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JAK Kinase Inhibition Abrogates STAT3 Activation and Head and Neck Squamous Cell Carcinoma Tumor Growth[1,2]

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Abstract

Aberrant activation of the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) 3 has been implicated in cell proliferation and survival of many cancers including head and neck squamous cell carcinoma (HNSCC). AZD1480, an orally active pharmacologic inhibitor of JAK1/JAK2, has been tested in several cancer models. In the present study, the *in vitro* and *in vivo* effects of AZD1480 were evaluated in HNSCC preclinical models to test the potential use of JAK kinase inhibition for HNSCC therapy. AZD1480 treatment decreased HNSCC proliferation in HNSCC cell lines with half maximal effective concentration (EC_{50}) values ranging from 0.9 to 4 μM in conjunction with reduction of $pSTAT3_{Tyr705}$ expression. *In vivo* antitumor efficacy of AZD1480 was demonstrated in patient-derived xenograft (PDX) models derived from two independent HNSCC tumors. Oral administration of AZD1480 reduced tumor growth in conjunction with decreased $pSTAT3_{Tyr705}$ expression that was observed in both PDX models. These findings suggest that the JAK1/2 inhibitors abrogate STAT3 signaling and may be effective in HNSCC treatment approaches.

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Introduction

Activation of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway has been detected in many human cancers [1–3]. JAKs are a family of cytoplasmic tyrosine kinases, comprised of four members—JAK1, JAK2, JAK3, and Tyk2 [4]. JAK activation occurs upon binding of a ligand to cell surface receptors, which phosphorylates tyrosine residues on the receptor and creates sites for interaction with proteins that contain phosphotyrosine binding SH2 domains [4]. The STATs are a family of downstream transcription factors of JAKs and other kinases and include STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6 [5]. STATs contain a conserved tyrosine residue near the C-terminus that is phosphorylated by JAKs, leading to the formation of homo-STAT or hetero-STAT dimers, tyrosine phosphorylation, and subsequent nuclear translocation [6]. In the nucleus, STATs serve as transcription factors initiating the transcription of downstream target genes [7]. Abnormalities of the JAK/STAT pathway contribute directly to cellular transformation [8], increased cell proliferation, survival, angiogenesis, and immune system evasion [7].

Cumulative evidence implicates STAT3 in cancer development and progression. Elevated STAT3 activity has been associated with increased morbidity and mortality in several cancers including multiple myeloma, leukemia, lymphoma, and breast and head and neck squamous cell carcinoma (HNSCC) [9]. We recently reported that the JAK/STAT pathway is rarely mutated in HNSCC in contrast to activating JAK mutations that characterize hematopoietic

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conditions including myeloproliferative neoplasms and leukemias [10,11]. Several approaches have been used to target STAT3 for cancer therapy [7]. These include peptidomimetics, aptamers, antisense oligonucleotides, G quartets, STAT3 decoys, dominant-negative mutants of STAT3, and small molecule tyrosine kinase inhibitors [12,13]. To date, a decoy oligonucleotide is the only STAT3 selective inhibitor, which has demonstrated biologic activity in HNSCC patients in a phase 0 clinical trial [14]. However, challenges in drug delivery have limited the clinical translation of transcription factor decoys [14].

JAK2 activating mutations and chromosomal translocations have identified JAK2 as a target for the treatment of myelofibrosis and may be a molecular target in several other cancers [4,9]. Given the paucity of small molecule STAT3-selective therapies, JAK inhibitors can be used to target STAT3 activation for cancer treatment. AZD1480 is a potent, ATP-competitive small-molecule inhibitor of JAK2 kinase [15]. AZD1480 demonstrated antitumor activity in several cancer models. In multiple myeloma cells, AZD1480 abrogated Interleukin -6 (IL-6)–induced activation of JAK2 and tyrosine phosphorylation of STAT3 [16]. In glioblastoma, AZD1480 suppressed STAT3 activation *in vitro* and inhibited the growth of xenograft tumors *in vivo*[17]. In the present study, the *in vitro* and *in vivo* efficacy of AZD1480 was evaluated in HNSCC preclinical models for the first time. *In vitro*, AZD1480 decreased HNSCC cell proliferation as well as STAT3 phosphorylation. *In vivo*, AZD1480 significantly slowed tumor growth in heterotopic xenograft tumor models derived from two HNSCC patient tumors in conjunction with downmodulation of pSTAT3 expression. These results suggest that JAK kinase inhibition may be a promising approach to target STAT3 signaling pathways in solid tumors, including HNSCC, which lack genetic JAK/STAT alterations.

Materials and Methods

Cell Lines and Reagents

HNSCC cell lines UMSCC-1, UM-22B, UM-22A, Cal33, PCI-15B, HN5, OSC19, PCI-52, and 686LN were genotypically validated using the AmpFlSTR Profiler Plus kit from PE Biosystems (Foster City, CA) according to the manufacturer's instructions. AZD1480 was obtained from Astra Zeneca, Waltham, MA.

Cell Culture

HNSCC cell lines UMSCC-1, UM-22B, and UM-22A (a generous gift from Dr Thomas E. Carey, University of Michigan), Cal33 (a kind gift from Jean Louis Fischel, Centre Antoine Lacassagne, Nice, France), PCI-52 and PCI-15B (created at the University of Pittsburgh), and HN5 and OSC19 were cultured in Dulbecco's modified Eagle's medium (Mediatech, Inc, Herndon, VA) containing 10% heat-inactivated FBS at 37°C with 5% CO_2. 686 LN (a kind gift from Georgia Chen, University of Emory, Atlanta, GA) was maintained in Dulbecco's modified Eagle's medium/F12 media (1:1) from Gibco (Carlsbad, CA) containing 10% heat-inactivated FBS (ISC BioExpress, Kaysville, UT).

