

# Constitutive Activation of Signal Transducers and Activators of Transcription 3 Correlates with Cyclin D1 Overexpression and May Provide a Novel Prognostic Marker in Head and Neck Squamous Cell Carcinoma<sup>1</sup>

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## Abstract

The precise mechanism responsible for the frequent overexpression of cyclin D1 in human head and neck squamous cell carcinoma (HNSCC) is not known. In view of the fact that signal transducers and activators of transcription 3 (Stat3) is often activated in HNSCC cells, we examined the effects of Stat3 on cyclin D1 expression and cell proliferation in the YCU-H891 HNSCC cell line that displays constitutive activation of Stat3. Expression of a dominant negative Stat3 construct in YCU-H891 cells inhibited proliferation, cyclin D1 promoter activity, and cellular levels of cyclin D1 mRNA and protein. The levels of the antiapoptotic Bcl-2 and Bcl-X<sub>L</sub> proteins were also inhibited. In 51 primary tumor samples from patients with squamous cell carcinoma of the p.o. tongue, there was a significant correlation between increased levels of the activated form of Stat3, phosphorylated-Stat3, and increased levels of cyclin D1 ( $P < 0.0001$ ). Increased tumor levels of phosphorylated-Stat3 were also associated with lower survival rates ( $P < 0.01$ ). This study provides the first evidence that in HNSCC, constitutive activation of Stat3 plays a causative role in overexpression of cyclin D1, and in clinical studies, Stat3 activation may provide a novel prognostic factor. Furthermore, agents that target Stat3 may be useful in the treatment of HNSCC.

## Introduction

Overexpression of the G<sub>1</sub> cell cycle control protein cyclin D1, and or the related mRNA, occurs in >50% of cases of human HNSCC<sup>3</sup> (1, 2), and overexpression of this protein is a marker of poor prognosis in this disease (1). Furthermore, *in vitro* and *in vivo* introduction of an antisense cyclin D1 sequence into HNSCC cells inhibits their growth and tumorigenicity, induces apoptosis, and also enhances their sensitivity to chemotherapeutic agents (3, 4). In ~20% of the cases of HNSCC, the *cyclin D1* gene is amplified (1, 5). However, in the majority of these cases, the gene is not amplified, suggesting that the increased expression of cyclin D1 is because of defects at the level of gene transcription. A similar discrepancy between cyclin D1 overexpression and gene amplification has also been reported in breast and colon cancers (6, 7). Recent studies indicate that aberrant  $\beta$ -catenin/

T-cell factor transcriptional activity up-regulates cyclin D1 expression in the latter two malignancies (6, 7). However, abnormalities in the  $\beta$ -catenin/T-cell factor pathway have not been reported in HNSCC. Autocrine activation of the TGF- $\alpha$ /EGFR pathway is observed frequently in HNSCC, and recent studies indicate that a transcription factor, Stat 3, is an important downstream target of this pathway (8). Indeed, Stat3 is activated frequently in both primary human HNSCC, as well as in normal mucosa from these cancer patients compared with control normal mucosa from patients without cancer (9). A constitutively active Stat3 construct can up-regulate cyclin D1 expression at the level of transcription in rodent fibroblast cell lines (10). Possible correlation between activation of Stat3 and increased levels of the cyclin D1 protein was demonstrated in human ovarian carcinoma cell lines (11). Furthermore, in human hematopoietic cells, Stat5 directly activates the cyclin D1 promoter via a *c-sis*-inducible element-like element (12). Therefore, in the present study we examined, both *in vitro* and in clinical samples, the possibility that activation of Stat3 plays an important role in overexpression of cyclin D1 and tumor progression in HNSCC. In the *in vitro* studies, we determined the effects of a dominant negative Stat3 on cell proliferation and cyclin D1 expression in the HNSCC YCU-H891 cell line, in which Stat3 is constitutively activated. In the clinical studies, we examined by immunohistochemistry the levels of phospho-Stat3, the activated form of Stat3, and cyclin D1 in 51 primary tumor samples obtained from patients with SCC of the p.o. tongue. Taken together, these results provide evidence that activation of Stat3 plays an important role in the expression of cyclin D1 in HNSCC and that activation of Stat3 may provide a novel prognostic marker in this disease.

