DUSP1 phosphatase regulates the proinflammatory milieu in head and neck squamous cell carcinoma

Xiaoyi Zhang¹, J. Madison Hyer², Hong Yu¹, Nisha J. D'Silva³, Keith L. Kirkwood¹

1. Department of Oral Health Sciences and Center for Oral Health Research, Medical University of South Carolina (MUSC)
2. Department of Public Health Sciences, MUSC
3. Department of Periodontics and Oral Medicine, University of Michigan School of Dentistry

Running title: DUSP1 regulates inflammation in HNSCC

Keywords: DUSP1, Il-1b, head and neck cancer, inflammation, 4-nitroquinoline 1-oxide

Financial Support:
R01DE018290, R01DE021423 and P30GM103331 (KLK)
R01DE022567 (NJD)
F30CA165518 (XZ)
UL1RR029882, UL1TR000062, P30CA138313, C06RR015455 (MUSC)

Corresponding author:
Keith L. Kirkwood, DDS, PhD
173 Ashley Avenue
BSB 254A, MSC 507
Charleston, SC 29425
Phone: (843) 792-0969
Fax: (843) 792-6626
Email: klkirk@musc.edu

Conflicts of interest: The authors disclose no potential conflicts of interest.

Word count: 2,999 words; 4 figures; 6 supplemental figures
Abstract

DUSP1 is a dual-specificity phosphatase that regulates mitogen-activated protein (MAP) kinase activity. Studies have associated loss of DUSP1 expression with certain cancers, but there has been no report of a mechanism by which this supports tumor progression. In this study, we found DUSP1 mRNA and protein decreased in human head and neck squamous cell carcinoma tissues compared to adjacent non-tumor controls. To evaluate the impact of this difference, we compared the susceptibility of Dusp-1 deficient mice to oral squamous carcinogenesis induced by 4-nitroquinoline 1-oxide. Dusp1-deficient mice displayed enhanced disease progression, characterized by advanced onset, histological stage, and tumor burden. In a syngeneic model of tumor progression, subcutaneous injection of EO771 cells formed faster-growing tumors in Dusp1-deficient mice, an effect abrogated by inhibition of p38 MAP kinase with SB203580. Histological and quantitative assessments demonstrated increased inflammation and deregulated chemokine and cytokine expression in Dusp1-deficient tumor tissues. Specifically, pro-inflammatory cytokine IL-1β was elevated. IL-1β production was recapitulated ex vivo in primary bone marrow-derived macrophages from Dusp1-deficient mice. Together, our results clearly establish the role of Dusp1 as a tumor suppressor gene that regulates cancer-associated inflammation.
Introduction

Inflammatory cells and their secreted products influence cancer cell growth, angiogenesis, tissue remodeling, and more (1). Chronic inflammation is known to enhance neoplastic processes, as in gastrointestinal and hepatocellular carcinomas (2). However, in other tumor types including head and neck squamous cell carcinoma (HNSCC), studies have identified immunosuppressive states characterized by infiltrating regulatory T and myeloid-derived suppressor cells (3). This dichotomy between enhanced pro-tumorigenic inflammation and suppressed anti-tumor immunity highlights the need to understand complex functions of inflammatory cells in the tumor microenvironment and identify therapeutic targets in these distinctive pathways.

DUSP1 is a dual-specificity Thr/Tyr phosphatase that regulates mitogen-activated protein kinase (MAPK) signaling pathways by dephosphorylating p38 and JNK MAPKs (4). Dusp1-deficient mice are highly susceptible to immune challenge, such as LPS in sepsis and inflammatory bone loss models, due to prolonged MAPK activity and exuberant cytokine production (5,6). In bladder, prostate, and colon cancers, Duspl expression is elevated in early stages of disease, but lost in a majority of poorly differentiated and metastatic tissues (7). In the present study, we assess the impact of Dusp1 deficiency in a murine model of carcinogen-induced oral cancer. Tumor progression was also enhanced in a syngeneic subcutaneous injection of breast cancer cells, rescued by p38 inhibition. Increased susceptibility and disease burden of Dusp1-deficient mice, in addition to development of a highly inflammatory microenvironment, suggest DUSP1 plays a key role in negatively regulating tumor-associated inflammation, in part through expression of the inflammatory cytokine IL-1β. To our knowledge, this is the first study to demonstrate a tumor suppressive role of DUSP1.
Materials and methods

