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Autore/i: Marioni G, Nucci R, Marino F, Cappellesso R, Pillon M, Zanoletti E, Giacomelli L, Franchella S, Billo P, Pares

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Evaluation of the Prognostic Role of pSTAT3 Expression in Temporal Bone Squamous Cell Carcinoma

*Gino Marioni, †Raoul Nucci, ‡Filippo Marino, ‡Rocco Cappellesso,
§Marta Pillon, ||Elisabetta Zanoletti, ‡Luciano Giacomelli, *Sebastiano Franchella,
¶Paola Billo, †Roberto Pareschi, and ||Alessandro Martini

*Department of Neurosciences, Otolaryngology Section, Padova University Hospital, Padova;
†Otorhinolaryngology Division, Legnano Hospital, Legnano; ‡Department of Medicine DIMED, §Pediatric
Onco-Hematology Unit, and ||Department of Neurosciences, Otorhinolaryngology Unit, Padova University Hospital,
Padova; and ¶Anatomic Pathology Division, Legnano Hospital, Legnano, Italy

Objective: Temporal bone squamous cell carcinoma (SCC) accounts for less than 0.2% of all head and neck tumors. Although some progress has been made in treating this aggressive tumor, the prognosis in advanced cases remains poor. More effective therapeutic strategies need to be considered, including receptor-mediated carcinoma-targeted therapy. Phosphorylated STAT3 (pSTAT3) regulates many genes that are necessarily expressed in cancer initiation, development, and progression, being involved in proliferation, anti-apoptosis, invasion, angiogenesis, and immune surveillance evasion. The aim of the present study was to preliminarily investigate the potential prognostic role of pSTAT3 expression in temporal bone SCC.

Study Design: Retrospective clinicopathologic investigation.

Setting: Tertiary referral centers.

Patients: Twenty-five consecutively operated patients with primary temporal bone SCC.

Intervention: pSTAT3 immunohistochemical expression in primary temporal bone SCCs was assessed with the aid of computer-based image analysis.

Main Outcome Measures: Conventional clinicopathologic parameters and pSTAT3 expression were correlated with SCC prognosis.

Results: pT, stage, and surgical margin status were significantly related with recurrence rate ($p = 0.002$, $p = 0.01$, and $p = 0.047$, respectively) and disease-free survival (DFS) ($p = 0.0049$, $p = 0.031$, and $p = 0.035$, respectively). pT classification was also related with disease-specific survival (DSS) ($p = 0.035$). The SCC recurrence rate did not correlate with pSTAT3 expression. Statistical analyses ruled out any significant difference in DFS or DSS when patients were stratified by pSTAT3 expression ($>80.0\%$ or $\leq 80.0\%$).

Conclusion: Despite our preliminary results, the role of pSTAT3 in temporal bone SCC warrants further investigation in larger series because there is increasing evidence in preclinical models to indicate that inhibiting STAT3 phosphorylation can be a useful addition to different anticancer strategies.

Key Words: Prognosis—pSTAT3—Squamous cell carcinoma—Targeted therapy—Temporal bone.

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The estimated worldwide incidence of head and neck cancer for the year 2008, calculated by the International Agency for Research on Cancer (IARC) for 182 countries in 5 continents, was more than 630,000 new cases (making it the seventh most common human cancer) (1). Temporal bone squamous cell carcinoma (SCC) accounts

for less than 0.2% of all tumors of the head and neck (2), but it is a distinctly aggressive malignancy. Although some progress has been made in the treatment of this disease over the decades, the prognosis remains poor (3), particularly for advanced cases. Multimodal therapy is considered essential for advanced tumors. Novel therapeutic agents are needed, designed specifically to target temporal bone SCC. Targeting strategies should be used as an adjuvant for malignancies being treated with surgery, radiotherapy, and conventional chemotherapy with a view to improving tumor response and disease-specific survival rates, without adding to the burden of treatment-related toxicity.

Address correspondence and reprint requests to Gino Marioni, M.D., Department of Neurosciences, Otolaryngology Section, Via Giustiniani 2, 35128 Padova, Italy; e-mail: gino.marioni@unipd.it

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Advances in our knowledge of the molecular mechanisms behind the development and progression of temporal bone SCC are needed with a view to identifying novel biomarkers that might help us to predict its biological behavior and prove suitable targets for therapy. Such biomarkers are usually proteins aberrantly and selectively expressed in cancers. The family of signal transducers and activators of transcription (STAT) consists of 7 proteins: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6, which are mapped to different human chromosomal regions (4). The STAT family of transcription factors transduce signals from a variety of extracellular stimuli and are important mediators of inflammation, cell survival, differentiation, and proliferation. In response to stimuli, STATs are phosphorylated by the Janus-activated kinase, which activates STAT inducing dimerization and nuclear translocation, where STATs bind to specific enhancer elements in target genes (5). STAT2, STAT4, and STAT6 only seem to be activated in normal conditions, whereas STAT1, STAT3, and STAT5 have an important role in cancer development (STAT1 as a tumor suppressor and STAT3 and STAT5 as oncogenes) (4).

