

STAT-Related Profiles Are Associated with Patient Response to Targeted Treatments in Locally Advanced SCCHN¹

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Abstract

The anti-epidermal growth factor receptor antibody cetuximab (Erbix, CTX) is currently used for the treatment of locally advanced squamous cell carcinoma of the head and neck (LA-SCCHN), as yet with modest effectiveness, prompting for the identification of response predictors to this treatment and for the targeting of additional pathways implicated in this disease. Within this scope, we investigated the effect of SRC/STAT pathway components on LA-SCCHN patient outcome. *SRC*, *STAT1*, *STAT3*, *STAT5A*, *STAT5B*, *ANXA1*, *CAV1*, *IGFBP2*, *EPHA2*, *EPHB2*, and *MSN* relative gene expression, as well as Stat protein activation, were assessed on LA-SCCHN tumor tissues from 35 patients treated with combined radiotherapy (RT) and CTX-based regimens. Stat1, Stat3, and Stat5 proteins were usually found activated in neoplastic nuclei (70.4%, 85.7%, and 70.8%, respectively). Activated Stat3 and Stat5 were associated with each other ($P = .017$) and with a *CAV1*_{high}/*MSN*_{high}/*IGFBP2*_{low} profile. All patients with tumors expressing high *STAT5A/EPHA2* experienced a complete response on RT-CTX-based treatments (12/15 complete responders, $P < .0001$) and a longer progression-free survival ($P = .024$). Few tumors expressed high *ANXA1/CAV1/EPHA2* and low *IGFBP2*, a putative dasatinib response-related profile, whereas high *ANXA1* was associated with shorter overall survival ($P = .003$). In conclusion, Stat activation is common in LA-SCCHN, where overexpression of *STAT5A* and *EPHA2* may predict for response to RT-CTX treatments. The *STAT5A/EPHA2* profile seems of particular interest for validation in larger cohorts and in multiple tumor types because markers for the positive selection of patients to benefit from CTX-containing treatments are currently lacking.

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Introduction

Squamous cell carcinoma of the head and neck (SCCHN) is a disease potentially curable with surgery and radiotherapy (RT) alone when diagnosed at early stages, as is the case for approximately one-third of patients. When diagnosed at advanced stages, however, SCCHN has a high risk for locoregional recurrence and metastasis on surgery and RT or tumors may be nonresectable. Treatment options for patients with locally advanced SCCHN (LA-SCCHN) include RT with concomitant administration of cisplatin or cetuximab ([CTX], Erbitux), a monoclonal antibody that blocks epidermal growth factor receptor (EGFR) activation, or both [1,2]. CTX in combination with RT has received US Food and Drug Administration approval for the treatment of patients with LA-SCCHN [3] based on clinical trial results showing that the drug reduces mortality and may control disease in collectively 50% of the cases [4], whereas it may also be effective in patients with recurrent and/or metastatic SCCHN who fail on platinum therapy [5]. EGFR targeting represents a successful proof-of-concept paradigm regarding the tumorigenic contribution of this receptor in SCCHN, as established in both clinical and experimental settings [6,7]. However, EGFR targeting still remains inefficient for a large proportion of SCCHN patients. Hence, resolving the mechanisms of intrinsic and acquired resistance to CTX in this type of tumors and identifying predictors of response to this drug are largely needed [7,8].

EGFR signaling is accomplished through three main pathways (RAS-MAPK, PI3K-AKT, and SRC-STAT), all of which function aberrantly in most carcinomas mostly on a ground of genetic alterations (mutations, gene amplifications/deletions) in the member molecules. In colorectal and non-small cell lung cancers (NSCLCs), EGFR-activating mutations are associated with response, whereas *KRAS* and *BRAF* mutations are associated with resistance to therapeutic EGFR antibodies and kinase inhibitors [9–11]. SCCHNs, however, do not in general carry *KRAS* and *BRAF* mutations [12] or classic activating EGFR mutations. Further, although the AKT pathway is usually activated in SCCHN [13], there are as yet no solid data to associate this parameter with disease outcome or treatment response. The third signaling pathway downstream of EGFR seems of particular interest in understanding tumorigenesis and response to anti-EGFR treatments in several carcinoma models. Three of the seven known signal transducers and activators of transcription (STATs) have mostly been studied in SCCHNs, namely, *STAT1*, *STAT3*, as well as *STAT5A* and *STAT5B*, which are genes closely neighbored to each other and to *STAT3*. STATs are transcription factors that may play oncogenic roles in the development of SCCHN [14], where Stat proteins may be activated by EGFR and/or SRC [15] or by non-EGFR pathways [16]. STATs act through receptor tyrosine kinases or cytokines [14], whereas their effects on gene transcription and cell function are described as distinct and nonredundant [17,18].