Animal models

Protocols were approved by Medical University of South Carolina (MUSC) Institutional Animal Care and Use Committee. Dusp1⁻/⁻ and Dusp1⁺/⁺ mice, on C57/129 mixed genetic background. Male and female mice aged 6-8 wk old and weighing 18-22g were treated with 4-nitroquinoline 1-oxide (4NQO), as previously described (8). Animals were euthanized at week 32 or when greater than 20% weight loss was documented. Dusp1⁻/⁻ and Dusp1⁺/⁺ mice backcrossed to C57BL/6 background for 10 generations were used for subcutaneous syngeneic tumor cell injections. Mice aged 6-8 wks received 5 x 10⁵ EO771 cells in 100μL PBS in a subcutaneous injection over the right flank. Tumor volume (l x w²/2) was measured with digital calipers every two days, and animals were euthanized if tumor size exceeded 1,500mm³. After tumors reached >50mm³ (day 7), animals received daily intraperitoneal injections of 5mg/kg SB203580 or dimethylsulfoxide vehicle.

Histological and FACS analyses

Formalin-fixed paraffin-embedded tissues were used for histology and immunohistochemistry. Two pathologists, blinded to experimental group, evaluated sections for pathology and inflammation. Inflammation was scored on a 0–4 scale (0, normal mucosa; 1, minimal inflammation (occasional scattered granulocytes and leukocytes); 2, mild inflammation (scattered granulocytes with occasional infiltrates); 3, moderate inflammation (scattered granulocytes with patchy infiltrates); and 4, severe inflammation (multiple extensive areas with abundant granulocytes and marked infiltrates), as previously described (9).
Immunohistochemistry was performed in EDTA antigen retrieval buffer, as previously described (6), using antibodies against CD11b (Abcam), F4/80 (Abcam), and Ly6G (Novus Biologicals). DAB-positive cells in tumor-positive fields of view were acquired at 10X magnification and quantified using Viziopharm acquisition and analysis software (Hoersholm, Denmark), and expressed as the number of positive cells in each histologically defined area. Single cell suspensions were generated from spleen and cervical lymph nodes from tumor-bearing mice for flow cytometry. Cells were stained according to manufacturer’s protocol against CD11b-APC, Ly6C-FITC, Ly6G-Pacific Blue, and F4/80-PE with at least 10,000 events analyzed by Miltenyi MACSQuant cytometer and software.

**Primary bone marrow-derived macrophage (BMDM) isolation**

Bone marrow cells were obtained from 8-12 week-old mice, as previously described (6). Macrophages were primed with LPS from Aggregatibacter actinomycetemcomitans (strain Y4, serotype B) followed by treatment with ATP or nigericin (Sigma-Aldrich), as previously described (10).

**Human tissue samples**

Snap-frozen tissues from head and neck squamous cell carcinoma patients were acquired from MUSC Hollings Cancer Center Tissue Biorepository, according to protocols approved by MUSC Institutional Review Board. Tissue pathology and tumor percentage within representative frozen sections were verified by Biorepository research pathology.

**Western blotting**
Tissue and cell lysates were processed as previously described (11). Primary antibodies were directed against DUSP1 (Millipore), caspase 1 p20 (Millipore) and p10 (Santa Cruz), Il-1β (Abcam), GAPDH, and α-tubulin (Cell Signaling). Secondary antibodies were horseradish peroxidase-conjugated anti-rabbit antibodies (Cell Signaling).

**Gene expression**

Gene expression was determined using probe-primer TaqMan sets for delta-delta Ct calculations: Ptprc, Il1b, Gapdh, 16s rRNA, DUSP1, and ACTB. The nCounter analysis system (NanoString Technologies, Seattle, WA) was used to quantify gene expression in a custom panel. Each sample profile was normalized to geometric mean of 4 reference genes, Eif4a2, Oaz1, Ncln, and Gfra2, chosen for lowest variance across experimental samples.

**Statistical analyses**

For matched human tissues, a paired t-test was performed. When comparing effect of genotype, a two-sample t-test was performed using the Satterthwaite method for unequal variances, Mann-Whitney U-test in absence of normal distribution, or pooled method for all other comparisons. For distributions of categorical scores, a Fischer’s Exact test was used. A repeated measures mixed model, with random intercept and time effect, was used to test effect of genotype on weight gain and inhibitor treatment by genotype interaction. Survival analyses (event = tumor volume ≥ 1500mm³) with Kaplan-Meier estimates were performed using a log-rank test. Adjusted p-values using Tukey-Kramer multiple comparisons method are provided when comparing least square mean estimates. All analyses were performed using SAS 9.4©.
Results and Discussion

*DUSP1 expression is decreased in HNSCC samples.*

DUSP1 mRNA and protein expression were lost in human oral squamous cell carcinomas compared to adjacent non-tumor control tissues (Figure 1A-B). These findings were consistent with reports of DUSP1 down-regulation or loss in other cancers (7). Expression of the proform of IL-1β, an mRNA previously identified in salivary exosomes from oral cancer patients (12), was also elevated in eight of the eleven pairs of HNSCC tissues (Figure 1B).