Unlike the situation in normal cells, in which phosphorylation is transient, a constitutive STAT3 activation has been reported in several primary cancers, including prostate, breast, lung, colon, liver, pancreas, and also head and neck carcinomas (6,7). Activated (phosphorylated) STAT3 regulates many genes that have to be expressed in the initiation, development, and progression of cancer, including proliferation, anti-apoptosis, invasion, angiogenesis, and immune surveillance evasion (4). In particular, phosphorylated-STAT3 (pSTAT3) has recently been described as a downstream effector of EGFR, interleukin 6, and Src family kinases in head and neck carcinoma (8–10). pSTAT3 over-expression occurs in a variety of malignancies, suggesting that STAT3 may be not only prognostically significant but also a target for therapy, and several strategies are being developed to target the STAT3 signaling pathway.

The present study has been the first designed to immunohistochemically investigate serine-phosphorylated STAT3 expression in patients with primary temporal bone SCC. The aim of the present study was to preliminarily evaluate the potential prognostic role of pSTAT3 expression in temporal bone SCC.

MATERIALS AND METHODS

The study was conducted on 25 consecutive patients with primary external auditory canal/temporal bone SCC, 15 women and 10 men, who were a mean 63.3 ± 8.2 years old. Auricular cancers spreading secondarily to the temporal bone were not considered. All patients underwent micro-otoscopy with biopsy, contrast-enhanced computerized tomography (CT), and/or magnetic resonance imaging of the temporal bone, neck ultrasonography (with or without fine-needle aspiration cytology), chest X-ray, and liver ultrasonography. Positron emission tomography was used in selected cases.

All 25 patients underwent temporal bone surgery performed by the same surgeon (R. P.) at the Otorhinolaryngology Division of Legnano Hospital from 1996 to 2011. The procedure involved the following: lateral temporal bone resection in 8 cases, subtotal temporal bone resection in 5, extended lateral temporal bone resection in 4, extended petrosectomy in 3, subtotal petrosectomy in 3, and limited surgical excision in 2. Cases classified as cT3-T4 had piecemeal resections with intraoperative evaluation of margins instead of en bloc resections. When the tumor infiltrated the bone, the temporal bone was drilled to expose the underlying dura, and en bloc temporal bone resection was replaced with tumor removal and petrosectomy to various extents. Seventeen patients underwent cervical lymph node dissection, and 16 had an ipsilateral parotidectomy. Postoperative radiotherapy (RT) was administered in 22 cases (in conventional once-daily fractions of 2 Gy for a total dose ranging from 60 to 66 Gy).

Basing the pT-stage on the revised Pittsburgh staging system (11,12), temporal SCCs were classified as pT1 in 3 cases, pT2 in 4, pT3 in 4, and pT4 in 14. The surgical margins were negative at pathology in 11 cases and positive in 14 (3 pT3 and 11 pT4). The regional lymph nodes were classified pathologically as pN₀ in 12 cases and pN+ in 5. No distant metastases (M) were detected at the time of diagnosis. The temporal bone SCCs showed a variable degree of differentiation; only 3 tumors were well differentiated (G1), resembling normal keratinizing squamous epithelium. Most cases (n = 13) displayed a moderate differentiation (G2), with a combination of areas rich in keratin pearls and zones of cells with mild nuclear pleomorphism arranged in large cohesive sheets or cords; and 9 tumors were poorly differentiated (G3), with immature cells scattered in irregular strands or forming small islands (4 of these G3 cases contained areas with a minimal keratinization).

Patients were followed up clinically on a regular basis and the time to recurrence was recorded. The mean follow-up was 46.0 ± 34.1 months (median, 42 mo).

Immunohistochemistry

Immunohistochemical analyses were performed on 4- to 5-mm-thick, formalin-fixed, and paraffin-embedded (FFPE) sections from each tumor sample with the anti-phospho-ser727 STAT3 primary antibody (rabbit polyclonal, SAB Signalway Antibody, Pearland, TX, USA; working dilution 1:200, 30 min, citrate buffer) according to the manufacturer's instructions. All sections were processed using the sensitive Bond Polymer Refine Detection kit—a biotin-free, polymeric horseradish peroxidase-linker antibody conjugate system—in an automated immunostainer (Bond maX, Menarini, Florence, Italy), as described elsewhere (13). Sections were then slightly counterstained with hematoxylin. Sections incubated without the primary antibody served as a negative control, whereas samples of breast carcinoma were used as a positive control. Within each section, cancer cell nuclei and cytoplasm were graded by intensity of the immunoreaction (0, absent, 1+, weak, 2+, moderate, and 3+, strong). Slides were scored independently by 2 pathologists (F. M. and R. C.), and a consensus was reached to score the intensity of the reaction.