Immunoblot Analysis

A panel of HNSCC cell lines including UMSCC-1, UM-22B, UM-22A, Cal33, PCI-15B, HN5, OSC19, PCI-52, and 686LN was seeded in 10-cm plates (1×10^6 cells), and after 24 hours, cells were harvested to obtain cell lysates. Forty micrograms of protein/lane was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and probed with rabbit anti–phospho-JAK1 and JAK2 polyclonal antibodies (Millipore, Billerica, MA), rabbit anti–phospho-STAT3 monoclonal antibody, and STAT3 polyclonal antibodies (Cell Signaling Technology, Inc, Danvers, MA). β-Tubulin (Abcam, Cambridge, MA) was used as a loading control.

The Cancer Genome Atlas Analyses

mRNA expression data, mutation data, and gene copy number data on 279 HNSCC tumors were downloaded from The Cancer Genome Atlas (TCGA) Data Portal using R version 3.02. Tumors with JAK copy number alteration were compared to tumors without JAK copy number alteration using an unpaired *t* test with Welch's correlation in Graphpad Prism 6.

Dose-Response Studies

HNSCC cell lines were treated with varying concentrations of AZD1480 for 72 hours. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assays were performed to determine percent cell viability.

siRNA Transfection

JAK2 siRNA was obtained from Dharmacon (Lafayette, CO), whereas the control siRNA was obtained from Thermo Scientific (Pittsburgh, PA). siRNA transfection was performed using Lipofectamine RNAi/MAX from Invitrogen (Grand Island, NY) following the manufacturer's instructions with a final siRNA concentration of 5 pmol/well. Protein was extracted 48 and 72 hours after transfection and immunoblotted for $pSTAT3_{Tyr705}$ and total STAT3. β-Tubulin was used as a loading control. Cell proliferation assays were performed on days 1, 3, and 6 after transfection.

Dose-Dependent Effect of AZD1480 in HNSCC Cell Lines

HNSCC cell lines (UMSCC-1, Cal33, and HN5) were plated, and after 24 hours of plating, cells were serum starved for an additional 24 hours and treated with increasing concentrations of AZD1480. Fifteen minutes before the end of 24 hours, cells were stimulated with IL-6 (50 ng/ml) cells. At the end of the 24-hour treatment time, cells were harvested to obtain cell lysates. Forty micrograms of protein/lane was subjected to electrophoresis and immunoblotted for $pSTAT3_{Tyr705}$ and total STAT3, Bcl-X_L, and cyclin D1. β-Tubulin was used as a loading control.

Dose-Dependent Effect of AZD1480 on $pSTAT3_{Tyr705}$ in HNSCC Cell Lines

HNSCC cell lines UMSCC-1 and Cal33 were plated, and after 24 hours of plating, cells were treated with increasing concentrations of AZD1480. At the end of the 24-hour treatment time, cells were harvested to obtain cell lysates. Forty micrograms of protein/lane was subjected to electrophoresis and immunoblotted for $pSTAT3_{Tyr705}$ and STAT3. β-Tubulin was used as a loading control.

Dose-Dependent Effect of AZD1480 on $pSTAT5_{Tyr694}$ in Cal33 Cell Lines

Cal33 cell lines were plated, and after 24 hours of plating, cells were serum starved for an additional 24 hours and treated with increasing concentrations of AZD1480. Fifteen minutes before the end of 24 hours, cells were stimulated with IL-6 (50 ng/ml) cells. At the end of the 24-hour treatment time, cells were harvested to obtain cell lysates. Forty micrograms of protein/lane was subjected to electrophoresis and immunoblotted for $pSTAT5_{Tyr694}$. β-Tubulin was used as a loading control.

Immunoblot Analysis and In Vivo Patient-Derived Xenograft Studies

Human tumors were obtained under the auspices of an Institutional Review Board (IRB)-approved protocol with informed consent. The tumor tissues were processed for Western blot analyses as described previously [18] to look at the expression of pJAK1, pJAK2, JAK1, and JAK2. β-Tubulin was used as a loading control. For the *in vivo* experiment, nonobese diabetic/severe combined immunodeficiency (NOD SCID) gamma mice (4-6 weeks old; 20 g; The Jackson Laboratory, Bar Harbor, Maine) were implanted with tumors subcutaneously from two different HNSCC patients. Before implantation, each mouse was anesthetized using isoflurane, and the area for the incision was prepared using iodine and alcohol preparation pads. A half centimeter incision or smaller was made, and a 25-mg tumor section was placed under the skin in both the right and left flanks. The wound was closed with Vetbond Tissue Adhesive, and a single injection of ketoprofen was given intramuscularly at a dose of 5 mg/kg. The animal was monitored until it was fully mobile and had recovered all normal functions. Once the tumors were palpable, mice were randomized and treated with AZD1480 or saline as a vehicle. Four groups (two groups of five mice with two tumors on each side from one patient and another two groups of five mice with two tumors on each side from a second patient) received either AZD1480 at a dose of 30 mg/kg or saline through oral gavage, twice daily, and at the end of the treatment period, tumors were harvested and lysates were collected for determination of $pSTAT3_{Tyr705}$ and STAT3 expression. Animal care was in strict compliance with institutional guidelines established by the University of Pittsburgh, the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, 1996), and the Association for Assessment and Accreditation of Laboratory Animal Care International. In addition, lysates from HNSCC patient tumors were collected and probed for expression of pJAK1, pJAK2, JAK1, and JAK2 expression.