## Materials and Methods

**Cell Lines, Cell Culture, and Materials.** The human HNSCC cell line YCU-H891, which was derived originally from a carcinoma of the hypopharynx, was generously provided by Dr. M. Tsukuda and has been described in our previous studies (13, 14). In this cell line, the EGFR, Stat3, and ERK proteins are constitutively activated (13, 14). The establishment of two clonal derivatives of YCU-H891 cells, Stt3DN66 and Stat3DN99, that stably express a dominant negative Stat3 protein, and a vector control cell line, is described elsewhere (14). All of the cell lines were maintained in a 5% CO<sub>2</sub> atmosphere at 37°C in RPMI 1640 with 10% FBS (Life Technology, Grand Island, NY). The medium for Stat3DN66, Stat3DN99, and vector control cells also contained 400  $\mu$ g/ml G418. TGF- $\alpha$  and PD98059 were obtained from Life Technology. PD98059 was dissolved in DMSO. The cyclin D1 luciferase reporter plasmid-1745CD1LUC was constructed and provided by Dr. R. Pestell (15). The dominant negative HA-tagged Stat three-dimensional constructs were provided by Dr. T. Hirano (16) and used to obtain the Stat3DN66 and Stat3DN99 derivatives, as described previously (14).

**Luciferase Reporter Assays.** These assays were done essentially as described previously (13). Briefly, triplicate samples of  $1 \times 10^5$  cells in 35-mm plates were transfected using lipofectin (Life Technology). One  $\mu$ g of the

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<sup>3</sup> The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; Stat, signal transducers and activators of transcription; p-Stat, phosphorylated-signal transducer and activator of transcription; RT-PCR, reverse transcription-PCR; SCC, squamous cell carcinoma; TGF, transforming growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; HA, hemagglutinin peptide; FBS, fetal bovine serum.

reporter plasmid and 10 ng of the p-cytomegalovirus- $\beta$ -gal plasmid DNA (used as a control) were cotransfected in opti-MEM I medium (Life Technology). After 16 h, the medium was changed to fresh serum-free RPMI 1640. The cells were then incubated for 24 h, in the presence or absence of 50 ng/ml of TGF- $\alpha$ , and luciferase activity was then determined in cell extracts, using the luciferase assay system (Promega, Madison, WI).  $\beta$ -gal activities were also determined using the  $\beta$ -galactosidase enzyme assay system (Promega). Luciferase activities were then normalized with respect to  $\beta$ -gal activities.

**RNA Extraction and Semiquantitative RT-PCR.** Total RNA was extracted with a single step method using the TRIzol reagent (Life Technology). RT-PCR was conducted using a SUPERSRIPT One-Step RT-PCR system (Life Technology), in a total volume of a 25- $\mu$ l reaction mixture containing 12.5  $\mu$ l of 2  $\times$  reaction mix, 2  $\mu$ l (1  $\mu$ g) of template RNA, 0.25  $\mu$ l of sense primer (20  $\mu$ M), 0.25  $\mu$ l of antisense primer (20  $\mu$ M), 0.4  $\mu$ l of reverse transcription/Platinum Taq Mix, and 9.6  $\mu$ l of distilled water. Initially, cDNA was generated at 50°C for 30 min. PCR was then conducted for 15, 20, 25, and 30 cycles in a thermal controller (Programmable Thermal Controller; MJ Research, Inc., Watertown, MA), and the optimal cycle number for quantification (25 cycles) was determined. Each amplification cycle consisted of 0.5 min at 94°C for denaturation, 0.5 min at 55°C for primer annealing, and 1 min at 72°C for extension. The sequences of PCR primers were as follows: cyclin D1 sense primer, 5'-CCC TCG GTG TCC TAC TTC AAA-3'; cyclin D1 antisense primer, 5'-CAC CTC CTC CTC CTC TTC;  $\beta$ -actin sense primer, 5'-CCA GGC ACC AGG GCG TGA TG-3'; and  $\beta$ -actin antisense primer, 5'-CGG CCA GCC AGG TCC AGA CG-3'. The sizes of the amplicons for cyclin D1 and  $\beta$ -actin were 726 and 436 bp, respectively. Twelve  $\mu$ l of each PCR product were then electrophoresed on 2% agarose gels, and the intensities of the specific bands were analyzed.

