactivation. Therefore, tumors that harbor *PTPR* (especially

PTPRT/D) mutations may be most amenable to treatment with STAT3 pathway inhibitors that are currently in preclinical and clinical development.

The frequency of *PTPR* mutations is unexpectedly high across all solid tumor types analyzed to date. The disperse distribution and lack of hotspot mutations in putative tumor suppressor *PTPR* genes suggests that these mutations likely represent loss-of-function events that typically affect tumor suppressive proteins rather than gain-of-function in oncogenic proteins in cancer. While this mutation pattern is consistent with that reported for select *PTPR*s in colorectal cancers [122], this work represents the most comprehensive analysis of somatic mutations of the *PTPR* family across all human cancers sequenced to date.

Constitutive STAT3 activation is frequently found in nearly all human cancers, and expression levels of pSTAT3 are often associated with poor prognosis [123-125]. Many mechanisms driving STAT3 activation in cancer have been elucidated, with much focus in recent decades on mutational activation of kinases upstream of STAT3, including EGFR, BCR-ABL, SRC-family kinases, and many others. Unfortunately, with a few notable exceptions, tyrosine kinase inhibitors have met with several clinical challenges, including a lack of predictive biomarkers for optimal selection of patients likely to benefit as well as the emergence of resistance mechanisms.

Direct exome sequencing of thousands of patient tumors has not identified common or consistent STAT3 mutational events in solid tumors, indicating that STAT3 mutation itself cannot account for any large fraction of cancers with overactive STAT3 signaling, and precluding the use of STAT3 mutation as a biomarker in large numbers of patients. Furthermore, solid tumors, including HNSCC, exhibit a lack of activating mutations in kinases upstream of STAT3 [114]. Thus, activating mutations of STAT3, or of direct upstream positive

regulators, are unlikely to be the key genetic factors driving STAT3 hyperactivation in human cancers, including HNSCC.

Somatic alterations of negative regulators of STAT3 remain incompletely understood in cancers, including HNSCC. As PTPRT/D have been reported to be direct negative regulators of STAT3 [20, 126], we sought to determine the impact of *PTPRT/D* alterations on STAT3 signaling and sensitivity to STAT3 inhibition in HNSCC tumors and preclinical models. Here we have investigated the hypothesis that *PTPRT/D* mutation leads to loss of function and subsequent upregulation of pSTAT3 expression, concomitant with increased sensitivity to pharmacologic STAT3 pathway inhibition.

Herein we first determined that tumor suppressor *PTPR* genes are associated with increased STAT3 activation in primary HNSCC tumors. *PTPRT/D* mutations occur frequently in HNSCC and across cancers, with *PTPRT* representing the single most frequently mutated phosphatase in HNSCC and across all cancers. These mutations are dispersed throughout the gene and protein, with no emergent hotspots, consistent with a pattern generally associated with tumor suppressors rather than oncogenes and concurrent with our hypothesis.

Both *PTPRT/D* have previously been shown to directly dephosphorylate pSTAT3. We have now demonstrated that WT PTPRT/D have the capacity to downregulate STAT3 signaling and growth/survival in HNSCC cells, while most mutants do not, further supporting a loss of function hypothesis. Interestingly, mutations occurring in both the extracellular and intracellular regions of PTPRD appear to decrease phosphatase activity (likely through undetermined allosteric mechanisms), while the one extracellular domain mutation of PTPRT that was tested did not. This may suggest that extracellular mutations of PTPRD, but not PTPRT, manifest through increased STAT3 activation, while only intracellular PTPRT mutations contribute in this

way. Instead, extracellular PTPRT mutations may manifest through STAT3-independent mechanisms, which could include modulation of cell-cell interactions, motility, and invasion.

Because of the proximal and direct interaction between *PTPRT/D* mutation and pSTAT3 overexpression, we hypothesized that loss of function of these proteins by somatic mutation may lead to increased sensitivity to STAT3 pathway inhibition. Here we have demonstrated that *PTPRT/D* mutation is indeed associated with increased sensitivity to the JAK/STAT3 inhibitor JSI-124 in HNSCC cells, indicating that these mutations may ultimately serve as predictive biomarkers for STAT3-targeted therapeutics.

In conclusion, *PTPR* mutational events are relatively common in primary HNSCC, as well as in many other solid tumors as revealed by large-scale whole-exome sequencing studies. We have demonstrated that tumor-specific mutational events in the *PTPRT/D* genes can serve as direct “drivers” for tumor growth by inducing hyperactivation of STAT3, a potent oncogenic transcription factor and PTPRT/D substrate. STAT3 pathway inhibitors are under active investigation in human cancers, and it is biologically plausible that select *PTPRT/D* mutations may identify tumors that may be particularly responsive to treatment with STAT3 pathway inhibitors.

3.0 FREQUENT PROMOTER HYPERMETHYLATION OF PTPRT INCREASES STAT3 ACTIVATION AND SENSITIVITY TO STAT3 INHIBITION IN HEAD AND NECK CANCER

3.1 INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is an invasive malignancy with more than 45,000 expected diagnoses and more than 8,000 expected deaths in 2015 in the United States alone. [127] Most cancers, including HNSCC, are characterized by constitutive activation of signal transducer and activator of transcription 3 (STAT3) via phosphorylation of tyrosine 705 (Y705). STAT3 is a transcription factor and potent oncoprotein that activates or maintains many cancer phenotypes including abnormal growth, proliferation, angiogenesis, and invasion as well as evasion of apoptosis and the anti-tumor immune response. [128] While aberrant activation of kinases upstream of STAT3 likely contributes to constitutive activation of STAT3 in cancer, the role of loss-of-function of downstream regulators, especially protein tyrosine phosphatases (PTPs), remains incompletely understood.

The PTP superfamily is a large group of enzymes that, in close coordination with tyrosine kinases, tightly regulates diverse signaling pathways by catalyzing the removal of a phosphate group from specific signaling proteins. The largest class of PTPs in the human genome is the

receptor-like PTP (PTPR) family, which contains 21 distinct members. PTPR family members span the membrane once and contain one or two intracellular catalytic domains, as well as a modular extracellular region that typically contains several protein-protein interaction domains. A role for PTPRs in the context of cancer is increasingly apparent, with many members implicated as oncogenes or tumor suppressor genes. [113] As described in Chapter 2, we recently reported that members of the PTPR family are frequently somatically mutated across all solid tumors analyzed, including HNSCC, with PTPR type T (PTPRT) representing the single most commonly mutated PTP in HNSCC and across all cancers. [119] Importantly, pSTAT3 is a validated direct substrate of PTPRT, and loss-of-function mutations of PTPRT lead to increased pSTAT3 expression and enhanced HNSCC cell growth/survival. [17, 119] As these loss-of-function PTPRT mutations are found in relatively few HNSCC tumors (5.6% of tumors analyzed), we sought to determine if loss of PTPRT expression by aberrant promoter methylation contributes to pSTAT3 overexpression and sensitivity to STAT3 inhibition in HNSCC.

Herein, we describe the high frequency of aberrant *PTPRT* promoter hypermethylation in HNSCC and other cancers. We demonstrate that *PTPRT* promoter methylation is significantly associated with downregulation of *PTPRT* expression, with an associated increase in expression of the PTPRT substrate pSTAT3 in HNSCC. We show that this methylation is reversible, leading to specific downregulation of pSTAT3 in HNSCC cells. Further, we demonstrate a correlation between *PTPRT* promoter methylation and sensitivity to STAT3 inhibition in HNSCC cell lines, suggesting that *PTPRT* methylation may serve as a predictive biomarker of responsiveness to STAT3 inhibitors currently in clinical development.

3.2 MATERIALS AND METHODS

3.2.1 Genomic and Proteomic Data and Analysis

TCGA methylation and RNA-Seq data were obtained through R software via the CGDS-R package or through the TCGA data matrix (https://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm). Reverse-phase protein array data were obtained from The Cancer Proteome Atlas (TCPA; http://app1.bioinformatics.mdanderson.org/tcpa/_design/basic/index.html). Statistical calculations were performed using GraphPad Prism 5 software (GraphPad, La Jolla, CA) unless otherwise noted.