Image Analysis

All samples were assessed for pSTAT3 expression on an image analysis (IA) workstation consisting of a conventional Zeiss Axioskop light microscope (Zeiss, Jena, Germany) with a color digital, Peltier-cooled video camera (MicroPublisher 5.0 RTV; QImaging, Surrey, Canada) connected to a personal computer with the Image-Pro Plus, Version 7 for Windows image analysis program (Media Cybernetics Inc., Bethesda, USA). In all cases,

five 1378×954 micron areas of tumor tissue were examined blindly with a 495-point sampling grid superimposed by the program on the image acquired with a 50X field of view and analyzed using a customized subroutine (Winrec; Immagini e Computer, Milano, Italy) for interactively disclosing and counting the points intercepting the positive and negative areas. The proportion of the positive area was calculated and recorded as a percentage (%), in accordance with stereologic best practice.

Statistical Analysis

The statistical tests applied were Fisher's exact test and Student's *t* test corrected for unequal variance, as appropriate. We chose the median value of 80.0% as the cutoff for binarizing the continuous variable, pSTAT3 expression; this cutoff did not suffer from any subjectivity because it was the median value of the pooled pSTAT3 expression levels. Disease-free survival (DFS) was expressed as the number of months elapsing from the date of completing treatment to locoregional recurrence occurrence. The log-rank test and the Kaplan-Meier survival function were used to assess DFS, reported in months for patients stratified according to the selected variables. Disease-specific survival (DSS) was expressed as the percentage of patients who have survived the disease at the latest follow-up.

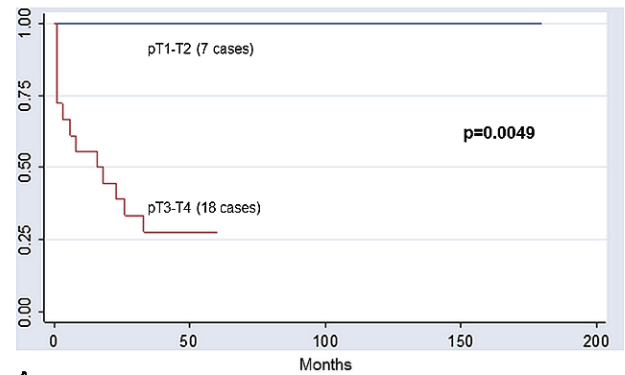
$p < 0.05$ was considered significant. The STATA 8.1 statistical package (Stata Corp., College Station, TX, USA) was used for all analyses.

RESULTS

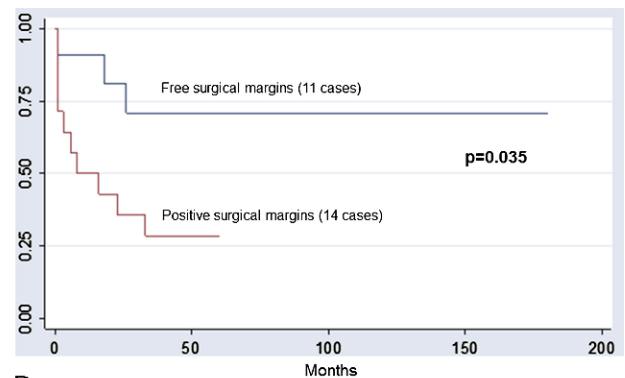
Patients' Clinical Outcome

Thirteen patients developed local SCC recurrences after a mean 10.6 ± 10.8 months (median, 6 mo). Eight of the 25 patients died of their disease, and 3 died of other causes. Fisher's exact test found significant differences in the distributions by pT (pT1–T2 versus pT3–T4) ($p = 0.002$), stage ($p = 0.01$), and surgical margin status (free versus positive) ($p = 0.047$) but not for lymph node status (cN₀ or pN₀ versus pN+, $p = 1$), or grade ($p = 0.10$) when patients were grouped according to whether their disease recurred locally after treatment. The log-rank test showed a significant difference in DFS (in months) when patients were stratified by pT (pT1–T2 versus pT3–T4) ($p = 0.0049$) (Fig. 1A), stage ($p = 0.031$), and surgical margin status (positive versus free) ($p = 0.035$) (Fig. 1B), but this was not the case for N status ($p = 0.84$) or grade ($p = 0.14$). DSS was 100% (7 of 7 cases) for T1–T2 patients and 44.4% (8 of 18 cases) for T3–T4 cases. Only pT classification (pT1–T2 versus pT3–T4) was significantly related to DSS (log-rank test, $p = 0.035$), whereas stage (log-rank test, $p = 0.11$), surgical margins (log-rank test, $p = 0.41$), lymph node status (log-rank test, $p = 0.17$), and grading (log-rank test, $p = 0.21$) were not. Table 1 describes the main demographic, clinicopathologic, and prognostic information for each patient.