**Protein Extraction and Immunoblotting.** Protein extraction and immunoblotting were done essentially as described previously (13, 14). The following primary antibodies were used: cyclin D1 (M-20), Bcl-2 (N-19), and Bcl-X<sub>L</sub> (H-62) from Santa Cruz Biotechnology (Santa Cruz, CA) and actin (20-33) from Sigma (St. Louis, MO). Specific protein bands were detected using the enhanced chemiluminescence system (Amersham International, Buckinghamshire, United Kingdom).

**ERK Phosphorylation Assays.** A percentage (40–50%) of confluent cells was cultured in RPMI 1640 minus serum for 16 h. Cells were pretreated with the indicated concentration of PD98659 for 30 min and then stimulated with 50 ng/ml TGF- $\alpha$  for 24 h. Proteins were extracted and immunoblotted with a phospho-ERK1/2 antibody (9106S; Cell Signaling, Beverly, MA).

**Proliferation Assays.** Growth curves were generated in medium containing either 10% serum or no serum. Cells (50,000) were seeded into 35-mm dishes in RPMI medium plus 10% FBS. After 24 h, half of the cultures was grown in 10% FBS and the other half in the absence of 10% FBS. The numbers of cell were then determined, in triplicate assays, during the subsequent 6 days, using a Coulter Counter (Beckman Coulter, Fullerton, CA). The mean values were used to generate growth curves.

**Tissue Specimens.** Initial biopsy specimens (51) were obtained from patients with SCC of the p.o. tongue, who subsequently underwent treatment with curative intent at the Division of Head and Neck of the National Kyushu Cancer Center in Japan, from 1992 to 1999. Clinical stage was determined according to the 1997 Edition of Tumor-Node-Metastasis Classification of Malignant tumors (Union Internationale Contre le Cancer) and Stage I, 11; Stage II, 25; Stage III, 1; and Stage IV, 14.

**Immunohistochemical Staining.** Cyclin D1 staining was done essentially as described previously (2). For p-Stat3 staining, we used the method described by Campbell *et al.* (17). The cyclin D1 antibody (NYC-CYCLIN D1-GM) was obtained from Novocastra (Newcastle, United Kingdom), and the phospho (Tyr705)-Stat3 antibody (#9131) was from Cell Signaling. The efficacy of the p-Stat3 antibody was confirmed using YCU-H891 cells (Fig. 2A), and, therefore, these cells were used as a positive control for p-Stat3 immunostaining. The degree of staining was measured as the percentage of positively stained nuclei in 200 tumor cells, as determined by two independent investigators, without knowledge of the specific cases. Cyclin D1 staining was regarded as positive when  $\geq 5\%$  of the tumor cells were stained. p-Stat3 staining was scored negative, 0–4%; weak, 5–29%; moderate, 30–49%; and strong,  $\geq 50\%$ . Strong staining was regarded as positive.

**Statistical Analyses.** The association between p-Stat3 and cyclin D1 expression was analyzed by both  $\chi^2$  test and Pearson's correlation coefficient.

The relationship between either the levels of p-Stat3 or cyclin D1 and clinicopathological factors was assessed with the  $\chi^2$  test. For survival analysis, the Kaplan-Meier method was used, and the statistical significance was analyzed by the Log-rank test. To determine whether the prognostic value of Stat3 is independent of cyclin D1 expression, T stage, the presence of nodal metastasis, or the clinical stage, the influence of these factors on patient survival was analyzed by the multivariate Cox proportional hazards method. Differences were considered to be statistically significant when:  $P < 0.05$ .