3.2.2 Methylation-Specific Polymerase Chain Reaction (MSP)

Two-millimeter diameter cores were obtained from formalin-fixed, paraffin-embedded HNSCC tumors under the auspices of a protocol approved by the Institutional Review Board of the University of Pittsburgh. Tumor DNA was isolated with the QIAamp DNA FFPE Tissue Kit, while the QIAamp DNA Mini Kit was used for isolation of DNA from cell lines, both according to the manufacturer's instructions (Qiagen, Hilden, Germany). DNA concentrations were determined using a Beckman Coulter spectrophotometer (Pasadena, CA). Bisulfite conversion of 1 μg of DNA per sample was performed using the EpiTect® Bisulfite Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). 1 μL of bisultfite-converted DNA eluate was used for MSP. MSP primers were designed using MethPrimer software [129] and purchased from Sigma-Aldrich (St. Louis, MO). MSP was performed with the EpiTect® MSP Kit (Qiagen,

Hilden, Germany). After reaction completion, products were analyzed by gel electrophoresis on a 2.5% agarose gel. Images were taken using the ChemiDoc™ XRS+ System and Image Lab™ software (Bio-Rad Laboratories, Inc., Hercules, CA). Densitometry was performed using ImageJ and the fractional methylation (beta value) for a particular tumor or cell line was calculated as (methylation signal) / (methylation signal + unmethylation signal).

3.2.3 Cell Culture

Cal27 and Detroit 562 cells were obtained from ATCC (Manassas, VA). 686LN cells were obtained from Georgia Chen at MD Anderson Cancer Center (Houston, TX). BICR 18 and PE/CA-PJ49 cells were obtained from Sigma-Aldrich (St. Louis, MO). UMSCC cell lines were obtained from Thomas E. Cary at the University of Michigan (Ann Arbor, MI). HSC-2 cells were obtained from Hideo Niwa at Nihon University (Tokyo, Japan). Cal27, Detroit 562, HSC-2, UMSCC 47, and UMSCC 22A were maintained in DMEM (Corning, Corning, NY) containing 10% fetal bovine serum (FBS; Gemini Bio-Products, West Sacramento, CA). UMSCC 1 were maintained in DMEM containing 10% FBS and 0.4 µg/mL hydrocortisone (Sigma-Aldrich, St. Louis, MO). BICR 18 were maintained DMEM containing 10% FBS, 0.4 µg/mL hydrocortisone, and 2 mM L-glutamine (Life Technologies, Carlsbad, CA). 686LN were maintained in DMEM/F12 (Life Technologies, Carlsbad, CA) containing 10% FBS. PE/CA-PJ49 were maintained in Iscove's DMEM (Corning, Corning, NY) containing 10% FBS and 2 mM L-glutamine. All cells were genotypically verified using the AmpFSTR Identifiler PCR Amplification Kit according to the manufacturer's instructions (Life Technologies, Carlsbad, CA) and maintained at 37°C and 5% CO_2.

3.2.4 5-Azacytidine (5-aza) and shRNA Treatment

Cal27 cells were plated at 250,000 cells per well on 6-well plates and incubated overnight. Medium was then replaced with complete medium containing 1 μM 5-aza (or 50% acetic acid in water as vehicle). 24 hours later, cells were transfected with 2 μg of shRNA targeted toward *PTPRT* (shPTPRT) or non-targeted scrambled sequence (shScr) with 6 μL of FuGENE® HD (Promega, Fitchburg, WI) in 200 μL of Opti-MEM® (Life Technologies, Carlsbad, CA) added directly to medium containing 5-aza (or vehicle). Cells were incubated for an additional 24 hours before DNA, RNA, and protein were harvested for analysis.

3.2.5 Western Blotting

Whole cell lysates were prepared in lysis buffer containing phosphatase and protease inhibitors using cell scrapers and sonication followed by centrifugation to remove cell debris. Lysate concentrations were determined with the Bio-Rad Protein Assay reagent (Bio-Rad Laboratories, Inc., Hercules, CA). 40 μg of protein was mixed with the appropriate volume of 4X loading dye containing β-mercaptoethanol and incubated in boiling water for 5 minutes. Samples were loaded into 8-10% polyacrylamide gel containing sodium dodecyl sulfate and allowed to resolve at ~125 V. Gels were blotted onto nitrocellulose membranes by semi-dry transfer at 21 V for 50 min. Blots were blocked in 5% milk for 1 hr at room temperature, then incubated with primary antibody in 1% milk overnight at 4°C with agitation. The next day, primary antibody was rinsed off followed by incubation in secondary antibody conjugated to horseradish peroxidase in 5% milk for 1 hr at room temperature. Blots were then imaged using Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Dallas, TX) and the ChemiDoc™ XRS+ System and Image

Lab™ software (Bio-Rad Laboratories, Inc., Hercules, CA). Primary antibodies for pSTAT3 and STAT3 were purchased from Cell Signaling Technology, Inc. (Boston, MA; typical dilution 1:1000-1:3000). β-tubulin primary antibody was purchased from Abcam (Cambridge, MA; typical dilution 1:50,000). Secondary antibodies were purchased from BioRad (Hercules, CA; typical dilution 1:1000-1:3000). All milk and antibody solutions were made in Tris-buffered saline containing Tween-20 at pH 7.6. Blots were quantitated by densitometry using ImageJ software.

3.2.6 Drug Treatment

Cells were plated on 48-well plates at a density of 6800 cells per well and incubated overnight before treatment with increasing concentrations of Stattic (Sigma-Aldrich, St. Louis, MO), JSI-124 (Calbiochem, San Diego, CA), LY2784544 (Eli Lilly and Company, Indianpolis, IN), or DMSO alone as vehicle control in triplicate in the appropriate complete medium (see 3.2.3 Cell Culture). MTT assays were performed by adding 5 mg/mL MTT in PBS to aspirated wells after 72 hrs and incubating at 37°C for 10-30 minutes as appropriate for each cell line. MTT was then aspirated and replaced with an equal volume of DMSO. 100 μL of DMSO was transferred from each well to a 96-well plate for data collection. Data were fit to a sigmoidal curve and EC_{50} values were determined using GraphPad Prism 5 (GraphPad, La Jolla, CA).

3.2.7 Animals

Mice were used in accordance with a protocol approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh, and were housed in a facility certified by the

American Association for the Accreditation of Laboratory Animal Care. 5-6 week old female Foxn1 nude mice were purchased from Harlan Laboratories (Indianapolis, IN). 16 mice were inoculated with 3×10^6 BICR 18 cells on the left flank, and 3×10^6 UMSCC 1 cells on the right flank. After 10 days of growth, UMSCC 1 cells had formed palpable tumors in 15/16 mice, while BICR 18 had formed none. At this time, 8 mice were randomized to receive 50 mg/kg Stattic in 1% Tween-80 in PBS by oral gavage five times per week as previously reported in an ovarian cancer model. [130] Seven mice received no treatment as control. Tumor sizes were blindly measured by caliper in two dimensions and volumes calculated as: Vol = (large measurement) × (small measurement)^2. Mice were sacrificed after measurements were taken on day 9 of treatment.