When the surgical approach was considered, ipsilateral parotidectomy and neck dissection did not correlate significantly with recurrence (Fisher's exact test, $p = 0.68$ and $p = 1$, respectively), DFS (log-rank test, $p = 0.68$ and $p = 0.22$, respectively), or DSS (log-rank test, $p = 0.16$ and $p = 0.07$, respectively).



A



B

FIG. 1. Disease-free survival in temporal bone SCC patients estimated from pT stage (A) and pathologic status of surgical margins (B), time (abscissa) calculated in months.

The status of the surgical margins correlated significantly with both pT (Fisher's exact test, $p = 0.001$) and stage (Fisher's exact test, $p = 0.009$).

pSTAT3 Expression, Intensity of Nuclear Reaction, and Clinicopathologic Features in Temporal Bone SCC

An initial analysis was performed on pSTAT3 staining in normal epidermis to qualitatively and quantitatively assess its common pattern of expression. In stratified squamous epithelium, pSTAT3 showed a diffuse nuclear immunoreaction in the basal and spinous layers, and scant staining in the granular layer, and none in the keratin layer (Fig. 2A).

All of the 25 temporal bone SCCs showed some pSTAT3 immunoreactivity. The intensity of the pSTAT3 nuclear reaction was classified as 1+ in 9 cases, 2+ in 8, and 3+ in 8 (Fig. 2B), whereas the cytoplasmic reaction was nil in 20 cases and 1+ in 5. Judging from the IA evidence, the mean nuclear pSTAT3 expression was $63.9 \pm 33.6\%$ (median 80.0%). In addition to the intensity of the immunoreaction and the number of positive cells, the expression pattern may also be important for the purposes of biomarker analysis. Several biomarkers are reportedly localized only in certain tumor areas (e.g., at the tumor's infiltrative edge). Immunolabeling

TABLE 1. Main demographic, clinicopathologic, and prognostic information for each patient with temporal bone squamous cell carcinoma

Patient no.	Sex	Age (yr)	Surgical treatment	pT	N-status	G	Surgical margins	Postoperative RT (yes/no)	SCC recurrence (yes/no)	DFS (mo)	Follow-up (mo)	Final status
1	M	56	LTBR	pT2	pN0	G2	Negative	Yes	No	180	180	AWD
2	M	62	STTBR	pT3	pN0	G1	Positive	Yes	No	60	60	AWD
3	M	68	STTBR	pT4	cN0	G2	Positive	Yes	Yes	1	24	DOC
4	M	71	LTBR	pT2	cN0	G2	Negative	Yes	No	72	72	AWD
5	M	75	ELTBR	pT2	pN1	G2	Negative	Yes	No	60	60	DOC
6	F	59	LTBR	pT4	pN1	G2	Positive	No	Yes	1	2	DOD
7	F	69	ELTBR	pT4	pN0	G2	Positive	Yes	Yes	16	42	DOD
8	F	55	ELTBR	pT4	pN0	G2	Positive	Yes	No	60	60	AWD
9	F	64	STP	pT3	pN0	G2	Positive	Yes	No	60	60	AWD
10	F	65	STP	pT4	cN0	G2	Positive	Yes	Yes	1	60	AWD
11	F	67	LTBR	pT1	pN0	G2	Negative	Yes	No	60	60	AWD
12	F	61	LTBR	pT4	pN1	G3	Positive	Yes	Yes	33	35	DOD
13	F	53	STTBR	pT4	pN0	G3	Negative	Yes	Yes	26	31	DOD
14	F	51	ELTBR	pT4	pN1	G3	Positive	Yes	Yes	8	19	DOD
15	M	63	STTBR	pT4	pN1	G3	Positive	Yes	No	60	60	AWD
16	F	72	LTBR	pT2	cN0	G2	Negative	Yes	No	60	60	AWD
17	F	66	STTBR	pT4	pN0	G2	Positive	Yes	Yes	6	19	DOD
18	F	47	LTBR	pT1	pN0	G1	Negative	Yes	No	60	60	AWD
19	M	55	EP	pT4	pN0	G3	Positive	Yes	Yes	23	34	DOD
20	M	63	LSE	pT3	cNo	G3	Negative	Yes	No	60	60	AWD
21	F	61	EP	pT4	pN0	G2	Positive	Yes	Yes	3	18	DOD
22	M	54	EP	pT4	pN0	G3	Negative	Yes	Yes	1	26	AWD
23	F	77	LTBR	pT4	cN0	G3	Negative	Yes	Yes	18	25	AWD
24	F	69	STP	pT1	cN0	G1	Negative	No	No	12	12	AWD
25	M	81	LSE	pT3	cN0	G3	Positive	No	Yes	1	10	DOC

AWD indicates alive without disease; DFS, disease free-survival; DOC, dead of other cause; DOD, dead of disease; ELTBR, extended lateral temporal bone resection; EP, extended petrosectomy; F, female; LSE, limited surgical excision; LTBR, lateral temporal bone resection; M, male; RT, radiotherapy; SCC, squamous cell carcinoma; STP, subtotal petrosectomy; STTBR, subtotal temporal bone resection.