## Results and Discussion

### Effects of Transient Expression of a Dominant Negative Mutant of Stat3, Stat3D, on Cyclin D1 Expression.

As mentioned in the "Introduction," cyclin D1 is overexpressed frequently in primary HNSCC (1), and it is also expressed at a high level in YCU-H891 cells (13). Therefore, it was of interest to determine whether Stat3 plays a role in controlling the transcription of cyclin D1 in these cells. For this purpose, we assessed the effect of a dominant negative mutant of Stat3, Stat3D, in transient transfection assays using a cyclin D1 promoter luciferase reporter. Because there is evidence that activation of the ras-ERK pathway can transcriptionally up-regulate cyclin D1 expression (18), we added the mitogen-activated protein/ERK kinase inhibitor PD98059 to some of these assays so that we could more specifically examine the role of activated Stat3. The optimal concentration of this inhibitor was first determined by Western blot analysis of the phosphorylated ERK protein (Fig. 1A). YCU-H891 cells were grown in serum minus medium for 16 h, pretreated with 0, 5, or 10  $\mu$ M PD98059 for 30 min, and then stimulated with 50 ng/ml TGF- $\alpha$  for an additional 24 h. We found that 10  $\mu$ M PD98059 strongly inhibited the phosphorylation of ERK1/2 that was stimulated by TGF- $\alpha$  (Fig. 1A). Therefore, we used this concentration of PD98059 in the cyclin D1 promoter luciferase assays and found that it did not inhibit these assays significantly, either in the absence or presence of TGF- $\alpha$  (Fig. 1A). However, the dominant negative Stat3D construct strongly inhibited both basal and TGF- $\alpha$ -stimulated cyclin D1 promoter activity (Fig. 1A).

### Effects of Stable Expression of Stat3D on Gene Expression in Derivatives of YCU-H891 Cells.

In view of the above results, we examined the effects of dominant negative Stat3 on cyclin D1 expression in two clonal derivatives of YCU-H891 cells (designated Stat3DN66 and Stat3DN99) that stably express the HA-tagged dominant negative Stat3D protein. The development of these derivatives and some of their properties are described elsewhere (14). We included in these studies a vector control cell line that had been transfected with only the empty vector pCAGGS-neo (14). In our previous study (14), we confirmed that the HA-tagged Stat3 D protein functions as a dominant negative in the two Stat3DN clones, by using a luciferase reporter construct that contains a Stat3-responsive element. The parental YCU-891 cells, the vector control cells, and the two Stat3D clones were transfected transiently with the cyclin D1 promoter luciferase reporter, with or without stimulation of the cells with TGF- $\alpha$  (Fig. 1B). In the Stat3DN66 cells, both basal and TGF- $\alpha$ -stimulated cyclin D1 promoter activity were markedly inhibited, when compared with the parental or vector control cells (Fig. 1B). In the Stat3DN99 cells, the basal activity of the cyclin D1 promoter was almost the same as that of the parental cells, but TGF- $\alpha$ -stimulated promoter activity was strongly inhibited (Fig. 1B).

Because dominant negative Stat3 strongly inhibited cyclin D1 promoter activity, we examined cellular levels of the endogenous cyclin D1 mRNA using semiquantitative RT-PCR. In both the Stat3DN66 and Stat3DN99 cells, the levels of cyclin D1 mRNA were markedly decreased when compared with the parental and vector control cells (Fig. 1C).