3.3 RESULTS

3.3.1 Frequent *PTPRT* Promoter Hypermethylation Leads to Decreased *PTPRT* mRNA Expression

To assess aberrant *PTPRT* promoter methylation in HNSCC, we analyzed TCGA data derived from the Illumina HumanMethylation450 platform, a quantitative assay that assesses methylation levels of more than 485,000 CG dinucleotides throughout the human genome. As promoter methylation is associated with decreased gene expression, we first determined which CG dinucleotide methylation event in the *PTPRT* promoter region was most negatively correlated with *PTPRT* mRNA expression (**Figure 10A**), and considered that methylation of this site was likely to most significantly contribute to reduced *PTPRT* expression. We then defined aberrant

hypermethylation as a fractional methylation level (beta value) at least three standard deviations above the mean methylation level of the same genetic locus in organ-matched normal tissue as determined by TCGA using the same assay. This analysis detected that 60.1% (256/426 tumors analyzed) of HNSCC tumors were aberrantly hypermethylated (**Figure 10B**). By this stringent measure, hypermethylated tumors exhibit significantly decreased *PTPRT* mRNA expression levels as determined by RNA-Seq (**Figure 10C**), suggesting the validity of the above definition and that *PTPRT* hypermethylation has the expected biologic effect. In contrast, copy number alterations of the *PTPRT* gene are relatively infrequent and are not significantly associated with altered *PTPRT* mRNA expression (**Figure 11**).

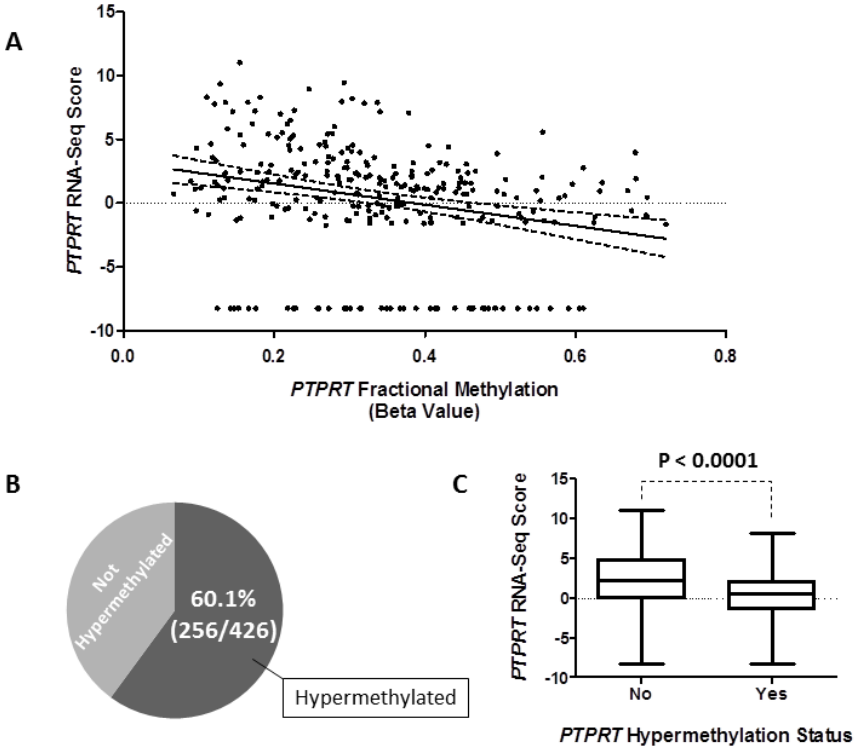


Figure 10. Frequent *PTPRT* promoter hypermethylation is associated with downregulation of *PTPRT* mRNA in HNSCC tumors. A) Methylation at the CG dinucleotide denoted cg04541293 significantly correlates with decreased *PTPRT* mRNA expression (n = 279, Pearson r = -0.2670, P < 0.0001, R^2 = 0.07131, 95% confidence interval depicted). B) *PTPRT* promoter hypermethylation (defined as a methylation level

greater than three standard deviations above the mean methylation level of the same genetic locus in organ-matched normal tissue samples) was assessed in 426 tumors from TCGA. C) *PTPRT* hypermethylation is significantly associated with downregulation of *PTPRT* mRNA (two-tailed unpaired t test). Whiskers represent minimal or maximal values. RNA-Seq Score in arbitrary units.

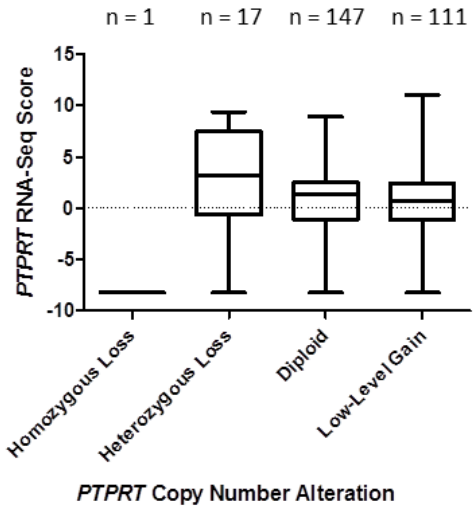


Figure 11. *PTPRT* copy number alterations are not significantly associated with altered mRNA expression in HNSCC. A Jonckheere-Terpstra test was performed using StatXact software (Cytel, Cambridge, MA). There are no cases of high-level amplification of *PTPRT* in the HNSCC tumors analyzed. Whiskers represent minimal or maximal values.

As human papilloma virus (HPV) infection is an etiologic and prognostic factor in a subset of HNSCC, we sought to determine if *PTPRT* promoter hypermethylation is associated with HPV status and observed no significant association (P = 1.00, Fisher's exact test; *PTPRT* promoter hypermethylation in 21/36 [58.3%] HPV-positive tumors versus 145/243 [59.7%] HPV-negative tumors), suggesting that HPV infection is not a driver of *PTPRT* promoter methylation. [131]

In order to validate TCGA findings in an independent HNSCC human cohort, we performed methylation-specific PCR (MSP) on 45 formalin-fixed, paraffin-embedded oral squamous cell cancers with primers directed at the promoter region of *PTPRT* (representative analysis in **Figure 12A**). We considered a tumor methylated when the methylation signal was more than 50% of the total signal as determined by densitometry. Using this semi-quantitative analysis, a similar high frequency of methylation was observed in this cohort (71.1%, 32/45 tumors analyzed; **Figure 12B**), further suggesting that *PTPRT* promoter methylation represents a common mechanism of *PTPRT* downregulation in HNSCC.

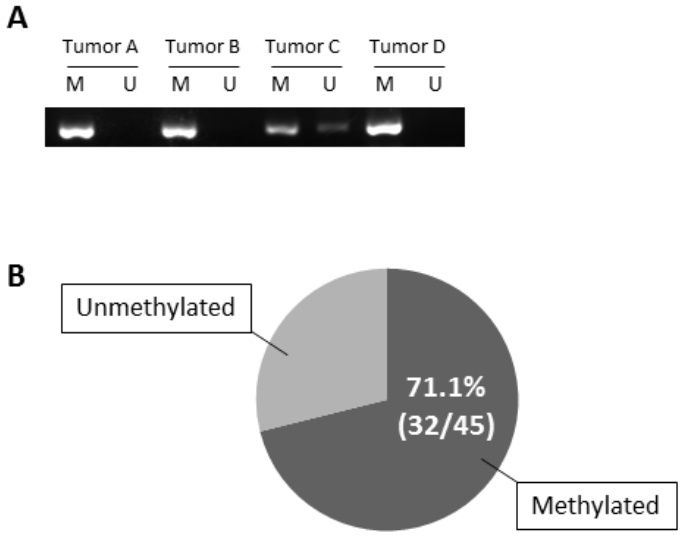
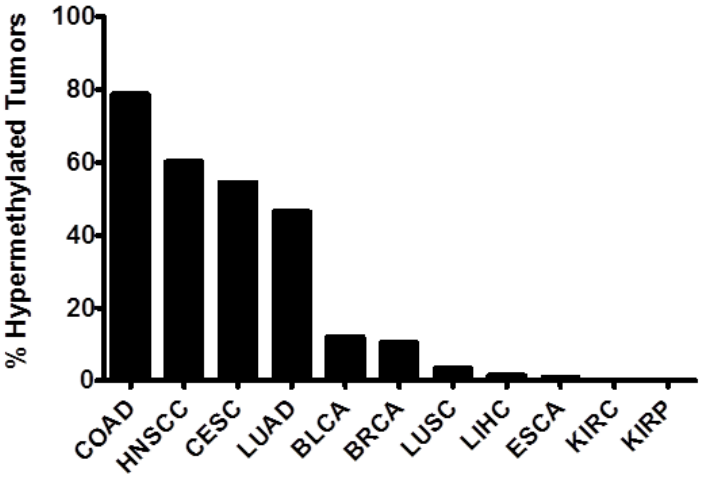


Figure 12. The *PTPRT* promoter is frequently methylated in an independent cohort of HNSCC tumors. A) Representative MSP analysis of the *PTPRT* promoter from four HNSCC tumors. M denotes primers amplifying methylated sequences, and U denotes primers amplifying unmethylated sequences. B) Summary of MSP analysis of 45 HNSCC tumors. A tumor is considered methylated when the methylation level is >50% of the total signal.