SRC family kinases (v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog, SFKs) and STATs have been associated with resistance to pharmaceutical EGFR targeting in SCCHN. In SCCHN cells in culture, a forced expression of dominant-mutant *STAT5* conferred resistance to erlotinib, a small-molecule EGFR inhibitor [19]. Further, elevated levels of active EGFR, MAPK, AKT/PKB, and *STAT3* were observed in a model of acquired resistance to CTX, whereas *STAT3* was found activated in resistant but not in the parental CTX-sensitive cells [20]. In the same line, SFKs were overactivated in CTX-resistant NSCLC cells and could be blocked by the Src inhibitor dasatinib [21]. SCCHN cell lines also have overactivated SFKs, *STAT3*, and *STAT5*

and can be growth-inhibited by dasatinib [22]. The commonly observed activation of the SRC/STAT pathway in experimental systems of SCCHN, especially in relevance to CTX resistance, has served as the rationale for proposing the clinical testing of Src inhibitors in these tumors [6,8].

Dasatinib (BMS-354825) has been developed as a specific Src/Abl inhibitor [23] and is currently in clinical use in imatinib-resistant, Philadelphia chromosome-positive leukemias [24,25], whereas it is also being tested in phase 1 and 2 studies in solid tumors [26,27]. In an attempt to predict for response to this drug, profiling of dasatinib-resistant and dasatinib-sensitive cell lines has been undertaken and dasatinib-response gene expression signatures have been described [28,29]. These signatures include Src-inducing and target proteins like the highly homologous closely neighbored caveolins (*CAV1* and *CAV2*), annexin 1A (*ANXA1*), the ephrin receptors A2 and B2 (*EPHA2* and *EPHB2*), moesin (*MSN*), insulin growth factor binding protein 2 (*IGFBP2*), and others. An expression signature of low *IGFBP2* and high *ANXA1*, *CAV1*, and *EPHA2* would predict sensitivity to dasatinib.

On the basis of the previously mentioned data, we investigated the effect of SRC/STAT-related gene expression and protein activation in LA-SCCHN tissues on patient outcome and response to CTX-based treatments. We also assessed the previously described dasatinib sensitivity profile because these patients might be candidates for receiving SRC inhibitors on failure of CTX in the near future. *STAT1*, *STAT3*, and *STAT5* were expressed, and the proteins were activated in most LA-SCCHN, whereas the dasatinib-response-predicting profile was encountered in few tumors. The most interesting finding of this study was that complete response to CTX was associated with high *STAT5A/EPHA2* messenger RNA (mRNA) expression in tumor tissues. If validated in larger cohorts, this profile may prove useful as a positive predictor for response to anti-EGFR antibodies not only in LA-SCCHN but also in other tumors where currently negative predictors are used for the exclusion of patients to receive this type of treatment.

Patients and Methods

A retrospective review of the medical records of 36 patients with newly diagnosed and histologically confirmed nonnasopharyngeal LA-SCCHN was undertaken. Twenty-three patients had been treated with concomitant chemoradiotherapy (CCRT) in five centers. Details about patient dose modifications, follow-up, and RT technique have been reported previously [30]. Further, 13 patients had been treated with CTX and RT only (CTX-RT). All adverse events were graded for this analysis according to the National Cancer Institute Common Terminology Criteria (version 3.0). The Radiation Therapy Oncology Group's criteria were used to assess RT-related toxicities.

After a median follow-up of 24.5 months, 11 deaths and 13 progressions were reported. Median survival has not yet been reached, but 1-year survival rate was 68%. Response to treatment was evaluated according to the RECIST criteria, as described in Fountzilias et al. [30]. Selected patient and tumor characteristics are shown in Table 1.

Tissues and Processing

Formalin-fixed paraffin-embedded tumor tissue from 36 patients was used for protein and gene analysis. Hematoxylin and eosin-stained sections were assessed by two pathologists for tumor tissue adequacy and were marked for tissue microarray (TMA) construction and for macrodissection where necessary (cases with <70% neoplastic cells). TMAs (two cores per case, 1.5 mm in diameter) were constructed with a manual arrayer (Beecher Instruments, Sun Prairie, WI).

Table 1. Selected LA-SCCHN Patient and Tumor Characteristics.

	N = 36		CCRT (n = 23)		CTX-RT (n = 13)	
Age, years						
Median (range)	64 (40-82)		65 (40-82)		60 (40-81)	
<60	n	%	n	%	n	%
≥60	16	44	12	33	4	11
≥60	20	56	11	31	9	25
Sex						
Man	30	83	18	50	12	33
Woman	6	17	5	14	1	3
Alcohol						
No	15	42	10	28	5	14
Yes	21	58	13	36	8	22
Smoking						
No	13	36	12	33	1	3
Yes	23	64	11	31	12	33
Performance status						
0	31	86	19	53	12	33
1	4	11	3	8	1	3
2	1	3	1	3	—	—
Primary tumor location						
Oropharynx	7	19	5	14	2	6
Hypopharynx	2	6	2	6	—	—
Larynx	15	42	6	17	9	25
Oral cavity	10	28	9	25	1	3
Paranasal sinuses	2	6	1	3	1	3
Histology grade						
Well differentiated	1	3	1	3	—	—
Moderately differentiated	10	28	5	14	5	14
Poorly differentiated	22	61	15	42	7	19
Undifferentiated	1	3	—	—	1	3
Unknown	2	6	2	6	—	—
Stage						
II	1	3	—	—	1	3
II	5	14	4	11	1	3
IVA	28	78	17	47	11	31
IVB	1	3	1	3	—	—
Unknown	1	3	1	3	—	—