*Dusp1 deficiency enhances susceptibility to carcinogen-induced oral cancer.*

The development of cancer results from the interplay between genomic alterations, such as those induced by carcinogen exposure, and a supportive tumor microenvironment. To understand how DUSP1 deficiency alters oral cancer development and progression, wild-type and Dusp1-deficient mice were subjected to a carcinogen-induced oral cancer model, previously optimized to generate heterogeneous oral lesions that evolve into squamous cell carcinomas (8). This model has been described as generating little inflammation with low levels of COX-2 expression and stromal infiltrate in C57BL/6 mice at the time of invasive SCC development (8,13). However, these studies propose an inflammatory microenvironment develops in response to carcinogen-induced oral tumorigenesis, enhanced by Dusp1 down-regulation, to promote disease progression. Although Dusp1-deficient mice are more susceptible to innate immune activation in sepsis and other models of acute inflammation (5,6), its phenotype in tumorigenesis is not well-defined.

Animals were exposed to 4NQO, a surrogate for chronic tobacco exposure, in drinking water for 16 weeks, and monitored for development of oral lesions (Figure 2A). Change in
weight, as an estimate of disease burden, was reduced in Dusp1-deficient mice over time (Figure 2B). Wild-type mice continued to gain weight, from 10-15 weeks, whereas weight gain in Dusp1-deficient mice stopped by 10 weeks, likely due to increasing tumor burden. Disease onset was accelerated in Dusp1-deficient mice by 2-3 weeks (Supplemental Figure 1). No difference in weight gain was noted in Dusp1-deficient mice in vehicle treatment group compared to wild-type controls (Supplemental Figure 2). At 32 weeks, significant elevation of total tumor burden and enhanced histological disease progression were found in Dusp1-deficient mice (Figure 2C). Wild-type mice lesions were distributed across the spectrum of hyperplasia, dysplasia, and in situ and invasive squamous cell carcinomas, whereas the majority of Dusp1-deficient lesions were invasive squamous cell carcinomas (Figure 2D).

**Dusp1 deficiency enhances tumor-associated inflammation.**

Enhanced leukocyte abundance was observed, both histologically and by *Ptprc* gene expression in Dusp1-deficient mice (Figure 3A). Nanostring analysis of tumor tissues did not reveal significant perturbations in markers of cellular infiltrate but suggested increased M2 macrophage polarization, (Supplemental Figure 3). *Ex vivo* analysis of primary bone marrow-derived macrophages from Dusp1-deficient mice did not reveal an intrinsic defect in M1 polarization or predilection toward M2 polarization (data not shown). Notably, mRNA levels of cytokines and chemokines *Il1b, Vegf, Cxcl1, Cxcl2, Ccl2,* and receptor *Ccr5* were highly elevated in Dusp1-deficient tissues, suggestive of predominantly monocyte-macrophage infiltrate (Figure 3B). Immunohistochemistry of tumor tissues identified a significance increase in Ly6G-positive infiltrates with a trend toward increased Cd11b-positive cells, and no difference in F4/80
expression (Figure 3C). Flow cytometry analysis of spleen and draining lymph nodes identified similar increases in Cd11b-positive cells with Ly6C and/or Ly6G expression.

To determine whether stromal Dusp1 deficiency enhances tumor progression, subcutaneous injections of the syngeneic breast adenocarcinoma EO771 cell line were implanted in wild-type and Dusp1-deficient female mice. Analysis of microarray datasets of human breast cancer tissues revealed patients, stratified into low DUSP1 expression, had significantly decreased progression-free survival (Supplemental Figure 4). Tumors in Dusp1-deficient animals grew more rapidly than in wild-type animals, an effect abrogated by treatment with the p38 inhibitor SB203580 (Figure 3D). Furthermore, survival was significantly decreased in Dusp1-deficient animals, rescued by inhibitor treatment (Figure 3D). Inhibitor treatment did not have a significant effect on tumor growth or survival in wild-type animals. These data demonstrate Dusp1 expression has a crucial tumor suppressive role in regulating stromal p38 MAPK activity and suggest downstream effects of its loss in HNSCC and other cancers are mediated through increased expression of inflammatory mediators.