Statistical Analyses

Statistical analyses of the *in vitro* cell line comparison studies as well as the *in vivo* antitumor efficacy of AZD1480 on tumor volume measurements on the last day of the xenograft experiment were compared by testing the difference, with a two-tailed Wilcoxon test.

Results

HNSCC Cell Lines Express Variable Levels of Phospho-JAK1, Phospho-JAK2, Phospho-JAK3, and Phospho-STAT3 Expression Levels and JAK2 Copy Number Correlates with JAK2 mRNA Expression in HNSCC Tumors

STAT3 activation has been studied in HNSCC downstream of several receptor and non-receptor kinases. The precise role of JAK kinases in HNSCC is incompletely understood. To determine the relative expression levels of total and phosphorylated forms of JAK1, JAK2, JAK3, and STAT3 in HNSCC cells, a panel of genotypically validated HNSCC cell lines including UMSCC-1, UM-22B, UM-22A, Cal33, PCI-15B, HN5, OSC19, PCI-52, and 686LN was evaluated for pJAK1, JAK1, pJAK2, JAK2, pJAK3, JAK3, $pSTAT3_{Tyr705}$, and STAT3 protein expression levels. As shown in Figure 1*A*, HNSCC cells demonstrated varying levels of pJAK1, pJAK2, pJAK3, and pSTAT3 in the cell lines tested. TCGA HNSCC samples were grouped according to their copy number alteration status. A copy number alteration of −2 is considered a homozygous deletion, −1 is a heterozygous deletion, 0 is no copy number alteration, 1 is a gain, and 2 is high level amplification. The copy number data sets were generated by the GISTIC or RAE copy number analysis algorithms in the TCGA portal. Both algorithms identify significantly altered regions of amplification or deletion. Although inconsistencies between copy number alteration (CNA) and gene expression profiles have been previously found in other cancer types [19], we compared RNAseq values of HNSCC tumors with altered gene copy number for the JAK family of proteins to HNSCC tumors without copy number change. As shown in Figure 1*B*, there is a significant correlation only between heterozygous deletion of JAK1 and JAK1 mRNA expression (CNA = −1, $P < .0001$). JAK1 is not commonly upregulated, and when it is, there is no apparent correlation between copy number alteration and mRNA expression ($P = .1438$). As shown in Figure 1*C*, JAK2 is commonly upregulated, and there is a significant correlation between copy number alteration and mRNA expression (CNA = 1, $P = .0168$; CNA = 2, $P = .0069$). There are also significant correlations between copy number losses and mRNA expression (CNA = −1, $P < .0001$; CNA = −2, $P < .0001$). We also compared RNAseq values of HNSCC tumors with JAK2 copy number alteration status of 1 to that of HNSCC tumors with copy number alteration status of 2 ($P = .0307$). Similarly to our JAK1 findings, JAK3 is rarely upregulated and high-level amplification is not significantly associated with increased JAK3 mRNA expression (Figure 1*D*; $P = .1070$).

AZD1480 Inhibits HNSCC Cell Line Proliferation In Vitro

AZD1480 has been reported to inhibit the proliferation of some solid tumor cell lines, whereas many others are clearly resistant to AZD1480 with concentration that inhibits growth by 50% (GI_{50}) values > 30 μM [15]. Dose-response experiments were performed in a panel of HNSCC cell lines to determine the relative sensitivity of HNSCC preclinical models. UMSCC-1, UM-22B, UM-22A, Cal33, PCI-15B, HN5, OSC19, PCI-52, and 686LN cells were treated with a range of doses of AZD1480 for 72 hours, and MTT assays were performed to assess cell viability. The half maximal effective concentration (EC_{50}) values of all the cell lines tested ranged from 0.9 to 3.8 μM, suggesting that the majority of HNSCC cells are sensitive to JAK1/2 inhibition (Table 1).

JAK2 Down-Regulation Attenuates HNSCC Proliferation

We next explored whether selective inhibition of JAK2 attenuated proliferation of HNSCC cells. UMSCC-1 and HN5 cells when transfected with JAK2 siRNA demonstrated knockdown of JAK2 expression after 48 and 72 hours, and proliferation of these HNSCC cells was reduced on treatment of the cells with JAK2 siRNA compared with control siRNA (Figure 2, *A* and *B*).