Expression Profiling

RNA was extracted from whole or macrodissected formalin-fixed paraffin-embedded sections by using an experimental method based on proprietary magnetic beads from Siemens Healthcare Diagnostics (Cologne, Germany), as previously described [31]. The method involves DNase I treatment for the degradation of contaminating DNA. Reverse transcription was accomplished with random hexamers and Superscript III followed by excess RNA removal with RNase H (Invitrogen, Paisley, UK), according to the instructions of the manufacturer. Exon spanning TaqMan MGB assays (premade; Applied Biosystems, Biosolutions,

Athens, Greece) were used to assess the relative expression of 12 genes in comparison to a housekeeping gene (β -glucuronidase [*GUSB*]). Target transcripts and assays are shown in Table 2. Except for the genes mentioned already, *PIAS3* (protein inhibitor of activated STAT 3) was included in the target transcript panel because its low expression or absence had been reported in association with Stat3 activation in gliomas [32]. Samples were assessed twice in 20- μ l reactions in separate runs along with no-template controls for 40 cycles under standard conditions in an ABI7500 real-time polymerase chain reaction (PCR) system and analyzed with the SDS v1.4 software (Applied Biosystems, Biosolutions) by keeping the reading threshold at 0.2 for all evaluations. Criteria for considering samples eligible for analysis were as follows: 1) for the identification of minimal quantities of amplifiable complementary DNA: *GUSB* CT values <36 and 2) for the evaluation of sample adequacy and PCR efficiency in consecutive runs: absolute difference of Δ CT ($CT_{target} - CT_{GUSB}$) values for the same sample was less than 0.5. By using these criteria, 31 of 36 samples were found eligible for further analysis. Relative expression of the target transcripts was assessed as the $2^{-\Delta\Delta CT}$ value (relative quantification value [RQ]) based on equal PCR efficiency for very short amplicons [33]. Mean RQs for each eligible sample were used for analysis.

Immunohistochemistry

Activation of Stat1, Stat3, Stat5, and AKT/PKB was assessed with antibodies against phosphorylated sites in the corresponding proteins. TMA sections were incubated with monoclonal antibodies (Cell Signaling, Boston, MA) diluted 1:50 against Stat1-phospho-Tyr701, clone 58D6 (Stat1), Stat3-phospho-Tyr705, clone D3A7 (Stat3), Stat5-phospho-Tyr694, clone C11C5 (Stat5), AKT-phospho-Thr308, clone 244F9H2 (Akt-T308), and AKT-phospho-Ser473, clone 736E11 (Akt-S473). Antigen epitopes were retrieved with a citrate mixture, pH 6.2 to 4, whereas the Envision system and diaminobenzidine were used for visualizing the antibody-antigen complex (all reagents from DAKO, Glostrup, Denmark).

Immunohistochemistry (IHC) markers were evaluated as negative and positive by using a 5% positivity cutoff. Tumors were characterized as positive for activated Stat1, Stat3, and Stat5 when they exhibited greater than 5% positive nuclei; as positive for phosphorylated Akt-T308, when greater than 5% cytoplasmic staining was observed; and as positive for phosphorylated Akt-S473, when greater than 5% of cells exhibited cytoplasmic and/or nuclear staining (scoring modified from Mizoguchi et al. [34] and Yamashita et al. [35]).

Table 2. Target Transcripts That Have Been Assessed for Relative Expression with FAM-TaqMan-MGB Assays.

Target Transcript	Chromosomal Location	Assay ID	RNA Reference (GenBank)	Position	Amplicon Length (bp)
<i>SRC</i>	20q12-q13	Hs00178494_m1	NM_198291.1, NM_005417.3	ex 7-8	70
<i>STAT1</i>	2q32.2	Hs01014005_m1	NM_139266.2, NM_007315.3	ex 3-4	67
<i>STAT3</i>	17q21.31	Hs01047580_m1	NM_213662.1, NM_139276.2, NM_003150.3	ex 3-4	87
<i>STAT5A</i>	17q11.2	Hs00234181_m1	L41142.1	ex 11-12	63
<i>STAT5B</i>	17q11.2	Hs00560035_m1	NM_012448.3	ex 2-3	91
<i>PIAS3</i>	1q21	Hs00180666_m1	NM_006099.3	ex 7-8	102
<i>ANXA1</i>	9q12-q21.2	Hs00167549_m1	NM_000700.1	ex 8-9	66
<i>CAVI</i>	7q31.1	Hs00971716_m1	NM_001753.3	ex 2-3	66
<i>EPHA2</i>	1p36	Hs00171656_m1	NM_004431.2	ex 16-17	55
<i>EPHB2</i>	1p36.1-p35	Hs00362096_m1	NM_017449.3, NM_004442.6	ex 3-4	67
				ex 3-4	67
<i>IGFBP2</i>	2q33-q34	Hs00167151_m1	NM_000597.2	ex 1-2	70
<i>MSN</i>	Xq11.2-q12	Hs01085677_g1	NM_002444.2	ex 10-11	61
<i>GUSB</i> (reference transcript)	7q21.11	4333767	NM_000181.1	ex 11-12	81