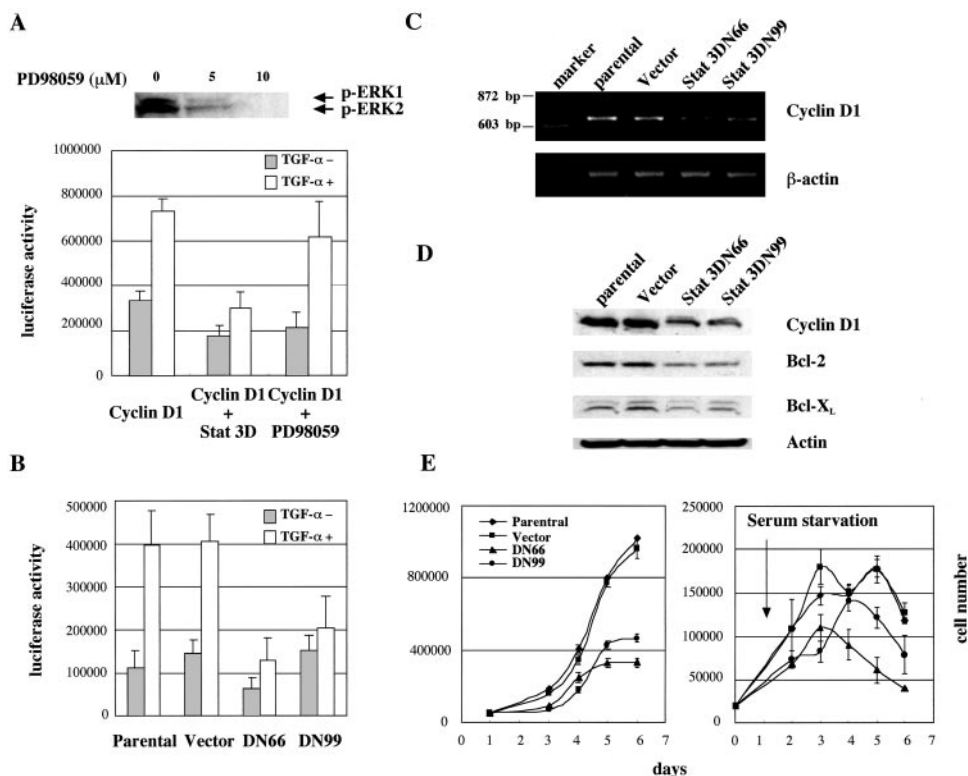


Fig. 1. A, determination of the optimal concentration of PD98059 to inhibit ERK1/2 phosphorylation (top panel) and reporter assays for cyclin D1 (bottom panel) in YCU-H891 cells. The cells were incubated in serum minus RPMI 1640 for 16 h, and then the indicated concentrations of PD98059 were added. Later (30 min), the cells were stimulated with 50 ng/ml TGF- $\alpha$  and then cultured for an additional 24 h. Proteins were then extracted and immunoblotted with the phospho-ERK1/2 antibody. In the reporter assays for cyclin D1, cells were transfected in opti-MEM I medium with the cyclin D1 promoter luciferase reporter plasmid plus or minus the Stat3D construct for 16 h. Then the medium was changed to serum minus RPMI 1640, and the cells were incubated for an additional 24 h in the absence (TGF- $\alpha$ -) or presence (TGF- $\alpha$ +) of TGF- $\alpha$ ,  $\pm$ 10  $\mu$ M PD98059, and extracts were analyzed for luciferase activity. B, reporter assays for cyclin D1 in YCU-H891 cells (Parental), empty vector-transfected cells (Vector), and two Stat3D stably transfected clones (Stat3DN66 and Stat3DN99). Cells were transfected with the cyclin D1 promoter luciferase reporter and incubated in the absence (TGF- $\alpha$ -) or presence (TGF- $\alpha$ +) of TGF- $\alpha$ , as described above. C, semiquantitative RT-PCR assays for cyclin D1 mRNA in extracts from YCU-H891 cells (Parental), vector control-transfected cells (Vector), and two Stat3D stably transfected clones (Stat3DN66 and Stat3DN99).  $\beta$ -actin was used as an internal control. D, immunoblot assays for cyclin D1, Bcl-2, and Bcl-X<sub>L</sub> proteins in YCU-H891 cells (Parental), vector control-transfected cells (Vector), and Stat3D stably transfected two clones (Stat3DN66 and Stat3DN99). An antibody for actin was used as a loading control. E, growth curves of YCU-H891 cells (Parental), empty vector-transfected cells (Vector), and two Stat3D stably transfected clones (DN66 and DN99). Cells were grown in medium containing 10% serum (left panel) or under conditions of serum starvation (right panel), beginning on day 1.

**Dominant Negative Stat3 Inhibits Cellular Levels of the Cyclin D1, Bcl-2, and Bcl-X<sub>L</sub> Proteins.** We did Western blot analysis of cell extracts to compare the levels of expression of the cyclin D1, Bcl-2, and Bcl-X<sub>L</sub> proteins in the parental, vector control, and two Stat3D clones (Fig. 1D). We found that the levels of the cyclin D1 protein were markedly reduced in both the Stat3DN66 and Stat3DN99 cells (Fig. 1D). These results, taken together with the above results in transient transfection cyclin D1 promoter activity assays (Figs. 1, A and B) and semiquantitative RT-PCR assays (Fig. 1C), provide strong evidence that activated wild-type Stat3 transcriptionally up-regulates cyclin D1 expression in YCU-H891 cells. We found that the levels of the Bcl-2 and Bcl-X<sub>L</sub> proteins were also reduced in both the Stat3DN66 and Stat3DN99 cells (Fig. 1D), providing evidence that activated wild-type Stat3 also stimulates the expression of these proteins.