Dose-Dependent Effect of AZD1480 on $pSTAT3_{Tyr705}$ Protein Expression in HNSCC Cell Lines

AZD1480 targets both JAK1 and JAK2 kinases. STAT3 is activated downstream of several kinases including JAKs. While there is little evidence to date to support a critical function of JAKs in HNSCC, cumulative evidence implicates STAT3 activation in HNSCC progression [2]. To determine the effect of AZD1480 on pSTAT3 expression, UMSCC-1, HN5, Cal33, 686LN, or PCI-15B cells were treated with increasing concentrations of AZD1480 for 24 hours, followed by immunoblot analysis for $pSTAT3_{Tyr705}$ and STAT3. AZD1480 abrogated IL-6–induced up-regulation of $pSTAT3_{Tyr705}$ in a dose-dependent manner in UMSCC-1, HN5, and Cal33 cells (Figure 3). Similar effects were seen on pSTAT3 expression in the

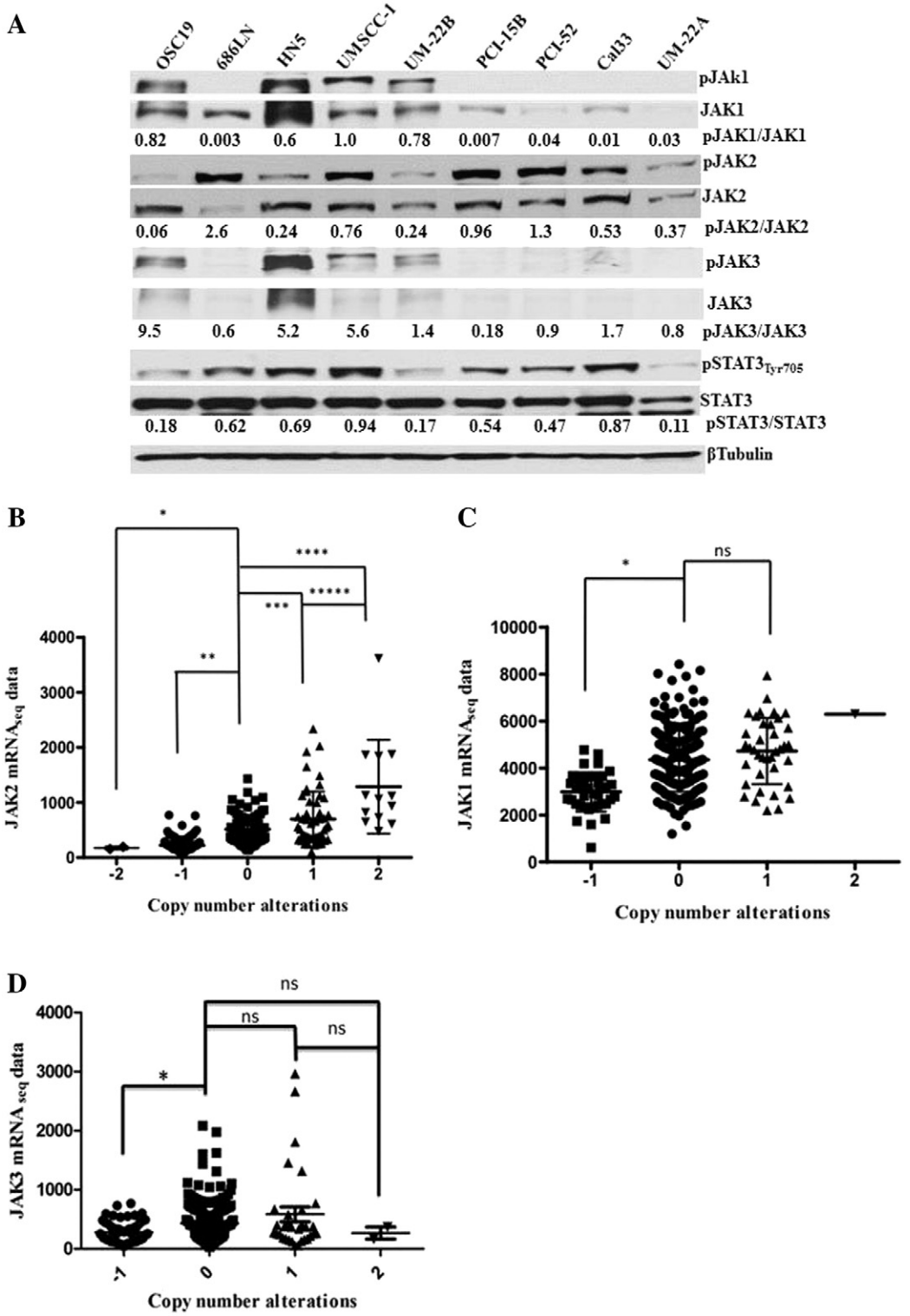

Figure 1. (**A**) HNSCC cell lines express varying levels of pJAK1, pJAK2, and pSTAT3. HNSCC cell lines (OSC19, 686LN, HN5, UMSCC-1, UM-22B, PCI-15B, PCI-52, Cal33, and UM-22A) were seeded in 10-cm plates (1×10^6 cells), and after 24 hours, cells were harvested to obtain cell lysates. Forty micrograms of protein/lane was subjected to electrophoresis and immunoblotted for pJAK1, pJAK2, pJAK3, and $pSTAT3_{Tyr705}$. β-Tubulin was used as a loading control. The numbers below the bands are a quantitative representation of pJAK1/β-tubulin, pJAK2/β-tubulin, pJAK3/β-tubulin, and $pSTAT3_{Tyr705}$/β-tubulin in HNSCC cell lines from three independent experiments. (**B**) Association between JAK2 mRNA expression and JAK2 copy number alteration in HNSCC tumors determined by GISTIC or RAE algorithm in the TCGA portal (*$P < .0001$; **$P < .0001$; ***$P = .0168$; ****$P = .0069$; *****$P = .0307$). *P* values were determined by unpaired *t* test with Welch's correlation. (**C**) Association between JAK1 mRNA expression and JAK1 copy number alteration in HNSCC tumors determined by GISTIC or RAE algorithm in the TCGA portal (*$P < .0001$). *P* value was determined by unpaired *t* test with Welch's correlation. (**D**) Association between JAK3 mRNA expression and JAK3 copy number alteration in HNSCC tumors determined by GISTIC or RAE algorithm in the TCGA portal (*$P < .0001$). *P* values were determined by unpaired *t* test with Welch's correlation.