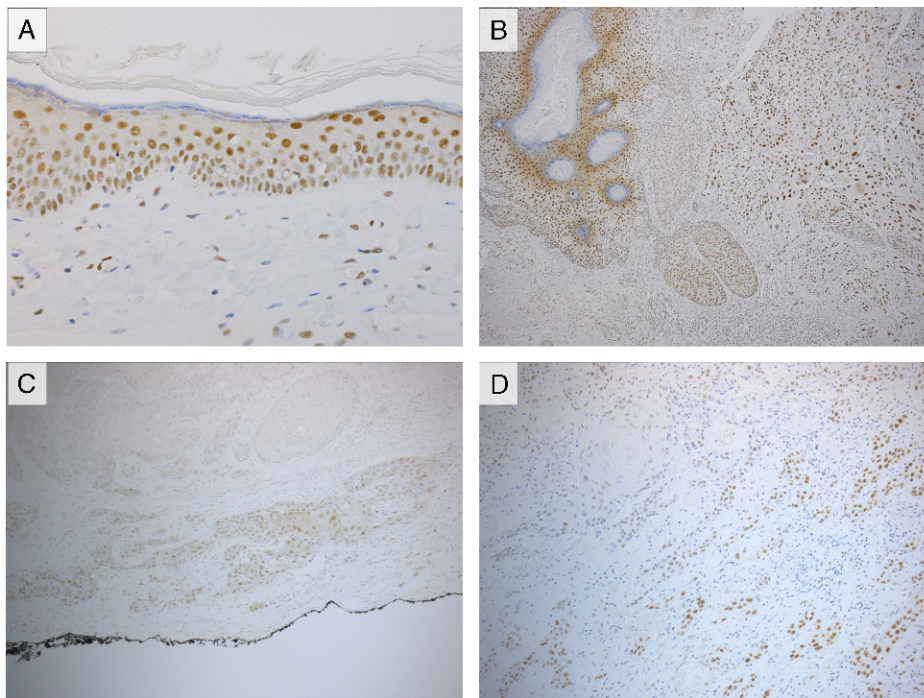


FIG. 2. Representative images of immunohistochemical reaction for pSTAT3 in the series considered. Normal stratified epithelium showing diffuse nuclear immunolabeling in both basal and spinous layers, a scant immunoreaction in the granular layer, and none in the keratin layer (A). Most cases showed a moderate-to-strong immunoreaction in a considerable proportion of carcinoma cells, as shown in the micrograph (B). Only a few cases showed a distribution of pSTAT3 immunolabeling at the tumor's edge (C; surgical margin identified by black ink). Tumor area with keratin pearls (top left) showing a weaker pSTAT3 expression than tumor zones with cells arranged in tiny strands (bottom right) (D). Original magnifications were $\times 200$, $\times 50$, $\times 50$, and $\times 40$, respectively.

for pSTAT3 also showed a patchy distribution in our series, although the edge of the tumor was only implicated in 7 cases (Fig. 2C). Overall, pSTAT3 expression was stronger in areas of the tumor where cells were arranged in tiny strands than in zones displaying large cohesive sheets of cells (mainly if associated with keratin pearls) (Fig. 2D). No specific pSTAT3 expression was found associated with lymphoplasmacytic infiltrate.

Statistical analysis ruled out any significant difference in pSTAT3 expression between pT1-T2 SCCs and pT3-T4 cases (Fisher's exact test, $p = 0.67$), between stage I-II and stage III-IV patients (Fisher's exact test, $p = 1$), between cN₀ or pN₀ and pN+ cases (Fisher's exact test, $p = 0.64$), or between G1-G2 and G3 disease (Fisher's exact test, $p = 1$). Considering the subcohorts of pT3-T4 patients with (13 cases) or without (5 cases) disease recurrence, Student's *t* test corrected for unequal variance ruled out any significant difference in mean pSTAT3 expressions ($p = 0.76$). Furthermore, the same statistical approach found that in patients with positive surgical margins, there was no significant difference in mean pSTAT3 expressions comparing the subcohort with (10 cases) or without (4 cases) disease recurrence (Student's *t* test corrected for unequal variance, $p = 0.19$). Table 2 summarizes the mean pSTAT3 expression in different clinicopathologic subgroups of temporal bone SCC patients. Fisher's exact test failed to identify any significant relationships between intensity of pSTAT3 nuclear or cytoplasmic reaction and pT classification ($p = 0.67$ and $p = 0.59$, respectively), lymph node status ($p = 0.12$ and $p = 1$, respectively), stage ($p = 1$ and $p = 1$, respectively), or pathologic grade ($p = 0.40$ and $p = 0.62$, respectively).

pSTAT3 Expression and Prognosis in Temporal Bone SCC

The recurrence rate did not correlate with pSTAT3 expression (Fisher's exact test, $p = 1$). The log-rank test

also ruled out any significant difference in DFS (in months) ($p = 0.67$) or DSS ($p = 0.39$) when patients were stratified by pSTAT3 expression ($>80.0\%$ or $\leq 80.0\%$). Statistical analysis revealed no significant association between pSTAT3 nuclear reaction intensity (1+ versus 2+ or 3+) and recurrence rate (Fisher's exact test, $p = 1$), DFS (log-rank, $p = 0.67$), or DSS (log-rank, $p = 0.27$), nor between pSTAT3 cytoplasmic reaction intensity (0 versus 1+) and recurrence rate (Fisher's exact test, $p = 1$), DFS (log-rank, $p = 0.44$), or DSS (log-rank, $p = 0.67$).