**Effects of Dominant Negative Stat3 on Cell Proliferation.** To assess biological effects of stable expression of the dominant negative Stat3 mutant, we compared the growth curves of the Stat3DN66 and Stat3DN99 clones to those of parental and vector control cells, when cells were grown in medium containing 10% serum and in serum minus medium (Fig. 1E). In the medium containing 10% serum, both of the clones that stably express the dominant negative Stat3 protein displayed slower growth rates and a lower saturation density than the parental or vector control cells (Fig. 1E). These studies are consistent with studies by Grandis *et al.* (19) in other HNSCC cell lines. In the

medium minus serum, the stat3DN66 and Stat3DN99 cells showed initial growth rates that were slower than those of the parental and vector control cells, and then both clones displayed a decline in cell numbers (Fig. 1E), presumably reflecting decreased viability in the absence of serum.

**The Levels of p-Stat3 Correlate with the Level of Cyclin D1, Nodal Metastasis, Clinical Stage, and Poor Prognosis in SCC Tumors of the p.o. Tongue.** Because the above-described *in vitro* assays provided evidence that constitutive activation of Stat3 up-regulates cyclin D1 expression and cell proliferation in HNSCC cells, it was of interest to determine whether in primary HNSCC tumors there is an association between activation of Stat3 and the overexpression of cyclin D1 and whether activation of Stat3 correlates with various clinicopathological parameters in patients with HNSCC. Samples of 51 individual primary SCC of the p.o. tongue, which had been obtained from patients before therapy, were analyzed by immunohistochemistry for nuclear staining with an antibody specific for phospho-Stat3, *i.e.*, the activated form of Stat3. In parallel studies, the same samples were analyzed by immunohistochemistry for nuclear staining for cyclin D1, using a cyclin D1-specific antibody. Representative examples of immunohistochemical staining are shown in Fig. 2A. Tumors were scored positive for p-Stat3 if they displayed strong staining, *i.e.*, if  $\geq$ 50% of the tumor cells displayed nuclear staining with the respective antibody and were scored positive for cyclin D1 if  $\geq$ 5% of the tumor cells displayed nuclear staining with