Table 1. EC_{50} Values for AZD1480 in HNSCC Cell Lines. HN5, Cal33, UM-22B, 686LN, UMSCC-1, UM-22A, OSC19, PCI-52, and PCI-15B Cells Were Treated with a Range of Concentrations of AZD1480 for 72 Hours. MTT Assays Were Then Performed and EC_{50} Values were Calculated. The Experiment was Performed Three Times with Similar Results. Results are Mean ± SD for All Three Experiments Combined

HNSCC Cell Line	EC_{50} (μM)
HN5	3.81 ± 1.99
Cal33	3.37 ± 0.75
UM-22B	2.66 ± 0.24
686LN	2.05 ± 1.33
UM SCC-1	1.67 ± 0.42
UM-22A	1.32 ± 0.39
OSC19	1.26 ± 0.20
PCI-52	1.00 ± 0.09
PCI-15B	0.99 ± 1.74

absence of exogenous IL-6 stimulation (Figure S1). JAK2 siRNA treatment also abrogated pSTAT3 expression in these cells (Figure S2).

In addition to STAT3, STAT5 is also constitutively activated in HNSCC cells [20]. To determine the effect of AZD1480 on pSTAT5 expression, Cal33 cells were treated with increasing concentrations of AZD1480 for 24 hours, followed by immunoblot analysis for $pSTAT5_{Tyr694}$. Similar to its effects on pSTAT3, AZD1480 abrogated IL-6–induced up-regulation of $pSTAT5_{Tyr694}$ in a dose-dependent manner in Cal33 cells (Figure S3).

AZD1480 Inhibits Growth and STAT3 Signaling In Vivo of HNSCC Patient-Derived Xenografts

Immortalized cell lines bear limited resemblance to human tumors. To determine the antitumor effects of AZD1480, we generated heterotopic tumorgraft models of primary HNSCC tumors and assessed the consequences of AZD1480 treatment in these models. Both patient-derived xenograft (PDX) models were derived from Human papillomavirus (HPV)-negative oral cavity tumors. As shown in Figure 4*A*, AZD1480 administration effectively abrogated the growth of both HNSCC tumorgrafts (30 mg/kg, administered twice daily through oral gavage) compared with saline (vehicle) treatment. At the end of treatment, tumors derived from patient 1 and treated with AZD1480 demonstrated a significant decrease in tumor volume (P = .0037) compared to the group treated with vehicle control. Tumors derived from patient 2, treated with AZD1480, demonstrated a decrease in tumor volume compared to the group treated with vehicle, although the difference did not reach statistical significance (P = .066). At the end of treatment, the tumors were harvested 3.5 hours after the last treatment and evaluated for pSTAT3 expression through immunoblot analysis (Figure 4*B*). Five of the nine tumors from patient 1 treated with AZD1480 were too small to be harvested for immunoblot analysis. Tumors from both patient 1 and patient 2 treated with AZD1480 demonstrated a significant decrease in $pSTAT3_{Tyr705}$ expression compared to the tumors treated with vehicle alone (P = .003 and P = .0006, respectively; Figure 4*C*).

Discussion

In the present study, the orally bioavailable JAK1/2 inhibitor AZD1480 was evaluated in HNSCC preclinical models to determine its antitumor efficacy. Evaluation of pJAK1, pJAK2, pJAK3, and pSTAT3 in a panel of nine immortalized HNSCC cell lines demonstrated variable endogenous expression levels of these proteins. Although JAK1 mutations have been detected in breast and gastric carcinomas [21], in TCGA, JAK1/2 mutations have been reported in a very small head and neck cancer patient population. Additionally, significant correlation of CNA and gene expression profiles in the TCGA HNSCC cohort is only seen for JAK2 and there is no evident correlation between CNA and gene expression for JAK1 or JAK3 in HNSCC. Discrepancies between CNA and gene expression levels