3.3.2 The *PTPRT* Promoter is Frequently Hypermethylated Across Human Cancers

We next sought to determine if *PTPRT* promoter hypermethylation is a common feature across cancer types. Further analysis of TCGA data reveals that the *PTPRT* promoter is frequently hypermethylated across a broad array of cancer types when hypermethylation is defined as a fractional methylation level (beta value) at least three standard deviations above the mean methylation level of the same genetic locus in organ-matched normal tissue samples. The highest incidence of *PTPRT* promoter hypermethylation occurs in colon adenocarcinoma (78.7%, 289/367 tumors analyzed), while HNSCC exhibits the second highest incidence (60.1%) among the cancers for which sufficient data were available for this analysis (**Figure 13A**). Of the cancers analyzed, four exhibit significant downregulation of *PTPRT* mRNA in hypermethylated tumors (HNSCC, colon adenocarcinoma, lung adenocarcinoma, and breast invasive carcinoma; $P < 0.05$), suggesting a functional role for aberrant *PTPRT* promoter methylation across several cancer types (**Figure 13B**).

A



B

Cancer	Percent Hypermethylation	Expression P Value	Normal (n)	Tumor (n)
COAD	78.7 (289/367)	**0.0274**	38	367
HNSCC	60.1 (256/426)	**<0.0001**	50	426
LUAD	46.4 (202/435)	**0.0163**	32	435
BRCA	10.5 (77/732)	**<0.0001**	96	732
CESC	54.5 (115/211)	0.1912	3	211
BLCA	12.0 (29/242)	0.2749	21	242
LUSC	3.3 (12/359)	0.1975	42	359
LIHC	1.5 (3/204)	NA	50	204
ESCA	1.1 (2/176)	NA	16	176
KIRC	0.0 (0/295)	NA	159	295
KIRP	0.0 (0/182)	NA	45	182

Figure 13. The *PTPRT* promoter is frequently hypermethylated across cancer types in association with downregulated *PTPRT* mRNA. COAD, colon adenocarcinoma; HNSCC, head and neck squamous cell carcinoma; LUAD, lung adenocarcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; BLCA, bladder urothelial carcinoma; LUSC, lung squamous cell carcinoma; LIHC, liver hepatocellular carcinoma; ESCA, esophageal carcinoma; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma. A) The *PTPRT* promoter is frequently hypermethylated in several cancers. B) Table summarizing hypermethylation across cancers. Expression P values represent the results of unpaired two-tailed t tests between hypermethylated and non-hypermethylated tumors, with those in bold denoting significant (P < 0.05) downregulation of *PTPRT* mRNA expression in hypermethylated tumors. Normal (n) and Tumor (n) denote the number of organ site-matched normal tissue or number of tumor samples used in this analysis, respectively.

3.3.3 *PTPRT* Expression is Inversely Associated with STAT3 Activation in HNSCC

As *PTPRT* promoter methylation leads to downregulation of *PTPRT* expression, we next sought to determine if *PTPRT* expression was inversely associated with pSTAT3 expression in HNSCC tumors. Analysis of TCGA and TCPA HNSCC data indicates that *PTPRT* mRNA expression is indeed inversely correlated with pSTAT3 expression in primary HNSCC tumors as determined by RPPA ($P < 0.008$) (**Figure 14A**). Indeed, representative immunohistochemical staining of an independent cohort of HNSCC tumors demonstrated that those with *PTPRT* promoter methylation, as determined by MSP, express elevated levels of pSTAT3 relative to tumors without *PTPRT* methylation (**Figure 14B**). Together, these findings suggest that *PTPRT* promoter methylation may be a frequent mechanism that contributes to STAT3 activation in HNSCC.

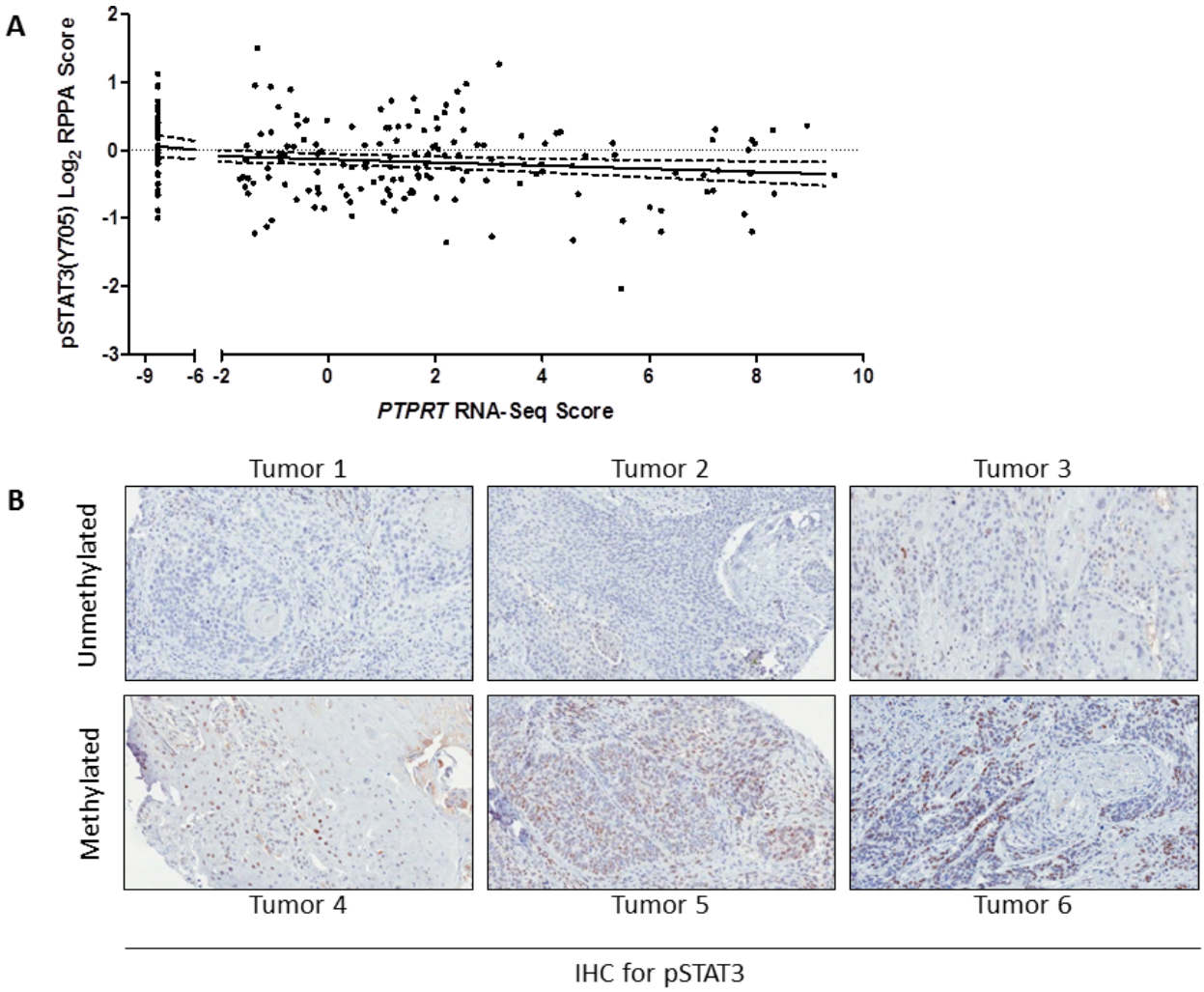


Figure 14. *PTPRT* promoter methylation and mRNA expression are associated with pSTAT3 expression. A) *PTPRT* mRNA expression is significantly correlated with pSTAT3 expression (n = 184, Pearson r = -0.1958, P < 0.008, R^2 = 0.03835, 95% confidence interval depicted). RNA-Seq Score in arbitrary units. B) Representative immunohistochemical analysis of pSTAT3 expression in six HNSCC tumors with the indicated *PTPRT* methylation status, performed as previously described. [119]