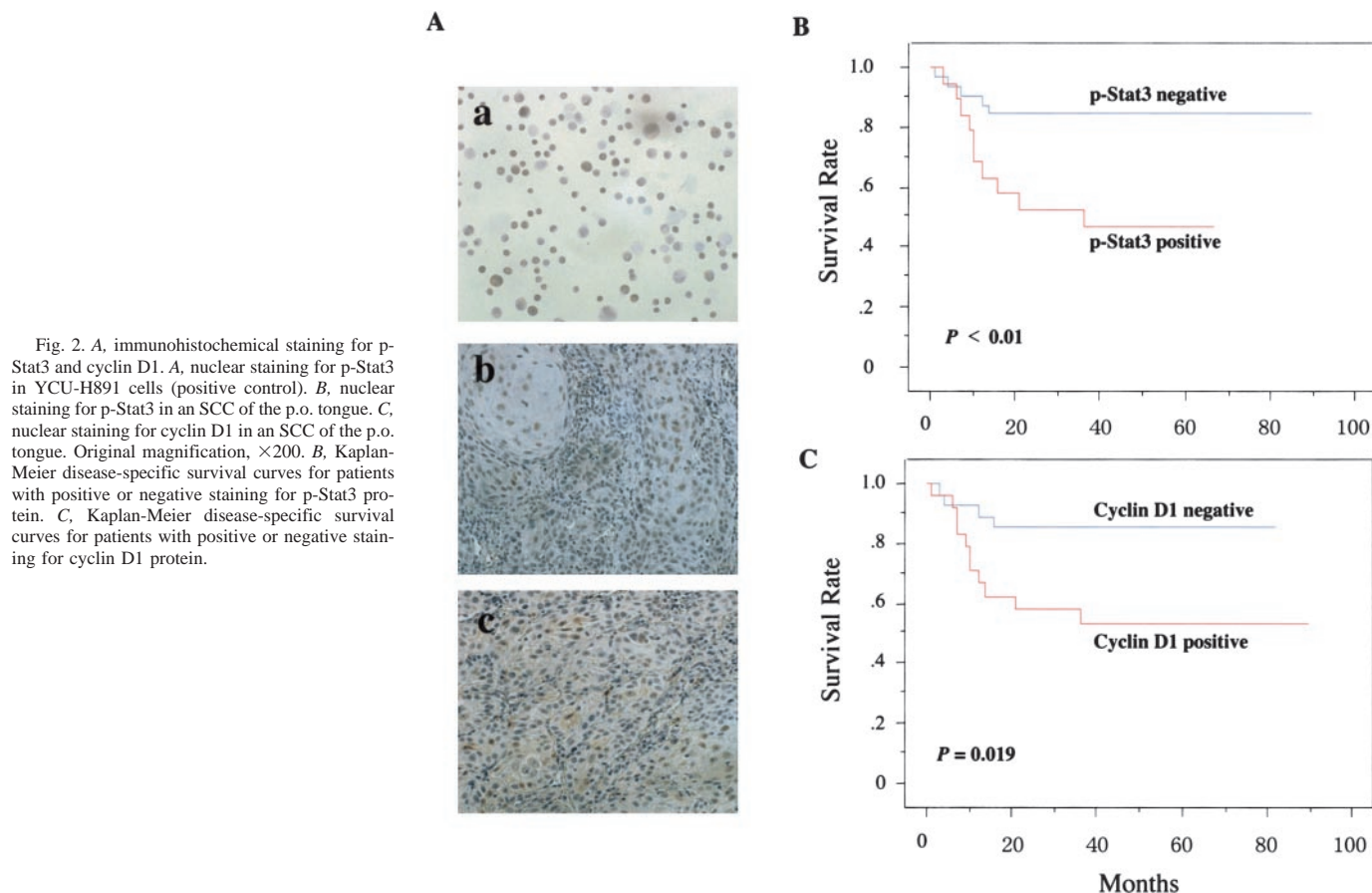


Fig. 2. A, immunohistochemical staining for p-Stat3 and cyclin D1. A, nuclear staining for p-Stat3 in YCU-H891 cells (positive control). B, nuclear staining for p-Stat3 in an SCC of the p.o. tongue. C, nuclear staining for cyclin D1 in an SCC of the p.o. tongue. Original magnification,  $\times 200$ . B, Kaplan-Meier disease-specific survival curves for patients with positive or negative staining for p-Stat3 protein. C, Kaplan-Meier disease-specific survival curves for patients with positive or negative staining for cyclin D1 protein.

the respective antibody (see “Materials and Methods”). Table 1 indicates that of the 51 tumors, 19 were positive for p-Stat3 (~37%). Of the remaining tumors, 19 were negative, 10 gave weak, and 3 gave moderate staining for p-Stat3 (data not shown). Table 1 indicates 24 of 51 tumors were positive for cyclin D1 (~47%). When these data were analyzed by the  $\chi^2$  test, there was a highly significant ( $P < 0.001$ ; Table 1) correlation between positive expression of p-Stat3 and positive expression of cyclin D1. Using Pearson’s correlation coefficient, we also found a significant association ( $r = 0.431$ ,  $P = 0.0014$ ) between these two parameters. These findings are consistent with our *in vitro* data (Fig. 1), indicating that activation of Stat3 enhances the expression of cyclin D1 in a HNSCC cell line.

When we examined possible correlations with various clinicopathological parameters, we found that increased levels of p-Stat3 significantly correlated with the existence of nodal metastasis ( $P = 0.016$ ) and the clinical stage ( $P = 0.03$ ) but not with tumor stage, whereas increased levels of cyclin D1 did not significantly correlate with any of these three parameters (Table 2). Kaplan-Meier survival curves indicated that patients positive for increased p-Stat3 demonstrated significantly ( $P < 0.01$ ) lower disease-specific survival rates (Fig. 2B). Increased cyclin D1 levels were also associated ( $P = 0.019$ ) with

Table 2 Correlations between nuclear p-Stat3 or cyclin D1 and clinicopathological parameters