Statistics

One of the major problems and challenges in studies dealing with relative gene expression is the reference system against which the obtained results are compared and analyzed. In this case, SCCHNs arise in a variety of epithelia with different functional properties, which makes it impossible to obtain one adequate reference tissue system for relative expression analysis. Ideally, normal counterparts of all tumors should be available for analysis, but this is practically not possible. Further, the commonly used pooled "normal" cell line template was considered inappropriate as well because transformation is required for normal epithelial cells to grow in culture, which *per se* abrogates normal control of survival and proliferation pathways. Hence, in this study, we considered it more pragmatic to analyze tumor samples only.

Categorical data were presented as counts and corresponding percentages, whereas continuous variables were summarized using medians and ranges. Correlations between the examined genes were calculated with the Spearman ρ correlation. To approach how high and low expression of the investigated genes associated with response to treatment, survival and progression-free survival (PFS), we used the extreme (upper and lower) quartile and median RQ values for each transcript target.

Comparisons between gene expression and clinicopathologic characteristics, as well as response to treatment were performed by using the χ^2 and Fisher exact test where appropriate. The Mann-Whitney test was performed to compare the distribution of each transcript target according to the status (positive/negative) of each IHC marker.

Survival was measured from treatment initiation until death from any cause or date of last contact. PFS was measured from the time of treatment initiation to verified disease progression, death, or last contact. Both survival and PFS were estimated using the Kaplan-Meier product-limit method and comparisons were performed using the log-rank test. To estimate the hazard ratios of gene expression and protein activation data for survival and PFS, univariate Cox regression analyses were performed. Hazard ratios are presented along with the corresponding *P* values from the Wald test. Follow-up was last updated in March 2010. All tests were two-sided, and the level of significance was set at $\alpha = 0.05$. Analysis was conducted by using the SPSS software for Windows, version 15 (SPSS Inc, Chicago, IL).

Results

SRC and *SRC*-Related Gene Expression in LA-SCCHNs

Relative expression results are shown in Table 3 and Figure 1. mRNA expression of *SRC*, *STAT1*, *STAT3*, and *STAT5A*, as well as of genes coding for proteins that are functionally related to Src and blocked by Src inhibitors, such as *ANXA1*, *CAVI*, *EPHA2*, and *IGFBP2*, was detectable in considerable although variable ratios *versus GUSB* in all

LA-SCCHN samples eligible for relative quantification analysis ($n = 31$). In comparison, some of these tumors expressed very low to undetectable levels of *PIAS3*, a *STAT3* inhibitor ($n = 3$); of *STAT5B*, the second *STAT5* component ($n = 3$); of *EPHB2*, a receptor for ephrin-B family members ($n = 1$); and of *MSN* (moesin), a cytoskeleton stabilizer ($n = 6$). Among all *STAT* members, *STAT1* was found to be expressed in relatively higher levels *versus GUSB*, whereas most LA-SCCHNs (20/31 [64.5%]) expressed *ANXA1* >15-fold than this housekeeping gene.

STAT3 was expressed in parallel with *STAT5B* ($P = .0019$), which might suggest common regulatory events for these two neighbored genes that are located on the same strand at 17q21. By contrast, *STAT5A*, which is located between *STAT3* and *STAT5B* but on the complementary strand, was expressed independently of these two genes and correlated with *SRC* expression ($P = .0024$). Thus, the two *STAT5* genes did not seem to share common activators in LA-SCCHN.

ANXA1 and *EPHA2* expression also correlated strongly with each other ($P < .0001$) but not with *SRC* or any *STAT* gene tested, providing evidence for separate transcription regulation of *ANXA1/EPHA2* and *SRC/STAT* genes in LA-SCCHN.

MSN expression varied in parallel with *CAVI* ($P < .0001$) and *EPHB2* ($P = .0191$). *MSN* mRNA also correlated with the expression of *STAT* family members, namely, with *STAT3* ($P = .0020$) and less with *STAT5B* ($P = .0302$) transcript levels. *PIAS3* expression was positively related to *STAT3* ($P = .0166$), whereas it was also strongly related to *MSN* ($P < .0001$) and *CAVI* ($P = .0081$). In all, the most significant above bivariate correlations suggest that 1) the negative regulatory loop for *STAT3* inhibition through *PIAS3* might be functional in LA-SCCHN, unlike to what seems to be the case in glioblastomas [32]; 2) transcription of *MSN*, *STAT3*, and its inhibitor *PIAS3* may be regulated by common signaling; 3) transcriptional activation of *CAVI* and *SRC/STAT* genes are not interrelated.