3.3.4 *PTPRT* Promoter Methylation is Reversible, Leading to *PTPRT*-Specific pSTAT3 Downregulation

To further investigate the mechanistic association between *PTPRT* promoter methylation and pSTAT3 expression, we designed an experiment involving shRNA targeted toward *PTPRT* and 5-aza, a non-specific DNA methyltransferase (DNMT) inhibitor that leads to genome-wide demethylation. We selected a HNSCC cell line (Cal27) that exhibits nearly complete methylation of the *PTPRT* promoter as determined by MSP. As expected, treatment of these Cal27 cells with 5-aza led to an increase in the unmethylated signal as determined by MSP(**Figure 15A**). This demethylation event was concurrent with restored expression of endogenous WT *PTPRT* as determined by RT-PCR, which was sufficient to lead to downregulation of pSTAT3 expression as determined by Western blot (**Figures 15B and 15C**). Transfection of Cal27 cells with shRNA directed against *PTPRT* (shPTPRT) following 5-aza treatment also resulted in an increase in the unmethylated signal as expected (**Figure 15A**), but the expression of *PTPRT* was significantly downregulated relative to 5-aza treatment alone, indicating that while the *PTPRT* promoter was demethylated by 5-aza, the shPTPRT treatment was sufficient to degrade the resulting *PTPRT* mRNA. Further, pSTAT3 expression remained unaffected under 5-azza plus shPTPRT conditions relative to 5-aza treatment alone (**Figures 15B and 15C**), suggesting that 5-aza-mediated downregulation of pSTAT3 is dependent upon demethylation and re-expression of *PTPRT* in these cells. These results provide mechanistic evidence that *PTPRT* promoter methylation contributes directly and proximally to STAT3 activation in HNSCC by downregulation of *PTPRT* expression.

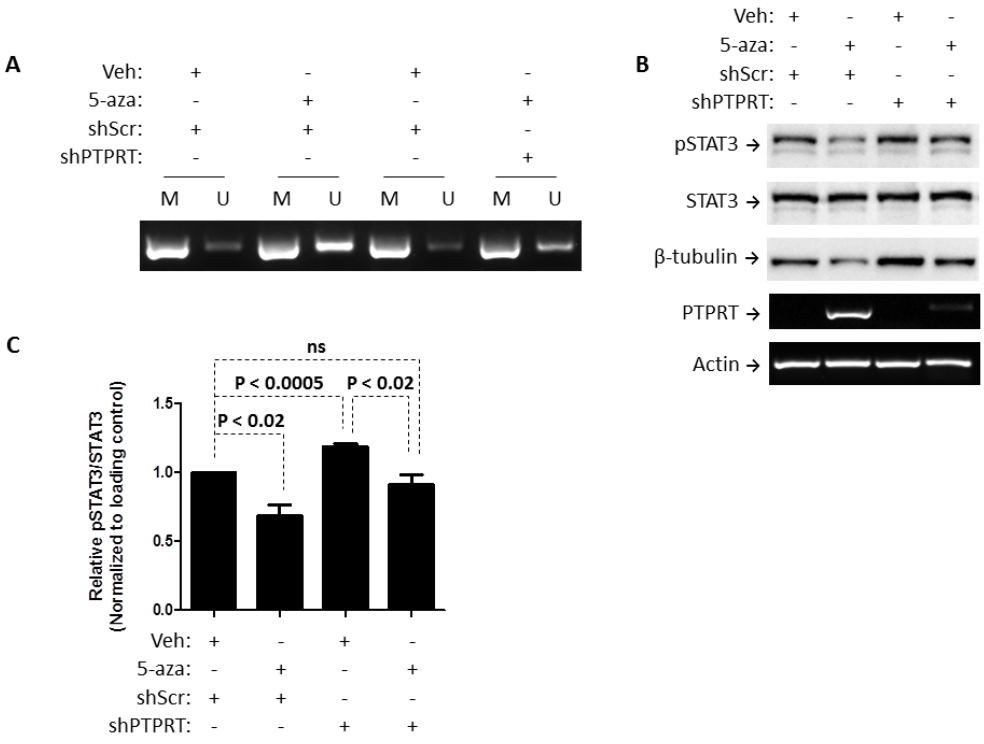
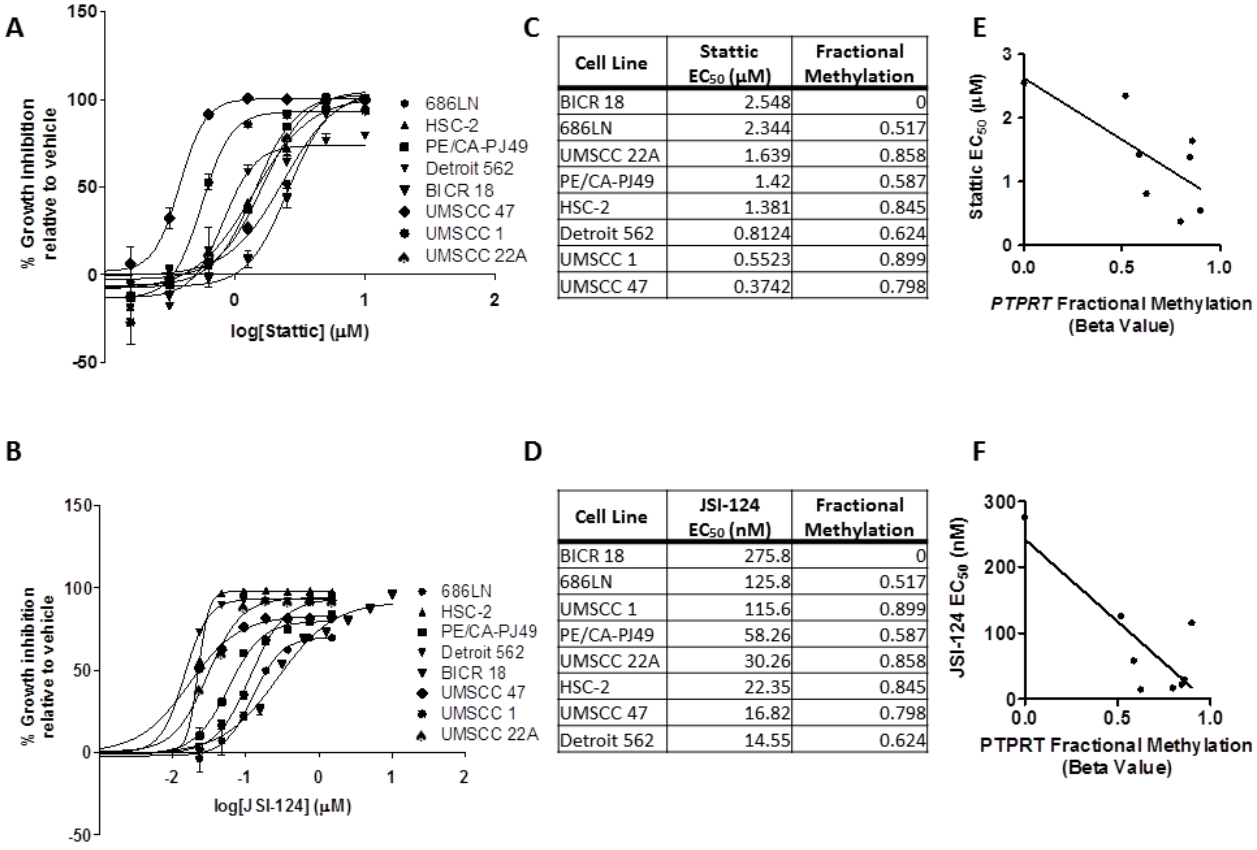


Figure 15. *PTPRT* promoter methylation is reduced by 5-azacytidine treatment, leading to *PTPRT*-specific pSTAT3 downregulation in HNSCC cells. A) MSP analysis of the *PTPRT* promoter in HNSCC cells (Cal27) following treatment with 1 μM 5-azacytidine (or vehicle) and shRNA targeted toward *PTPRT* (shPTPRT) or scrambled non-targeting shRNA (shScr). B) Western blot and RT-PCR analysis of Cal27 cells treated as indicated. C) Graphical representation of pSTAT3/STAT3 expression analyzed by Western blot following the indicated treatments (analyzed by unpaired two-tailed t tests, n = 3).