DISCUSSION

From a prognostic viewpoint, few attempts have been made in the available literature to go beyond merely clinical and conventional pathologic studies on temporal bone SCC. Immunohistochemical methods have rarely been used to assess the oncogenic and oncosuppressive biological mechanisms behind this malignancy. Sugimoto et al. (14) investigated the role of epithelial-mesenchymal transition in 16 cases of temporal bone SCC using immunohistochemistry to measure vimentin expression, finding that DSS was not significantly related to epithelial-mesenchymal transition. Our group very recently considered the expression of CD105 (a protein associated with proliferation of angiogenic endothelial cells) in 20 temporal bone SCCs (15). CD105 correlated significantly with the temporal bone SCC recurrence rate and DFS, meaning that it might be useful for detecting patients at higher risk of local SCC recurrence. The importance of these studies lies in that gaining a better understanding of the onco-genesis and progression of temporal bone SCC at molecular level may lead to the identification of biomarkers correlating with prognosis that could become the target of new therapies. STAT proteins are well-known transducers of signals from the cell membrane to the nucleus. Phosphorylated STAT3 dimerizes and translocates to the nucleus

TABLE 2. Image analysis-measured pSTAT3 expression and disease-free survival correlated with classical pathological variables in temporal bone squamous cell carcinoma

	No. of cases	SCC recurrence (no. of cases)		Mean disease-free survival (\pm SD) in months	pSTAT3 expression % (\pm SD)
		No	Yes		
pT1	3	3	0	44.0 \pm 22.6	73.7 \pm 32.3
pT2	4	4	0	93.0 \pm 50.5	43.7 \pm 30.3
pT3	4	3	1	45.3 \pm 25.5	65.5 \pm 26.5
pT4	14	2	12	18.4 \pm 19.8	67.1 \pm 34.4
pN+	5	2	3	24.1 \pm 22.4	63.4 \pm 31.7
N0 (cN ₀ + pN ₀) ^a	20	10	10	39.0 \pm 41.4	64.4 \pm 34.6
Stage I	3	3	0	44.0 \pm 22.6	73.7 \pm 32.3
Stage II	2	2	0	126.0 \pm 54.0	47.5 \pm 42.5
Stage III	6	5	1	50.2 \pm 22.0	57.0 \pm 24.8
Stage IV	14	2	12	18.4 \pm 19.8	67.1 \pm 34.4
G1	3	3	0	44.0 \pm 22.6	68.3 \pm 29.0
G2	13	7	6	44.6 \pm 48.0	61.8 \pm 37.1
G3	9	2	7	25.6 \pm 21.1	65.6 \pm 29.2
Positive surgical margins	14	4	10	23.8 \pm 24.6	69.6 \pm 33.3
Free surgical margins	11	8	3	55.4 \pm 45.7	56.6 \pm 32.5

^acN₀ 8 cases and pN₀ 12 cases.

to regulate the transcription of target genes. STAT3 dysregulation has been associated with transformation and progression in various cancers, participating in several known pathways of tumor angiogenesis, apoptosis, and cell cycle by regulating the expression of Bcl-2, Bcl-XL, Mcl-1, survivin, cyclin D2, p21waf1/cip1, and p27kip1 (16).

Compared with our previous series investigated in 2012 for the expression of CD105 (15), the current one included 5 additional cases, operated respectively in 1996, 1998, 1999, and 2011 (2 cases). In the present series, pSTAT3 nuclear immunolabeling was of moderate-to-strong intensity in a considerable proportion of the carcinoma cells in all cases. An ideal biomarker would be a protein aberrantly expressed at significantly higher levels in tumor tissues than in their normal counterpart, but all the temporal bone SCCs considered here showed a diffuse immunoreaction in the basal and spinous layers of the normal stratified squamous epithelium. The pattern of expression might be important because several biomarkers occur at specific tumor sites (e.g., at the tumor's infiltrative edge), but only 7 of our 25 cases expressed pSTAT3 at the tumor's edge, and the distribution of the pSTAT3 expression was generally patchy in all our temporal bone SCCs. In the case of the present series, a major limitation of such speculation lies in the positive surgical margins found in several cases because of the advanced local extension of the SCC at presentation (11 SCCs were pT4, and 3 were pT3). We found a sizable subgroup of patients (14/25 cases) whose surgical margins were histologically positive, so the real infiltrative edge of the tumor was probably not evaluated. pSTAT3 expression was also higher in SCC areas displaying cells arranged in tiny infiltrative strands than in areas showing large cohesive sheets of cells. This gives the impression that pSTAT3 might be associated with a particular localization within the SCC, and further, larger studies would need to investigate this hypothesis.