STAT1 and *IGFBP2* expression did not correlate with any other mRNA target tested here. No significant negative correlations were observed.

Activation of Stat and Akt/Pkb Proteins in LA-SCCHNs

The three Stat proteins investigated in this study were found activated (phosphorylated) in most LA-SCCHN tumors, as indicated by the specific nuclear staining for each target (Figure 2, A–C). Stat3 phosphorylation was the most frequent event, usually coinciding with Stat5 phosphorylation (Fisher exact test, $P = .017$). Stat1-positive cells were usually confined to the most differentiated areas of the tumor (Figure 2A). No significant association was observed between each one of these activated proteins with the clinical and histopathologic parameters presented in Table 1. Activation of these proteins, as assessed by IHC, was not related to the level of the corresponding mRNA expression.

Table 3. mRNA Expression Characteristics of All Genes Examined: Relative Quantification* Was Performed for Individual LA-SCCHN Tumors.

	<i>SRC</i>	<i>STAT1</i>	<i>STAT3</i>	<i>STAT5A</i>	<i>STAT5B</i>	<i>PIAS3</i>	<i>ANXA1</i>	<i>CAVI</i>	<i>EPHA2</i>	<i>EPHB2</i>	<i>IGFBP2</i>	<i>MSN</i>
Mean	1.1390	7.9610	2.1000	0.2745	0.1265	0.1153	24.5500	0.5973	0.9563	0.2101	2.1290	0.3232
SD	1.0540	6.8930	1.0680	0.2478	0.0841	0.1298	32.1900	0.8089	0.8034	0.2182	3.0930	0.3181
SE	0.1862	1.2190	0.1889	0.0438	0.0149	0.0230	5.6910	0.1430	0.1420	0.0386	0.5468	0.0562
Minimum	0.0699	0.5404	0.4121	0.0217	0.0001	0.0001	1.1240	0.0260	0.0513	0.0001	0.0475	0.0001
Maximum	5.8320	30.5500	4.8200	1.4790	0.3169	0.5141	183.8000	2.7740	2.9340	0.9526	11.9700	1.2300
25th percentile	0.5222	2.9450	1.3240	0.1465	0.0588	0.0284	7.1690	0.0830	0.3876	0.0764	0.3324	0.0943
Median	0.9758	7.0570	1.8700	0.2106	0.1202	0.0702	16.0100	0.2045	0.6922	0.1369	0.7533	0.2153
75th percentile	1.3820	10.1400	2.5760	0.3306	0.1956	0.1608	31.6900	0.9123	1.2230	0.2235	2.4610	0.5378

*Values were calculated with the $2^{-\Delta\text{CT}}$ method for target *versus GUSB*, where $\Delta\text{CT} = (\text{CT}_{\text{target}}) - (\text{CT}_{\text{GUSB}})$.

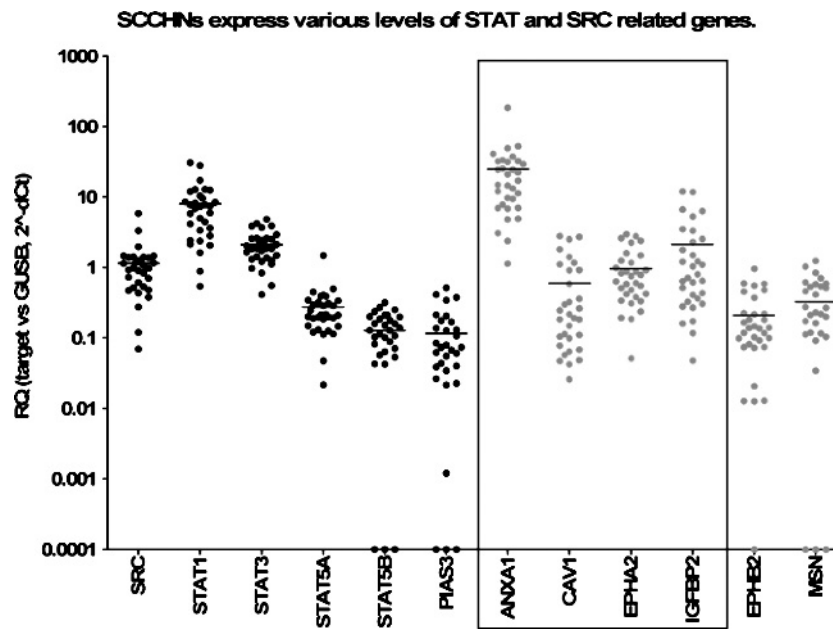


Figure 1. Relative expression of *STAT* and *SRC*-related genes in LA-SCCHN. The distribution of RQ values reflecting the presence of gene transcripts in comparison to those of *GUSB* (housekeeping gene) is presented in a logarithmic scale. Horizontal lines correspond to mean values per category; black dots, *SRC/STAT* genes; gray dots, genes producing *SRC* target proteins, some of which (box) are included in the proposed gene signature predicting for response to dasatinib.