**Dusp1 deficiency increases Il1b expression.**

A pair of tumor tissues from Dusp1-deficient and wild-type mice, matched for histological stage and inflammation score, were analyzed by targeted qPCR array to assess expression levels of 89 cytokines, chemokines, and receptors. The initial screen showed Il1b elevated 70-fold in the Dusp1-deficient sample (data not shown), then validated by qPCR in a larger set of tissues (Figure 4A). Western blot analyses of caspase-1 and Il-1β demonstrate high levels of both protein proforms, likely secondary to increased inflammatory infiltrate and Il1b transcript abundance in Dusp1-deficient mice (Figure 4B). A strength of 4NQO exposure is its
mirroring of human cancer progression from hyperplasia to invasive squamous cell carcinoma. In this model, Dusp1-deficient mice demonstrate accelerated tumorigenesis with increased inflammation and provide first evidence for a mechanistic link between loss of DUSP1 expression and advanced disease progression. Although a panel of increased cytokines and chemokines were identified by qPCR and Nanostring analyses, IL-1β may be a major contributor, particularly in HNSCC as previously suggested in patient and in vitro studies (14,15). The broad impact of Dusp1 deficiency on tumor progression in this model highlights the utility in identifying an upstream regulator of pro-tumorigenic inflammatory response for therapeutic targeting with greater impact than biologics against a single cytokine.

Treatment of primary bone marrow-derived macrophages from Dusp1-deficient mice with LPS (100ng/mL) resulted in significantly increased levels of Il1b mRNA (Figure 4C). Despite the striking level of pro-caspase-1 detected in Dusp1-deficient tumor tissues, no significant elevation of caspase-1 expression or cleavage product was detected in primary cultures (Figure 4D). This discrepancy may be due to differences in cellular constituents of tumor tissues between wild-type and Dusp1-deficient mice, as suggested by the increased numbers of inflammatory cells by histological scoring and immunohistochemistry (Figure 3), which can be normalized in ex vivo culture systems of primary macrophages.

Conclusions

Loss of DUSP1 expression has implications on biologic activities of many tumor cellular constituents, which are not fully dissected in these experiments. A recent survey of over 400 human HNSCC tissues revealed highly elevated p38 MAPK activity in nearly 80% of cases, with significant impact on cancer cell growth, lymphangiogenesis, and angiogenesis in xenograft
studies (16). Immunohistochemistry of 4NQO-induced tumors in both wild-type and Dusp1-deficient mice show the same trend of elevated expression of phospho-p38 but not ERK1/2 nor SAPK/JNK MAPKs in tumor epithelial tissues (Supplemental Figure 5). No differences in phospho-p38 staining were seen in wild-type of Dusp1-deficient tumor despite altered MAPK activity and expression in immunoblots of whole tissue lysates (Supplemental Figure 6), suggesting a more critical role of MAPK signaling in supporting stromal and immune cells. Furthermore, in a syngeneic model, inhibition of p38 by SB203580 significantly decreased tumor cell growth in Dusp1-deficient mice but not wild-type animals. Differential contributions of DUSP1 signaling in infiltrating immune cells versus supporting stromal cells, such as fibroblasts and endothelial cells, must be addressed in future studies.

Although numerous reports have identified down-regulation of DUSP1 across diverse tumor tissues, this is the first study to demonstrate a tumor suppressive role. Results from these in vivo and ex vivo studies also highlight a mechanism for up-regulation of pro-inflammatory cytokine IL-1β in oral cancer, previously shown to be elevated as mRNA in salivary exosomes (12) and secreted protein (14). Using a model mimicking genetic heterogeneity of human HNSCC following long-term carcinogen exposure, these findings support IL-1β’s role as potential biomarker. Additional work must be done to address its impact on disease progression.