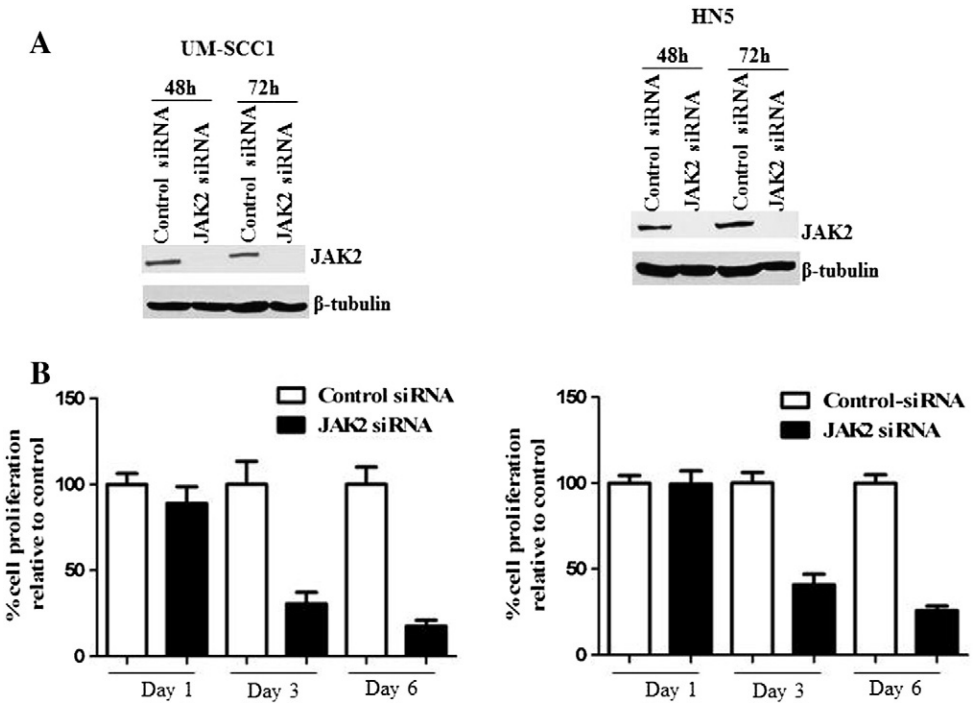

Figure 2. Proliferation of HNSCC cells upon knockdown of JAK2 by JAK2 siRNA. (**A**) UMSCC-1 and HN5 cells were transfected with JAK2 siRNA or control siRNA, and after 48 and 72 hours, cells were harvested for JAK2 expression. (**B**) Further cell proliferation was monitored upon JAK2 knockdown after days 1, 3, and 6. Results are from three separate experiments.

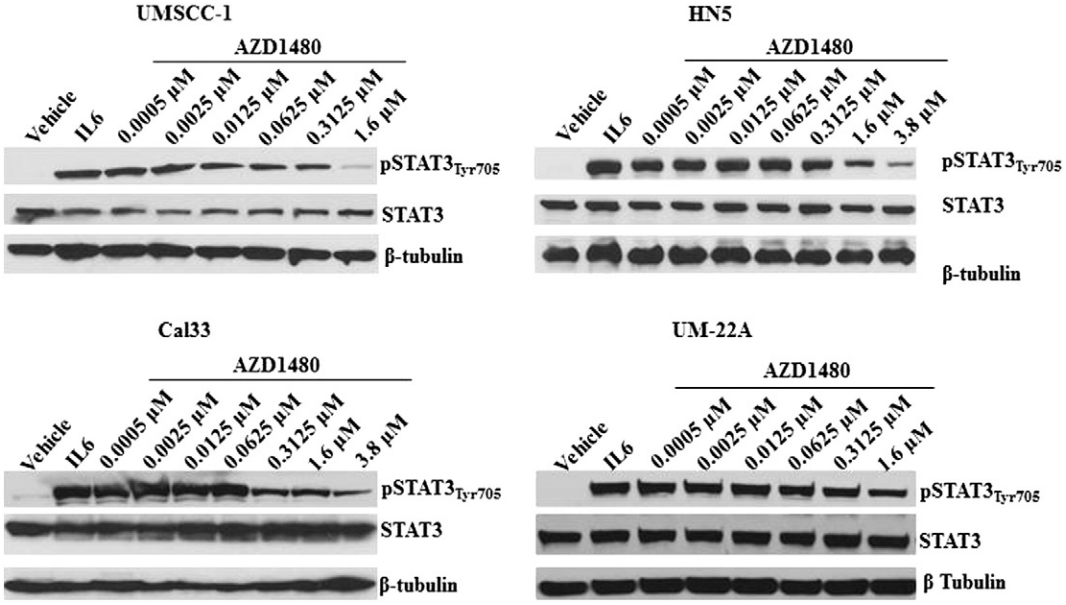

Figure 3. Effect of AZD1480 on $pSTAT3_{Tyr705}$ expression in HNSCC cell lines. UMSCC-1, HH5, Cal33, and UM-22A cells were treated with increasing concentrations of AZD1480. After 24 hours of treatment, cells were stimulated with IL-6 (50 ng/ml) for the last 15 minutes. At the end of the treatment, cells were harvested to obtain cell lysates. Forty micrograms of protein/lane was subjected to electrophoresis and immunoblotted for $pSTAT3_{Tyr705}$ and total STAT3. β-Tubulin was used as a loading control. The experiment was performed three times with similar results.

have been reported in hepatocellular and colorectal carcinomas, where copy number losses did not necessarily correlate with gene expression levels [19] and in oral squamous cell carcinoma (OSCC) where CNA could be attributed to only 31% of gene expression differences detected [22]. The exact mechanism for correlation of CNA and gene expression is unknown, and other factors, such as DNA methylation, or histone acetylation, methylation, and phosphorylation, may contribute. *In vitro* and *in vivo* studies with AZD1480 in HNSCC cell lines and PDXs demonstrated antiproliferative and antitumor efficacy in conjunction with inhibition of STAT3 phosphorylation. HNSCC cell lines treated with AZD1480 demonstrated EC_{50} values in the nanomolar or submicromolar range that did not correlate with endogenous pSTAT3 expression levels. Further, we also examined the effect of JAK2 siRNA on HNSCC cell proliferation and we did not find any correlation between sensitivity to JAK2 siRNA and response to AZD1480. Biochemical evaluation of AZD1480 in HNSCC cell lines showed dose-dependent decreases in pSTAT3 expression with increasing AZD1480 concentrations. Evaluation of *in vivo* antitumor efficacy in PDXs demonstrated abrogation of tumor volumes in conjunction with decreases in pSTAT3 expression in the tumors from mice treated with AZD1480 compared to vehicle control–treated animals. The responses of HNSCC PDXs to AZD1480 treatment underscores the heterogeneous responses likely to be observed in the clinic and emphasizes the need for predictive biomarkers to identify individuals who are most likely to benefit from this treatment strategy.