Discordance between mRNA and protein expression for *STAT3* and *STAT5A* has previously been reported [36,37], whereas activation of *Stat* proteins in SCCHN is induced by a number of signal transduction pathways that do not always depend on *STAT* mRNA expression (recently reviewed in Lai and Johnson [38]). Interestingly, however,

tumors positive for *Stat5* phosphorylation expressed relatively high *CAVI* and *MSN* but relatively low *IGFBP2* mRNA (Figure 3). Results in the same line were also obtained for *Stat3* phosphorylation; however, because of the very small number of cases without activated *Stat3*, comparisons between positive and negative cases for this marker

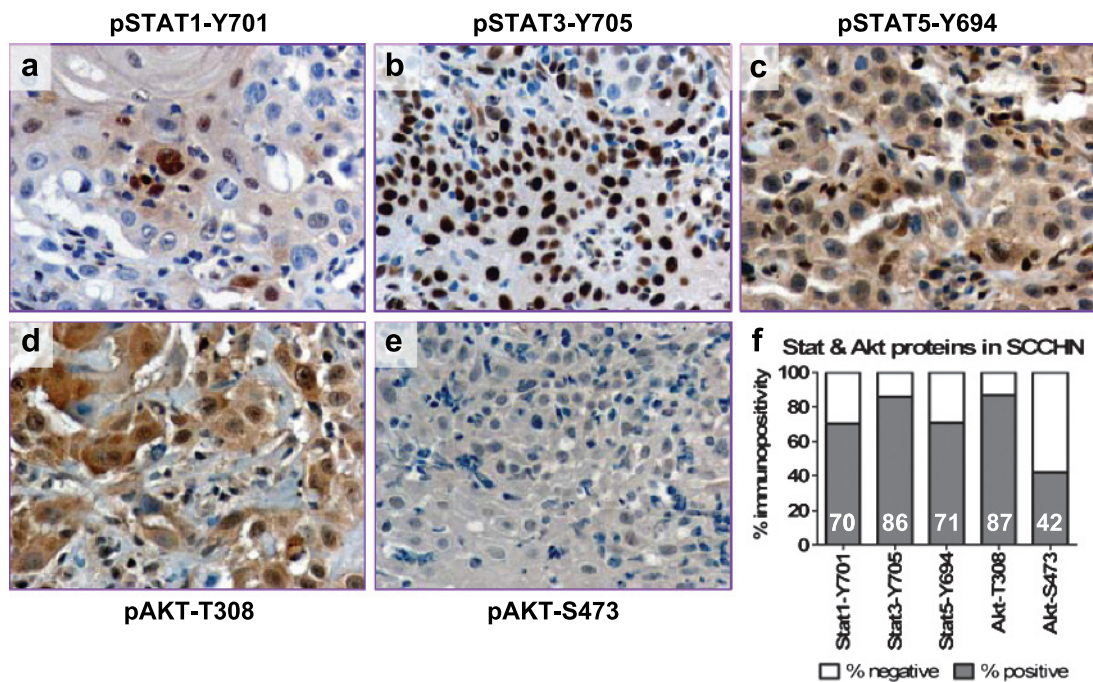


Figure 2. Immunohistochemical investigation of *Stat* and *Akt/PKB* activation in LA-SCCHN. Microphotographs A to E (all magnifications, $\times 200$): A typical case of a well-differentiated LA-SCCHN is shown (antibodies as indicated for each microphotograph). *Stat1*, *Stat3*, and *Stat5* proteins seem activated, whereas *Akt* (*Akt1-3*) proteins are phosphorylated at *Thr308* but not at *Ser473*. This profile was observed in most LA-SCCHN, as collectively shown in the graph in F. Numbers within bars correspond to the rate of immunopositivity obtained with each antibody.