	No.	p-Stat3 positive	<i>P</i>	cyclin D1 positive	<i>P</i>
T stage					
I and II	41	15 (37%)	0.84	20 (49%)	0.62
III and IV	10	4 (40%)		4 (40%)	
Nodal metastasis					
Negative	39	11 (28%)	0.016	17 (44%)	0.37
Positive	12	8 (67%)		7 (58%)	
Clinical stage					
I and II	36	10 (27%)	0.03	15 (41%)	0.23
III and IV	15	9 (60%)		9 (60%)	

poorer prognosis (Fig. 2C), which is consistent with a previous large-scale study of cyclin D1 immunostaining on SCC of the p.o. tongue (20, 21). In a multivariate Cox analysis, we found that p-Stat3 ( $P < 0.01$ ) was a predictor of poor prognosis, independent of cyclin D1 expression, T stage, the presence of nodal metastasis, or clinical stage. In this analysis, we found that cyclin D1 ( $P = 0.025$ ), T stage ( $P = 0.0005$ ), the presence of nodal metastases ( $P < 0.0001$ ), and clinical stage ( $P < 0.0001$ ) were also independent prognostic factors.

Thus, the present studies provide the first evidence that the frequent overexpression of cyclin D1 in HNSCC (see “Introduction”) may be attributable, at least in some cases, to increased activation of Stat3, which, in turn, is because of frequent autocrine activation of the TGF- $\alpha$ /EGFR pathway in HNSCC (8). Indeed, in a recent study (13), we found that in YCU-H891 cells, inhibition of EGFR activity by epigallocatechin-3-gallate, a major biologically active component of green tea, inhibits Stat3 activation and cyclin D1 promoter activity and decreases the cellular level of the cyclin D1 protein. There is also previous evidence that increased expression of cyclin D1 correlates with an aggressive phenotype in HNSCC (1). Therefore, aberrant

Table 1 Correlation between nuclear expression of p-Stat3 and cyclin D1

p-Stat 3 staining	cyclin D1 staining			<i>P</i>
	Negative <i>n</i> = 28	Positive <i>n</i> = 23	Total	
Negative <i>n</i> = 32	24 (47%)	8 (16%)	32 (63%)	<0.0001 <sup>a</sup>
Positive <i>n</i> = 19	3 (6%)	16 (31%)	19 (37%)	
Total	27 (53%)	24 (47%)	51 (100%)	

<sup>a</sup> $\chi^2$  test.

activation of the EGFR-Stat3 pathway in HNSCC (8) may contribute to the malignancy of these cancers, at least in part, through overexpression of cyclin D1. The precise mechanism by which activation of Stat3 enhances transcription from the cyclin D1 promoter remains to be determined.

Our previous finding that expression of a dominant negative Stat3 protein in YCU-H891 cells markedly increases their sensitivity to inhibition by 5-fluorouracil (14) may also be clinically relevant. This effect could be secondary to the above-described suppression of cyclin D1 overexpression and/or the associated decreased expression of the antiapoptotic proteins Bcl-2 and Bcl-X<sub>L</sub> (Fig. 1D). Our findings are consistent with previous studies in rodent fibroblast cell lines, indicating that Stat3 can transcriptionally up-regulate the expression of Bcl-X<sub>L</sub> (10). In addition, abrogation of Stat3 function causes down-regulation of the Bcl-2 and/or Bcl-X<sub>L</sub> proteins and thereby enhances cellular sensitivity to treatment with UV or mitomycin C in ras-transfected intestinal epithelial cells (22) and causes an increase in apoptosis in a human HNSCC xenograft model (9). Our clinical data provide the first evidence that there is a strong association between increased levels of the activated form of Stat3 (p-Stat3) and cyclin D1 overexpression in HNSCC (Table 1) and that activation of Stat3 might provide an independent prognostic factor in this and possibly other malignancies. It is of interest that p-Stat3, but not cyclin D1, was associated with the presence of nodal metastasis and clinical stage (Table 2). Therefore, activation of Stat3 may play an important role, independent of cyclin D1, in tumor invasion and nodal metastasis.

Obviously, these preliminary findings require confirmation in a larger series of cases and in other types of HNSCC. Nevertheless, our findings suggest that Stat3 and Stat3-related pathways of signal transduction may provide promising prognostic markers and molecular targets for the treatment of patients with HNSCC.

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## Constitutive Activation of Signal Transducers and Activators of Transcription 3 Correlates with Cyclin D1 Overexpression and May Provide a Novel Prognostic Marker in Head and Neck Squamous Cell Carcinoma

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