The contribution of persistent STAT3 activation to tumor progression, drug resistance, cell migration and invasion, angiogenesis, and metastasis of cancer has identified STAT3 as a therapeutic target in preclinical models of most malignancies [23,24]. Although a large number of STAT3 inhibitors have been reported to date, there is a paucity of selective small molecule STAT3 targeting agents under clinical development [25]. Given the challenges associated with developing small molecule inhibitors that directly inhibit the STAT3 transcription factor, targeting upstream activating kinases such as JAKs offers a pharmaceutically viable alternative to block STAT3 signaling. Several JAK1/2 inhibitors have been developed following the discovery of the JAK2 mutation (JAK2V617F) in myeloproliferative disorders [26]. A number of these JAK inhibitors have been assessed for their effectiveness in a variety of preclinical cancer models. A natural compound screen identified JSI-124 (cucurbitacin I), which is very effective at suppressing the levels of tyrosine-phosphorylated STAT3 and JAK2 but is unable to directly inhibit Src, JAK1, or JAK2 kinase activities in vitro, indicating a lack of clarity about the precise target of this compound. JSI-124 suppressed proliferation, induced DNA fragmentation, poly(ADP-ribose) polymerase cleavage, and caspase-3 activation in a dose-dependent manner in OSCC cell lines. JSI-124 also decreased expression of pSTAT3 expression as well as survivin, a downstream target of STAT3 signaling pathway. However, the *in vivo* antitumor efficacy of JSI-124 in OSCC has not been reported [27]. In nasopharyngeal cancer, administration of JSI-124 in nude mice before tumor cell inoculation suppressed *in vivo* tumor formation [28]. WP1066, a JAK2 inhibitor, demonstrated antitumor effects mediated, at least in part, by suppression of JAK2-STAT3 signaling in preclinical HNSCC models [3]. FLLL32, a novel small molecule inhibitor derived from curcumin, induced sensitized HNSCC cells to cisplatin *in vitro*[29].

In the absence of preclinical data on the effects of JAK1/2 inhibitors in HNSCC, we tested AZD1480 in HNSCC models to determine its antitumor properties. AZD1480, a pharmacologic inhibitor of JAK1/2, has been used as a therapeutic strategy to target STAT3 signaling in several cancers including glioblastoma [17], skin [16], and lymphoma [30], among others. The results of our study and others [31,32] demonstrate that AZD1480 affects the expression of pSTAT3 and pSTAT5. Others have reported that in addition to effects on pSTAT3, AZD1480 downregulates expression of STAT3