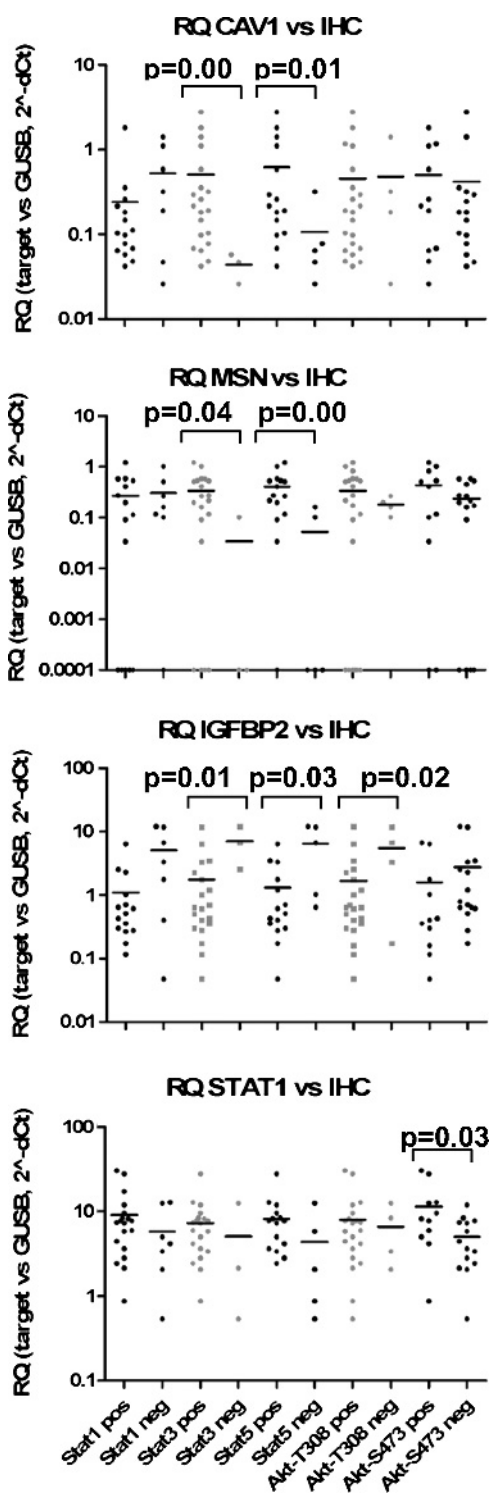


Figure 3. Relative expression of *CAV1*, *MSN*, *IGFBP2*, and *STAT1* in association with Stat and Akt protein phosphorylation in LA-SCCHN. Tumors were categorized as positive/negative for phosphorylated Stat1-Y701, Stat3-Y705, Stat5-Y694, Akt-T308, and Akt-S473. RQ values are shown in a logarithmic scale. Horizontal lines correspond to mean values per category. Categories (IHC status, x axis) in the lower graph are valid for all graphs. Statistically significant associations of gene expression with Stat/Akt phosphorylation status are shown (Mann-Whitney P , exact significance, two-sided).

might represent statistical artifacts and should definitely be validated in larger studies.

Akt/PKB was frequently found phosphorylated at Thr308 but less so at Ser473 (Figure 2, *D–F*). Immunopositive LA-SCCHNs for Akt-Ser473 showed increased STAT1 mRNA expression in comparison to Akt-Ser473–negative tumors (Figure 3). In the same line with Stat5 activation, tumors with phosphorylated Akt at Thr308 expressed lower levels of IGFBP2; however, this result was again limited by the very small number of Akt-Thr308–negative cases.

STAT and SRC-Related Expression Profiles in Association with Treatment Response

Patient follow-up data in association with the observed gene expression profiles are shown in Table 4. LA-SCCHN tumors expressing very high *ANXA1* levels (upper quartile) were associated with significantly shorter survival (hazard ratio [HR] = 8.03, 95% confidence interval [CI] = 4.32–11.74, $P = .0026$) and marginally with earlier relapse of disease (HR = 6.03, 95% CI = 3.81–8.25, $P = .0502$; Figure 4). No further significant association was observed on overall and PFS for any other parameter tested, including IHC-determined activation of Stat and Akt proteins.

Of 37 patients, 35 were assessable for response to CTX-based treatments; 17 (47%) were complete responders, 6 (17%) showed partial response, 2 (6%) had stable disease, and 10 (28%) developed progressive disease while treated. Among patients with disease progression, there was one patient who discontinued treatment because of grade 3 mucositis and died a few months later. One patient was not assessable for response because he died early of disseminated disease, and for the rest, no information for response was available. mRNA profiling data were obtained in 31 of the above 35 cases (Table 4 and Figure 5A).

All patients with tumors expressing very high levels of *STAT5A* and *EPHA2* mRNA (RQ values in the upper quartile) exhibited a complete response on CTX-based treatments; by contrast, none of the tumors in the group of non–complete responders (0/16) expressed very high *STAT5A* and *EPHA2* ($P = .0002$ each; Figure 5A). When assessing these two genes together at the same RQ value cutoff (upper quartile), 12 of 15 tumors in the complete responder group expressed very high *STAT5A* or *EPHA2* mRNA or both ($P < .0001$). This expression pattern was further associated with prolonged PFS for LA-SCCHN patients (Figure 5B). This significant association of *STAT5A* and *EPHA2* expression with best response to CTX-based treatment of LA-SCCHN was not maintained when assessing RQ values at lower cutoffs (median and lower quartile).

In addition, response to CTX-based treatment was negatively related to Stat1 protein phosphorylation ($P = .009$) because all patients bearing tumors without Stat1 activation were complete or partial responders ($n = 8$, almost half of the cases in the responders group). However, five of these tumors expressed very high *STAT5A* and two very high *EPHA2* levels; hence, it is questionable whether the absence of Stat1 activation contributed to tumor response in these cases.