3.3.5 *PTPRT* Promoter Methylation is Associated with Increased Sensitivity to STAT3 Inhibition in HNSCC Cells

Due to the proximal nature of *PTPRT* promoter hypermethylation and pSTAT3 overexpression, we hypothesized that such methylation may predict enahanced sensitivity to STAT3 pathway inhibition. To evaluate whether *PTPRT* promoter methylation may serve as a predictive

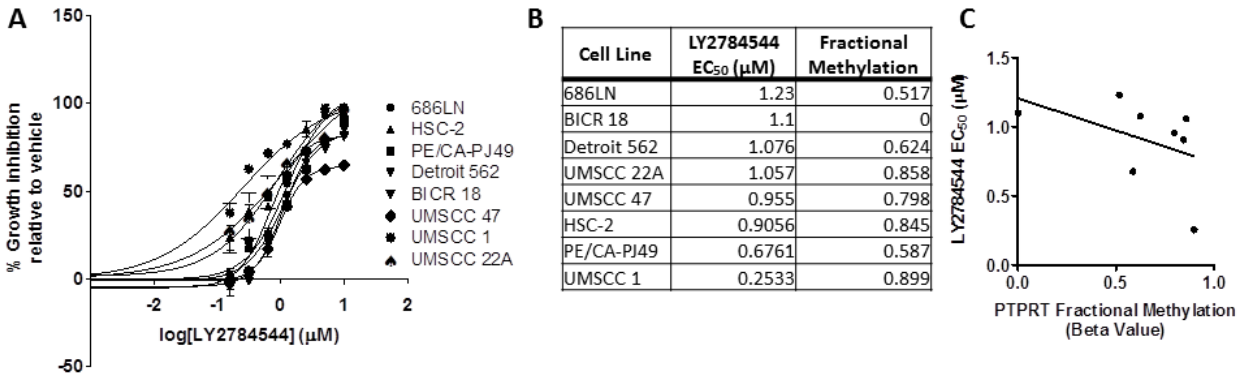
biomarker for STAT3 targeted therapies, we first conducted MTT assays to determine EC_{50} values for selective STAT3 inhibitors in a panel of 8 HNSCC cell lines (**Figures 16A and 16B**) that exhibit varying levels of *PTPRT* promoter methylation as determined by MSP (summarized in **Figures 16C and 16D**). **Figures 16E and 16F** illustrate that *PTPRT* promoter methylation and sensitivity to Stattic (a direct inhibitor of STAT3 dimerization and nuclear translocation by binding to the SH2 domain) or JSI-124 (a JAK/STAT3 pathway inhibitor) are significantly inversely correlated ($P < 0.05$), indicating that HNSCC cell lines with a high degree of *PTPRT* promoter methylation are most sensitive to STAT3 inhibition. A similar trend was observed with the JAK2 inhibitor LY2784544, though this observation was not statistically significant (**Figure 17**). Notably, we observe no direct correlation between *PTPRT* promoter methylation and pSTAT3 expression in this cell line panel, suggesting that high pSTAT3 expression *per se* may not be predictive of sensitivity to STAT3 inhibitors. Instead, *PTPRT* promoter methylation may lead to some degree of addiction to STAT3 signaling in HNSCC cells, thus leading to STAT3 inhibitor sensitization. These findings indicate that HNSCC tumors with a high degree of *PTPRT* promoter methylation may be most amenable to treatment with STAT3 inhibitors that are currently in preclinical and clinical development.



Cell Line	Stattic EC_{50} (μM)	Fractional Methylation
BICR 18	2.548	0
686LN	2.344	0.517
UMSCC 22A	1.639	0.858
PE/CA-PJ49	1.42	0.587
HSC-2	1.381	0.845
Detroit 562	0.8124	0.624
UMSCC 1	0.5523	0.899
UMSCC 47	0.3742	0.798

Cell Line	JSI-124 EC_{50} (nM)	Fractional Methylation
BICR 18	275.8	0
686LN	125.8	0.517
UMSCC 1	115.6	0.899
PE/CA-PJ49	58.26	0.587
UMSCC 22A	30.26	0.858
HSC-2	22.35	0.845
UMSCC 47	16.82	0.798
Detroit 562	14.55	0.624

Figure 16. Increased *PTPRT* promoter methylation is associated with increased sensitivity to STAT3 inhibition. A,B) Dose-response curves for 8 HNSCC cell lines treated with increasing concentrations of Stattic or JSI-124. MTT assays were performed after 72 hours of treatment. C,D) Tables of EC_{50} values and fractional methylation determined by MSP for the cell lines treated in A and B, sorted from least to most sensitive. E,F) The *PTPRT* fractional methylation level of the cells treated in A and B correlates with sensitivity to Stattic (n = 8, Pearson r = -0.7916, $P < 0.05$, $R^2 = 0.5178$) and JSI-124 (n = 8, Pearson r = -0.8224, $P < 0.02$, $R^2 = 0.6763$).



Cell Line	LY2784544 EC_{50} (μM)	Fractional Methylation
686LN	1.23	0.517
BICR 18	1.1	0
Detroit 562	1.076	0.624
UMSCC 22A	1.057	0.858
UMSCC 47	0.955	0.798
HSC-2	0.9056	0.845
PE/CA-PJ49	0.6761	0.587
UMSCC 1	0.2533	0.899

Figure 17. Increased *PTPRT* promoter methylation is associated with increased sensitivity to the JAK2 inhibitor LY2784544. A) Dose-response curves for 8 HNSCC cell lines treated with increasing concentrations of LY2784544. MTT assays were performed after 72 hours of treatment. B) Table of EC_{50} values and fractional methylation determined by MSP for the cell lines treated in A, sorted from least to most sensitive. C) Analysis of the correlation between *PTPRT* fractional methylation and sensitivity to LY2784544 reveals a trend toward higher sensitivity to LY2784544 in HNSCC cells with higher *PTPRT* methylation (n = 8, Pearson r = -0.4411, P < 0.3, R^2 = 0.1946).

We next sought to determine whether *PTPRT* methylation may serve as a predictive biomarker of sensitivity to STAT3 inhibition in a heterotopic tumorgraft model of HNSCC. We inoculated 16 nude mice with 3×10^6 BICR 18 cells (which exhibit total *PTPRT* unmethylation; β = 0) in one flank, and 3×10^6 UMSCC 1 cells (which exhibit near total *PTPRT* methylation; β = 0.899) in the other flank. After 10 days, the unmethylated cells had not formed any tumors, while the methylated cells had formed palpable masses in 15/16 mice (mean volume = 100.3 mm^3). As BICR 18 cells are the only HNSCC cells identified to date with total (or indeed, >50%) *PTPRT* unmethylation, we are therefore unable to determine the relative sensitivities of unmethylated versus methylated HNSCC cells *in vivo*. Nevertheless, starting on day 10 post-inoculation, 8 mice were randomized to receive 50 mg/kg Stattic by oral gavage five times per week, while 7 mice received no treatment. Serial tumor measurements were performed three times per week by

a blinded investigator. After 9 days of treatment, a large and statistically significant ($P < 0.01$) reduction in tumor volume was observed in the Stattic treatment arm relative to the no treatment group (**Figure 18**). Though we cannot determine relative sensitivities to this inhibitor between methylated and unmethylated tumorgrafts, these findings confirm that a *PTPRT*-methylated HNSCC heterotopic tumorgraft (UMSCC 1 cells) is indeed sensitive to STAT3 inhibition *in vivo*.