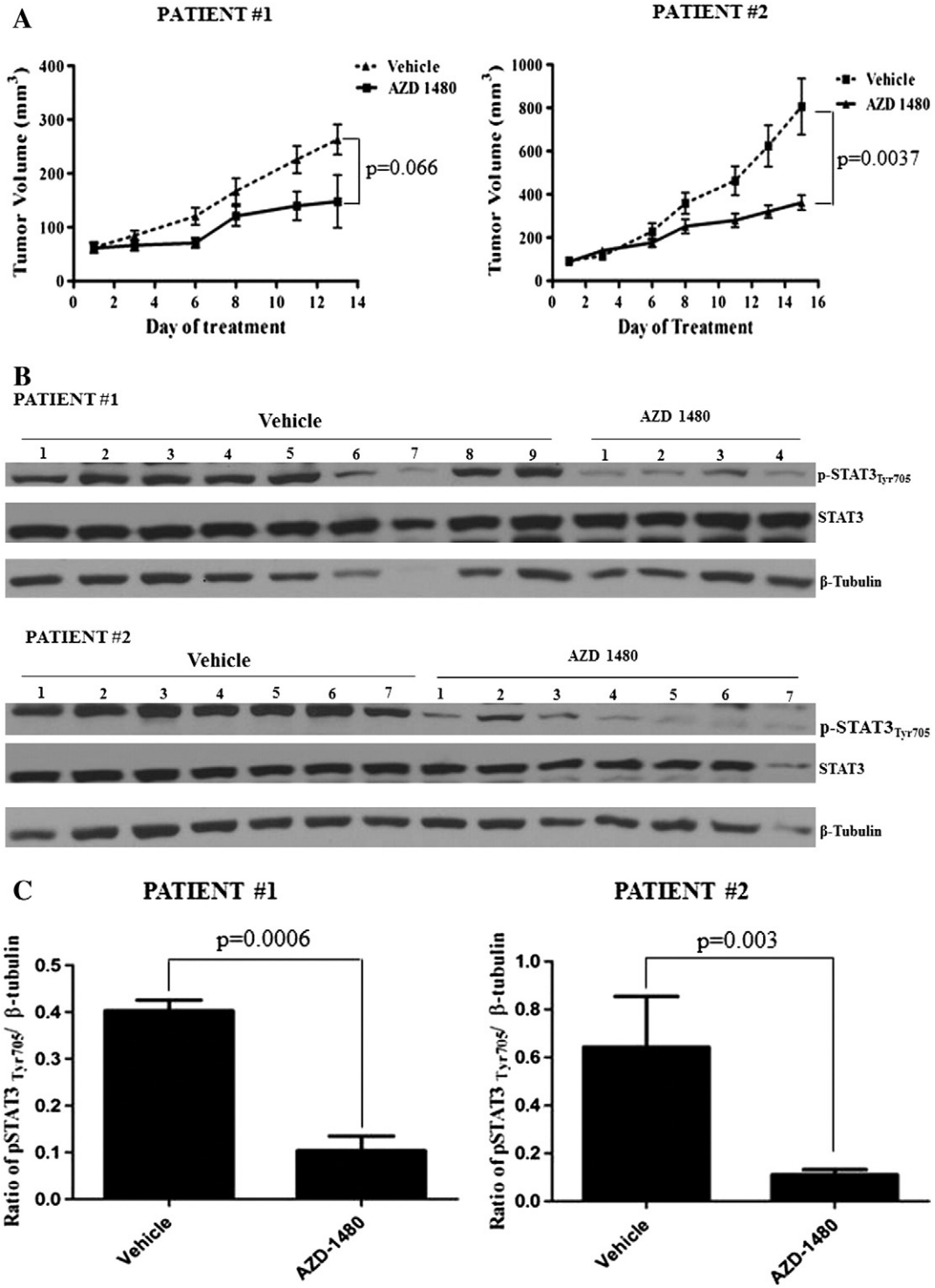

Figure 4. AZD1480 suppresses growth and expression of $pSTAT3_{Tyr705}$ target genes in HNSCC patient-derived heterotopic tumorgrafts. (**A**) HNSCC tumors from two individual patients were inoculated subcutaneously in the right and left flanks of NOD SCID gamma mice. Following the development of palpable tumors, mice were randomized and AZD1480 at a dose of 30 mg/kg was administered twice daily through oral gavage. Tumor volume measurements were obtained three times a week and measured to days 15 and 13 and are graphed. The nonparametric Wilcox Mann-Whitney test was used for tumor volume on day 15 to determine significance. (**B**) At the end of treatment (day 13 for patient 1 tumors and day 15 for patient 2 tumors), tumors were harvested, and whole-cell lysates were prepared and subjected to immunoblot analysis for $pSTAT3_{Tyr705}$ and STAT3. β-tubulin was used to assess protein loading. (**C**) The bar graph is a quantitative representation of the ratio of $pSTAT3_{Tyr705}$/β-tubulin ($P = .003$, patient 1 and $P = .066$, patient 2).

target proteins including *cyclin D1*, *survivin, Bcl-2*, and *Mcl1* [16,33]. Knockdown of JAK2 using siRNA from our studies and others [32] is associated with reduced expression of pSTAT3. In papillary thyroid carcinoma and medullary thyroid carcinoma, AZD1480 demonstrated reduced phospho-Y1062 receptor tyrosine kinase for members of the glial cell line-derived neurotrophic factor

(GDNF) family (RET) levels and Mammalian target of Rapamycin (mTOR) effector phospho-S6, while JAK1/2 down-regulation by siRNA did not affect cell growth nor RET and S6 activation [34]. In the present study, a lack of significant correlation between biochemical target inhibition and EC_{50} values suggests that expression levels of target(s) in the tumor do not predict sensitivity to AZD1480 and the capacity to predict therapy outcome does not merely depend on the expression of these STAT3 pathway proteins in tumor cells. PDXs are emerging as potentially more relevant preclinical models in many cancers including HNSCC [35,36]. Although they require a significant investment of resources and a commitment to transfer biospecimens rapidly from the operating room to the laboratory, these models offer an opportunity to assess putative therapeutic agents while preserving essential patient tumor characteristics. Such an approach was recently reported by our group and others identifying *PIK3CA* mutation as a predictive biomarker to Phosphoinositol-3-kinase (PI3K) pathway inhibitors in HNSCC [10,35]. Although the predictive biomarkers of response to JAK1/2 inhibitors including AZD1480 remain unknown, our results in PDX models may reflect different clinical responses of patients to agents in the clinic.

Cumulative evidence supports activation of STAT3 as an oncogenic pathway in many cancers including both solid tumors and hematopoietic malignancies. In the absence of a STAT3 selective targeting agent amenable to systemic administration, small molecule JAK inhibitors represent a plausible clinical strategy to abrogate STAT3 signaling. In the present study, we tested the effects of AZD1480, an orally active pharmacologic inhibitor of JAK1/JAK2, in preclinical HNSCC models including immortalized cell lines. AZD1480 was also tested in PDX models derived from primary HNSCC tumors. Our results suggest that JAK inhibition abrogates STAT3 activation with heterogeneous responses in both cell lines and animal models. Further evaluation of pJAK1/pJAK2 levels in the HNSCC patient tumors used for heterotopic tumorgraft demonstrated increased expression of pJAK2 in the PDX that was more sensitive to AZD1480 suggesting that baseline pJAK2 expression may contribute to mediating responses to JAK inhibitors.

Conclusion

Our studies suggest that the STAT3 protein can be targeted using AZD1480, the JAK1/2 inhibitor. AZD1480 is able to inhibit proliferation of the HNSCC cell lines, downmodulate pSTAT3 *in vitro* in a dose-dependent manner, as well as reduce tumor growth in HNSCC PDX models in conjunction with decrease in pSTAT3 expression in the PDX tumors.

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