In the present series of 25 temporal bone SCCs, the carcinoma recurrence rate did not correlate significantly with pSTAT3 expression, and statistical analyses ruled out any significant difference in DFS (in months) or DSS when patients were stratified by pSTAT3 expression ($>80.0\%$ or $\leq 80.0\%$). No significant associations emerged between pSTAT3 nuclear reaction intensity (1+ versus 2+ or 3+) and recurrence rate, DFS, or DSS. When Seethala et al. (17) investigated tyrosine-phosphorylated STAT immune expression in two independent cohorts of patients with head and neck SCC (61 and 69 cases, respectively), they concluded that pSTAT3 was unrelated to clinical outcome in either cohort. On the other hand, Macha et al. (16) studied a series of 94 oral SCCs and found that cases with higher nuclear tyrosine-phosphorylated STAT levels had a significantly shorter DFS than those not expressing nuclear pSTAT3; Cox's regression analysis also identified nuclear pSTAT3 as the most significant predictor of a poor prognosis.

The main strength of our study lies in the homogeneity of the series of patients considered because of the following: 1) they all had primary temporal bone SCC, 2)

they underwent surgery performed consecutively by the same team, and 3) only surgical specimens (not biopsies) of temporal bone SCC were assessed. We also used a computer-based IA system to ensure a highly accurate, precise, and reproducible analysis of the immunostained slides. On the other hand, the main weaknesses of our investigation concern the retrospective setting and the limited number of cases considered.

From a conventional pathologic viewpoint, pT staging in the present cohort of temporal bone SCCs correlated with recurrence rate ($p = 0.002$), DFS ($p = 0.0049$), and DSS ($p = 0.035$). Although there is no widely accepted staging system for temporal bone SCC, pT staging based on the revised Pittsburgh system was found consistent and applicable to our patients. Conversely, lymph node status did not correlate with prognosis in terms of recurrence rate, DFS, or DSS. Although nodal disease is generally considered a marker of the aggressiveness of temporal bone SCC, the prognostic value of lymph node involvement is reportedly limited (18). The small number of pN+ cases in the present series (5 cases [20%]) and the limited pathologic lymph node involvement (all cases were pN1) prevent us from making any meaningful comments on this issue. In our series, positive surgical margins at pathology (found in 14 of 25 cases) were identified in advanced-stage temporal bone SCCs, and surgical margin status correlated significantly with recurrence rate ($p = 0.047$) and DFS ($p = 0.035$) but not with DSS. Based on these preliminary results, and as already reported in larger series (19), radical SCC resection, achieving pathologically free margins, is an important prognostic issue. The need to try to achieve free surgical margins in cases of temporal bone SCC is confirmed in our series by the fact that any post-operative radiotherapy administered was unrelated to any of the prognostic parameters considered.

STAT3 may be activated by the phosphorylation of both tyrosine and serine. Indeed, tyrosine phosphorylation at residue 705 reportedly activates STAT3 and leads to its dimerization and translocation in the nucleus, where it promotes the transcription of target genes (20). Several authors have claimed that both tyrosine and serine phosphorylation are needed to maximize the activation of transcription by STAT3 (21–25). Other studies have suggested instead that serine phosphorylation at residue 727 of STAT3 is not necessary for its transcriptional activity. This issue is still debated, however, and several studies have produced evidence to refute said view (23,24,26,27). In this study, we only investigated phosphorylation in serine 727, so we cannot exclude a role for pSTAT3 in temporal bone SCC because the levels of phospho-tyrosine 705 STAT3 have yet to be analyzed. Further studies should focus on this issue to thoroughly clarify whether pSTAT3 has a relevant role in this tumor.