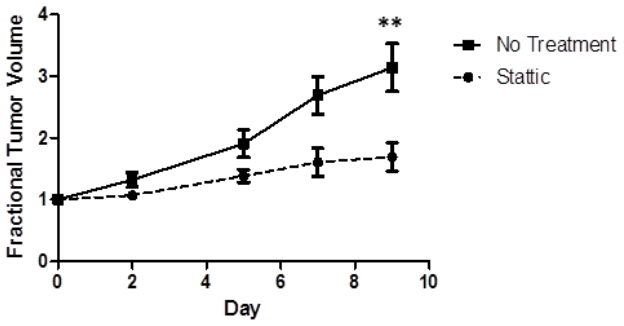


Figure 18. HNSCC cells with high *PTPRT* promoter methylation are sensitive to STAT3 inhibition *in vivo*. Mice bearing UMSCC 1 heterotopic tumorgrafts were treated with Stattic (50 mg/kg five days per week by oral gavage, n = 8) or left untreated (n = 7). Tumors in mice receiving Stattic exhibited significant growth inhibition relative to those in the untreated mice (P < 0.01, unpaired two-tailed t test).**

3.3.6 *PTPRD* Promoter Hypermethylation or Gene Copy Number Alterations are not Significantly Associated with PTPRD Loss of Function

As *PTPRD* also directly targets pSTAT3 and *PTPRD* mutations affect STAT3 signaling and cellular phenotypes similarly to *PTPRT* mutations, we additionally hypothesized that *PTPRD* promoter methylation or gene copy loss contributes to STAT3 overactivation in HNSCC. We defined aberrant promoter hypermethylation as previously described: a fractional methylation level (beta value) at least three standard deviations above the mean methylation level of the same

genetic locus in organ-matched normal tissue as determined by TCGA using the same methylation assay (Illumina HumanMethylation450). In contrast to frequent promoter hypermethylation of *PTPRT* **(Figure 10**), no cases of aberrant *PTPRD* promoter hypermethylation were observed in this cohort (0/426 tumors analyzed). This finding was confirmed in an independent cohort by MSP, where both tumor and patient-matched adjacent normal tissue exhibited *PTPRD* promoter methylation, suggesting that this methylation event may represent a feature of normal oral epithelium (**Figure 19**).

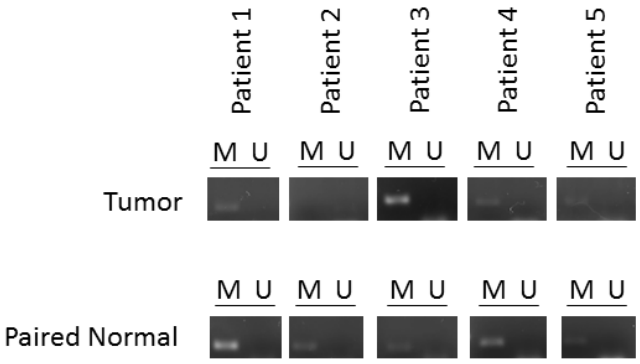


Figure 19. The *PTPRD* promoter is not hypermethylated in HNSCC tumors compared with adjacent normal mucosa. Five HNSCC tumors and matched normal mucosa from the same patients were collected and analyzed by MSP. M denotes primers amplifying methylated sequences, while U denotes primers amplifying unmethylated sequences.

Analysis of *PTPRD* copy number alterations as determined by TCGA across cancer types revealed that copy number loss is more common than gain in HNSCC and in all cancers analyzed, with the exception of colorectal and cervical cancers (**Figure 20A**). While this suggests that *PTPRD* copy number loss may contribute to loss of *PTPRD* expression and function, no significant association between *PTPRD* copy number alteration and mRNA was observed in HNSCC, similarly to that observed for *PTPRT* (**Figure 11**). This finding suggests

that gene copy loss does not significantly contribute to loss of expression or function of PTPRD in HNSCC (**Figure 20B**).

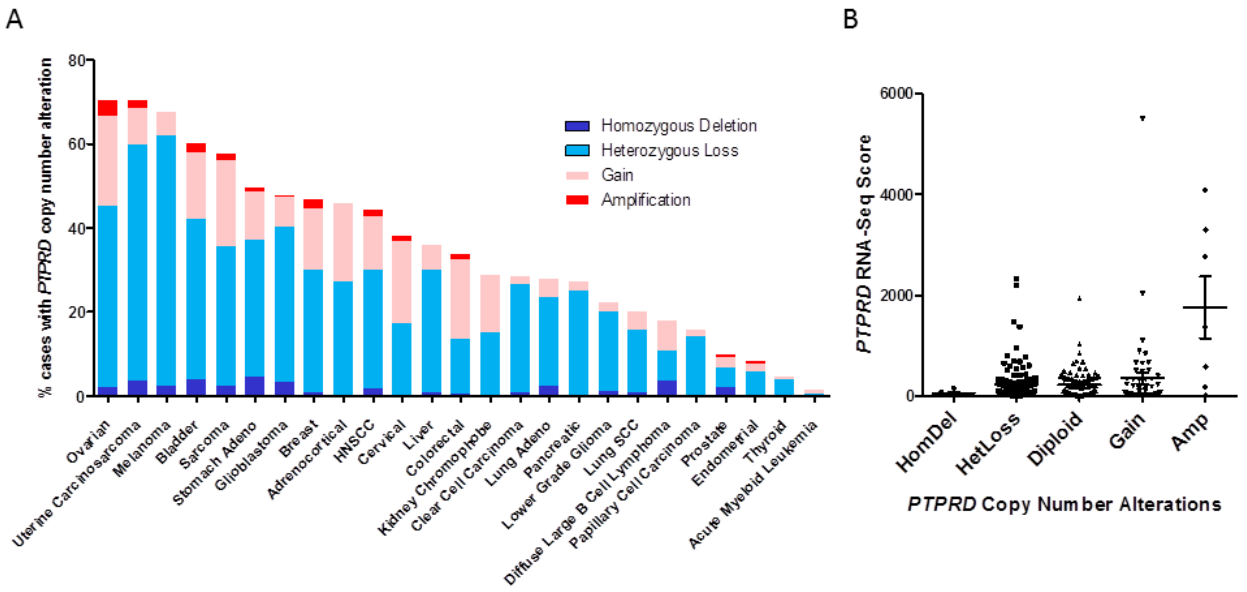


Figure 20. *PTPRD* copy number alterations are frequent across cancers but are not associated with *PTPRD* mRNA expression in HNSCC. (A) Copy number alteration of *PTPRD* in human cancers as determined by TCGA. (B) *PTPRD* copy number alterations do not correlate with altered *PTPRD* mRNA expression in HNSCC. A Jonckheere-Terpstra test was performed using StatXact software (Cytel, Cambridge, MA).

3.4 DISCUSSION

The survival rate for HNSCC patients has remained stagnant in recent decades despite advances in the understanding of the biological underpinnings of this disease and improved therapeutic strategies. In 2006, the Food and Drug Administration approved cetuximab, a monoclonal antibody targeting the epidermal growth factor receptor (EGFR), for the treatment of HNSCC

patients. [132] Unfortunately, cetuximab has produced limited success in the clinic, at least in part due to the lack of predictive biomarkers, including EGFR expression or copy number gain. [133] There is therefore a need for the identification of both novel targets for pharmacologic inhibition and biomarkers of sensitivity to those emerging therapeutics. STAT3 is a promising target for pharmacologic inhibition, and STAT3 inhibitors are being developed and tested in early phase clinical trials. [134] Additionally, we previously reported that STAT3 activation contributes to cetuximab resistance in HNSCC preclinical models and tumors from patients treated on cetuximab-containing protocols, indicating that direct inhibition of STAT3 may overcome certain mechanisms of resistance to available targeted therapeutics. [40] It is increasingly apparent that most molecular targeted agents will be most effective in subgroups of patients identified by specific genomic, epigenomic, and/or proteomic characteristics, including promoter methylation. The identification of biomarkers that identify those individuals most likely to benefit from such agents would allow the design of more scientifically rational clinical trials, and ultimately may provide broad clinical benefit to cancer patients.