In light of cytokine and chemokine findings discussed above, implications of these studies may extend well beyond HNSCC, as DUSP1 expression has been shown to be dysregulated in other cancers, such as breast, and may be predictive of response to targeted therapies, such as cetuximab (7,17). Genomic studies have not identified a common mutation or deletion, but a recent DNA methylation screen identified significantly increased promoter hypermethylation in over 80% of oral cancer tissues (18). Pharmaceutical intervention to induce
DUSP1 expression, with rosiglitazone and mapracorat, are currently being explored *in vitro* with promising results (19,20). In summary, results presented here demonstrate for the first time that DUSP1 plays an important role within the tumor microenvironment, regulating key cytokines such as IL-1β. Down-regulation of DUSP1 leads to enhanced disease progression. Thus, restoring DUSP1 expression represents a promising target of pharmacological intervention and merits further investigation as a suppressor of tumor-promoting inflammation.
References


Figure Legends

Figure 1. Human HNSCC tissues have decreased DUSP1 expression. A, DUSP1 gene expression in human oral squamous cell carcinoma and matched adjacent non-tumor tissues, by qPCR. n = 10, p = 0.0378, Student’s paired t-test. B, DUSP1 and IL1B protein expression in human HNSCC samples (T) and matched adjacent non-tumor tissues (N). DUSP1 bands were developed prior to adding GAPDH antibody.

Figure 2. Dusp1 deficiency enhances 4NQO-induced disease progression. A, Schematic of 4NQO-induced model of oral cancer with representative image of an oral lesion at 28 weeks. B, Disease burden, estimated by weight change. n = 37 per group, p < 0.001. C, Individual tumor burden n = 14-15, p = 0.0032. D, Neoplastic progression of tumors, scored on the indicated histologic scale.

Figure 3. Dusp1 deficiency enhances tumor-associated inflammation and disease progression. A, Tumor inflammation scores and expression of pan-leukocyte marker Ptprc by qPCR. n = 12-13 (vehicle), 31 per group (4NQO), p < 0.001 (inflammation), n = 7 per group, p = 0.0175, Mann-Whitney (Ptprc). B, Nanostring analysis of chemokine, cytokine, and receptor gene expression. n = 6 per group, p < 0.05. C, FACS analysis of myeloid-lineage cells in spleen and cervical lymph nodes from tumor-bearing mice, using markers CD11b, Ly6C, Ly6G, and F4/80. n = 6 per group. p < 0.05. Immunohistochemistry of F4/80, CD11b, and Ly6G-positive cells quantified in a 10X field of view of oral tumor sections. n = 17-18 (F4/80), 8 (CD11b), 8-9 (Ly6G). p = 0.0176 (Ly6G). D, Volumes of subcutaneous EO771 tumors with daily intraperitoneal injections.
of SB203580 (5mg/kg) or vehicle control, starting on day 7. Rate of tumor growth is increased in Dusp1-deficient vehicle-treated animals (p < 0.0001) and Dusp1-deficient SB203580-treated animals (p = 0.0098). Dusp1-deficient animals had decreased survival compared to wild-type animals (p = 0.0123). Survival was enhanced in Dusp1-deficient animals treated with SB203580 compared to vehicle treatment group (p = 0.0086). n = 6-8.

Figure 4. Dusp1 deficiency enhances Il-1β expression in tumor tissues and primary macrophages. A, Expression levels of Il1b mRNA, by qPCR. n = 7-8 per group, p = 0.003. B, Immunoblots of procaspase-1 and proIl-1b in tissue lysates. C, Expression of Il1b mRNA after LPS stimulation (n = 4, p = 0.003). D, Expression of Il1b and caspase-1 and cleavage products in macrophage lysates and supernatant.
Figure 1

A

B

N T N T N T N T N T N T

DUSP1
GAPDH
proIL1B
IL1B
Figure 2

A

4NQO in drinking water
Vehicle control

B

Regular water

C

D

Individual tumor burden (mm)

Number of mice

WT  KO

Invasive SCC
In Situ SCC
Dysplasia
Hyperplasia
Normal

WT  KO
Figure 3
Figure 4

A

B

Veh

WT

KO

4NQO

WT

KO

Caspase 1, 45kD

II-1b, 35kD

GAPDH

C

D

Ctrl

LPS

LPS/ATP

WT

KO

WT

KO

WT

KO

Caspase 1, 45kD

II-1b, 35kD

II-1b, 17kD

a-tubulin, 50kD

Lysates

Caspase 1, 20kD

II-1b, 35kD

II-1b, 17kD

Supernatants
DUSP1 phosphatase regulates the proinflammatory milieu in head and neck squamous cell carcinoma

Xiaoyi Zhang, Madison Hyer, Hong Yu, et al.

Cancer Res  Published OnlineFirst October 13, 2014.

Updated version  Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-14-1379

Supplementary Material  Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2014/10/11/0008-5472.CAN-14-1379.DC1

Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.