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REFERENCES

- Marioni G. Rationale behind survivin inhibition as a potential therapeutic strategy in head and neck carcinoma too. *Curr Oncol Rep* 2013;15:1–2.
- Ito M, Hatano M, Yoshizaki T. Prognostic factors for squamous cell carcinoma of the temporal bone: extensive bone involvement or extensive soft tissue involvement? *Acta Otolaryngol (Stockh)* 2009;129:1313–9.
- Madsen AR, Gundgaard MG, Hoff CM, et al. Cancer of the external auditory canal and middle ear in Denmark from 1992 to 2001. *Head Neck* 2008;30:1332–8.
- Wang X, Crowe PJ, Goldstein D, Yang JL. STAT3 inhibition, a novel approach to enhancing targeted therapy in human cancers (Review). *Int J Oncol* 2012;41:1181–91.
- Madoux F, Koenig M, Sessions H, et al. Modulators of STAT Transcription Factors for the Targeted Therapy of Cancer (STAT3 Inhibitors). Probe Reports from the NIH Molecular Libraries Program [Internet]. Bethesda, MD: National Center for Biotechnology Information (US), 2009 [updated March 25, 2011].
- Xu X, Kasembeli MM, Jiang X, Tweardy BJ, Tweardy DJ. Chemical probes that competitively and selectively inhibit Stat3 activation. *PLoS One* 2009;4:e4783.
- Dobi E, Monnier F, Kim S, et al. Impact of STAT3 phosphorylation on the clinical effectiveness of anti-EGFR-based therapy in patients with metastatic colorectal cancer. *Clin Colorectal Cancer* 2013;12:28–36.
- Grandis RJ, Melhem MF, Gooding WE, et al. Levels of TGF- α and EGFR protein in head and neck squamous cell carcinoma and patient survival. *J Natl Cancer Inst* 1998;90:824–32.
- Xi S, Zhang Q, Dyer KF, et al. Src kinases mediate STAT growth pathways in squamous cell carcinoma of the head and neck. *J Biol Chem* 2003;278:31574–83.
- Sriuranpong V, Park JI, Amornphimoltham P, Patel V, Nelkin BD, Gutkind JS. Epidermal growth factor receptor-independent constitutive activation of STAT3 in head and neck squamous cell carcinoma is mediated by the autocrine/paracrine stimulation of the interleukin 6/gp130 cytokine system. *Cancer Res* 2003;63:2948–56.
- Moody SA, Hirsch BE, Myers EN. Squamous cell carcinoma of the external auditory canal: an evaluation of a staging system. *Am J Otol* 2000;21:582–8.
- Hirsch BE. Staging system revision. *Arch Otolaryngol Head Neck Surg* 2002;128:93–9.
- Fassina A, Marino F, Siri M, et al. The miR-17–92 microRNA cluster: a novel diagnostic tool in large B-cell malignancies. *Lab Invest* 2012;92:1574–82.
- Sugimoto H, Ito M, Hatano M, Kondo S, Suzuki S, Yoshizaki T. Roles of epithelial-mesenchymal transition in squamous cell carcinoma of the temporal bone. *Otol Neurotol* 2011;32:483–7.
- Marioni G, Nucci R, Marino F, et al. Neoangiogenesis in temporal bone carcinoma: the prognostic role of CD105. *Otol Neurotol* 2012;33:843–8.
- Macha MA, Matta A, Kaur J, et al. Prognostic significance of nuclear pSTAT3 in oral cancer. *Head Neck* 2011;33:482–9.
- Seethala RR, Gooding WE, Handler PN, et al. Immunohistochemical analysis of phosphotyrosine signal transducer and activator of transcription 3 and epidermal growth factor receptor autocrine signaling pathways in head and neck cancers and metastatic lymph nodes. *Clin Cancer Res* 2008;14:1303–9.
- Zanoletti E, Danesi G. The problem of nodal disease in squamous cell carcinoma of the temporal bone. *Acta Otolaryngol (Stockh)* 2010;130:913–6.
- Yin M, Ishikawa K, Honda K, et al. Analysis of 95 cases of squamous cell carcinoma of the external and middle ear. *Auris Nasus Larynx* 2006;33:251–7.
- Darnell JE Jr. STATs and gene regulation. *Science* 1997;277:1630–5.
- Wen Z, Zhong Z, Darnell JE Jr. Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. *Cell* 1995;82:241–50.
- Ram PA, Park SH, Choi HK, Waxman DJ. Growth hormone activation of Stat 1, Stat 3, and Stat 5 in rat liver. Differential kinetics of hormone desensitization and growth hormone stimulation of both tyrosine phosphorylation and serine/threonine phosphorylation. *J Biol Chem* 1996;271:5929–40.
- Ceresa BP, Pessin JE. Insulin stimulates the serine phosphorylation of the signal transducer and activator of transcription (STAT3) isoform. *J Biol Chem* 1996;271:12121–4.
- Wen Z, Darnell JE Jr. Mapping of Stat3 serine phosphorylation to a single residue (727) and evidence that serine phosphorylation has no influence on DNA binding of Stat1 and Stat3. *Nucleic Acids Res* 1997;25:2062–7.
- Ng J, Cantrell D. STAT3 is a serine kinase target in T lymphocytes. Interleukin 2 and T cell antigen receptor signals converge upon serine 727. *J Biol Chem* 1997;272:24542–9.
- O'Rourke L, Shepherd PR. Biphasic regulation of extracellular-signal-regulated protein kinase by leptin in macrophages: role in regulating STAT3 Ser727 phosphorylation and DNA binding. *Biochem J* 2002;364(Pt 3):875–9.
- Shen Y, Schlessinger K, Zhu X, et al. Essential role of STAT3 in postnatal survival and growth revealed by mice lacking STAT3 serine 727 phosphorylation. *Mol Cell Biol* 2004;24:407–19.