Here we investigate the potential utility of *PTPRT* promoter methylation as a predictive biomarker of response to STAT3 inhibition in HNSCC preclinical models. We report that the *PTPRT* promoter is frequently hypermethylated in HNSCC (>60%) and several other cancer types in association with decreased *PTPRT* mRNA expression. Interestingly, a high rate of *PTPRT* promoter hypermethylation has previously been reported in other cancer types using alternate methodologies independent of TCGA. Colorectal cancer has the highest incidence of *PTPRT* promoter hypermethylation according to the present analysis (>78%), concordant with a previously published analysis of sporadic colorectal cancer, where nearly all tumors analyzed

were methylated at the *PTPRT* promoter in contrast to no observed methylation in matched normal tissues. [135] In addition, in hepatocellular carcinoma (a tumor type we were unable to analyze here due to unavailability of normal tissue in TCGA data), the *PTPRT* methylation level has been reported to progressively increase from adjacent tissue to tumor. [135, 136] Our present findings indicate that *PTPRT* promoter methylation may represent a common event across many cancer types, especially HNSCC, colorectal cancer, lung adenocarcinoma, and breast invasive carcinoma. These results suggest that the development of a therapeutic strategy informed by *PTPRT* promoter hypermethylation may be of wide clinical benefit.

To date, the functional signaling consequences downstream of *PTPRT* promoter hypermethylation have not been described, and as such, no strategies to mitigate the effects of this event have been proposed. Herein we demonstrate that the *PTPRT* promoter methylation is associated with a decrease in *PTPRT* mRNA expression in HNSCC. This downregulation is in turn associated with an increase in expression of the PTPRT substrate pSTAT3, indicating that *PTPRT* promoter methylation likely contributes to overexpression of PTPRT substrates, including pSTAT3. We further evaluated *PTPRT* promoter methylation in an independent cohort of HNSCC and observed a similarly high frequency to that observed in data available from TCGA, thus confirming the high incidence of this methylation event. We also demonstrate that *PTPRT* methylation significantly contributes to pSTAT3 upregulation in HNSCC cells with experiments that employ both 5-aza and shRNA to establish a direct mechanistic connection between these events.

Together, these studies suggest that HNSCC cells and tumors that exhibit high levels of *PTPRT* promoter methylation may be more dependent on STAT3 signaling for growth and

survival, thus offering the opportunity to exploit *PTPRT* methylation as a predictive biomarker of sensitivity to STAT3 inhibition. Here we have established an association between *PTPRT* promoter methylation and sensitivity to inhibitors of the STAT3 signaling pathway by calculating significant correlations between *PTPRT* promoter methylation in HNSCC cell lines as determined by MSP and EC_{50} values for the STAT3 inhibitor Stattic and STAT3 pathway inhibitors JSI-123 and LY2784544. These studies therefore provide a rationale for designing future clinical trials of STAT3-targeted therapeutics that select for patients with a high degree of *PTPRT* promoter methylation. Screening for high *PTPRT* promoter methylation may ultimately allow the identification of patients who are most likely to benefit from treatment with STAT3 targeting agents, leading to improved clinical outcomes.

4.0 DISCUSSION

Head and neck squamous cell carcinoma (HNSCC) is a frequently fatal malignancy that arises from the squamous epithelium of the upper aerodigestive tract. Current treatment modalities for this disease generally include surgery combined with some combination of chemotherapy, radiation, and/or cetuximab, a monoclonal antibody targeting EGFR that was approved for use in HNSCC by the FDA in 2006. These regimens are associated with significant co-morbidities, including skin reactions, mucositis, and surgical complications leading to diminished quality of life. [137-139] The major complication leading to low overall survival of HNSCC patients is frequent recurrence or formation of second primary tumors. These secondary tumors are often resistant to current therapies, and recurrent tumors in patients formerly treated on cetuximab-containing protocols have been reported to exhibit increased pSTAT3 expression, suggesting that STAT3 activation may represent an important and targetable mechanism of acquired cetuximab resistance in HNSCC patients. [40]

STAT3 is an oncogenic transcription factor that is frequently hyperactivated by tyrosine phosphorylation in primary and recurrent HNSCC. Aberrant constitutive STAT3 activation leads to potent activation or maintenance of several cancer phenotypes, including growth, survival, motility/invasion, angiogenesis, and evasion of apoptosis and the immune response. As such, STAT3 is a rational drug target for which targeted inhibitors are currently in clinical development. Importantly, biomarkers of response to such agents are currently lacking. It is

increasingly apparent that most targeted therapeutics will be most effective in tumors with particular and defined biologic characteristics, such as somatic mutation or promoter methylation of specific genes. The identification of such predictive biomarkers of exceptional response to STAT3 inhibitors may therefore lead to the design of more rational clinical trials, ultimately leading to improved patient outcomes in HNSCC.

While STAT3 is constitutively hyperactivated in most cancers, the mechanisms underlying this phenomenon remain incompletely understood. Somatic mutation of *STAT3* is rare, occurring with a frequency that is dramatically insufficient to explain the high frequency of observed STAT3 pathway overactivation. Instead, much effort has been expended to determine the contribution of mutational activation of kinases upstream of STAT3, including growth factor receptors such as EGFR and FGFR, as well as intracellular kinases such as SRC and JAK, among many others. Recent whole-exome sequencing studies across multiple institutions have now indicated that activating kinase mutations are rare in HNSCC, with the notable exception of *PIK3CA*. [116-118] We therefore sought to test the hypothesis that loss of function of proteins that normally inactivate STAT3, particularly phosphatases, will lead to STAT3 pathway activation and ultimately to increased sensitivity to STAT3 inhibition.

The PTPR family is the largest subgroup of the human protein tyrosine phosphatome. These enzymes, in close coordination with tyrosine kinases, regulate the phosphorylation status of signaling proteins, including STAT3. In particular, PTPRT and PTPRD have been demonstrated to directly target pSTAT3 in cell-free systems and several human cell types. [17, 20] Additionally, somatic mutation or promoter methylation of these genes has been reported in several cancer types, including GBM, colorectal cancer, and hepatocellular carcinoma, suggesting a contribution of loss of function of these proteins across cancer types. These prior

findings led us to further investigate the hypothesis that loss of function of PTPRT/D in particular will lead to increased phosphorylation of their common substrate pSTAT3, and ultimately lead to enhanced sensitivity to STAT3 inhibition.

The results presented herein support an important role for mutation or promoter methylation of *PTPRT/D* in HNSCC. We have shown that loss of function of PTPRT or PTPRD by somatic mutation or promoter hypermethylation occurs in ~10% or >60% of HNSCC tumors, respectively. These events lead to upregulation of pSTAT3, concomitant with upregulated cancer phenotypes such as growth, survival, proliferation, and escape from serum dependence. Importantly, several preclinical models indicate that *PTPRT/D* mutation or promoter methylation may predict enhanceed sensitivity to STAT3 pathway inhibitors. Further preclinical and clinical studies to more firmly establish these events as predictive biomarkers, including in knockout mouse models of oral carcinogenesis and a Phase 0 clinical trial expected to include 35 patients, are already underway. These studies may then inform the design of future trials that will include large numbers of patients selected for STAT3-targeted therapy based on the *PTPRT/D* status of their tumors. Such work may ultimately allow for the identification of patients who will be most likely to respond to STAT3 inhibitors, leading to improved patient outcomes in cancer.

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