Because it had been proposed that SRC pathway targeting could be of benefit for patients with LA-SCCHN, especially those not responding to CTX treatments [6,21], it seemed rational to investigate whether the previously described dasatinib response–predictive signature for solid tumors [28] would be expressed in our cases. Four of the six genes in this signature were analyzed here (*ANXA1*, *CAV1*, *EPHA2*, and *IGFBP2*), and profiles were obtained for all RQ value cutoffs (upper and lower quartiles, and median [Figure 4B]). Among the 31 patients

Table 4. Gene Expression in Comparison to LA-SCCHN Patient Outcome and Response to CTX-Containing Treatments.

Sample	Treatment	Overall Survival (mo)	Progression Free Survival (mo)	Response	<i>SRC</i>	<i>STAT1</i>	<i>STAT3</i>	<i>STAT5A</i>	<i>STAT5B</i>	<i>PIAS3</i>	<i>ANXA1</i>	<i>CAV1</i>	<i>EPHA2</i>	<i>EPHAB2</i>	<i>IGFBP2</i>	<i>MSN</i>
1	CCRT	31.87*	11.67	CR	433	7.255	2.155	0.215	0.101	0.127	41.098	0.098	1.159	0.369	0.515	0.588
2	CCRT	39.18*	39.18 [†]	CR	0.479	5.938	0.412	0.022	0.043	0.001	9.331	0.048	0.329	0.098	0.116	0.000
3	CCRT	23.08*	23.08 [†]	CR	0.070	10.346	1.423	0.112	0.000	0.061	29.081	0.110	2.581	0.116	1.197	0.112
4	CCRT	4.33	4.33	CR	1.375	2.377	1.829	0.496	0.042	0.067	2.369	0.906	0.421	0.021	0.323	0.230
5	CCRT	33.34*	33.34 [†]	CR	3.301	6.859	4.213	0.448	0.271	0.040	4.938	0.247	0.185	0.225	0.796	0.458
6	CCRT	31.67*	31.67 [†]	CR	1.184	17.268	3.848	1.479	0.160	0.043	20.966	0.113	0.602	0.139	0.269	0.115
7	CCRT	4.95	4.95	CR	1.252	2.809	2.556	0.199	0.237	0.107	52.527	0.042	2.757	0.013	0.619	0.091
8	CCRT	36.95*	36.95 [†]	CR	0.967	7.738	1.654	0.247	0.071	0.055	33.174	0.068	1.597	0.092	0.304	0.304
9	CCRT	24.33*	24.33 [†]	CR	1.154	2.413	2.509	0.389	0.317	0.039	32.111	0.296	2.249	0.073	3.456	0.219
10	CCRT	9.44	9.44	ED	0.376	2.135	2.118	0.114	0.057	0.023	49.351	0.057	0.981	0.182	2.521	0.000
11	CCRT	7.93	3.70	PD	1.402	7.408	2.439	0.126	0.064	0.137	3.031	0.357	0.550	0.586	2.280	0.580
12	CCRT	6.30	3.93	PD	5.832	5.808	2.583	0.291	0.103	0.000	37.453	0.078	0.787	0.000	0.649	0.000
13	CCRT	24.49*	4.13	PD	1.138	8.369	1.376	0.309	0.113	0.022	31.341	0.184	0.633	0.166	0.173	0.201
14	CCRT	26.62*	4.16	PD	1.456	3.613	1.253	0.316	0.000	0.000	24.218	0.104	0.912	0.013	0.279	0.000
15	CCRT	10.98	4.85	PD	0.920	4.426	3.665	0.335	0.210	0.085	24.251	0.184	0.751	0.012	0.641	0.174
16	CCRT	31.87*	31.87 [†]	SD	0.898	30.548	2.918	0.197	0.089	0.213	17.113	1.163	0.826	0.478	0.160	0.837
17	CCRT	25.93*	25.93 [†]	CR	0.119	7.749	0.962	0.211	0.139	0.074	14.591	0.149	0.868	0.075	0.711	0.275
18	CCRT	23.87*	23.87 [†]	CR	0.535	0.540	1.208	0.201	0.053	0.034	11.259	0.047	0.051	0.074	11.967	0.000
19	CCRT	20.69	11.67	CR	0.274	0.880	0.554	0.146	0.000	0.000	6.945	0.064	0.307	0.220	1.028	0.000
20	CTX-RT	13.38*	13.38 [†]	CR	1.385	12.510	1.112	0.286	0.199	0.130	6.974	0.026	0.480	0.099	6.639	0.103
21	CTX-RT	12.85*	12.85 [†]	CR	0.700	5.007	1.487	0.390	0.251	0.026	13.251	0.191	0.335	0.081	0.405	0.118
22	CTX-RT	12.79*	12.79 [†]	CR	1.456	27.819	1.729	0.404	0.127	169	22.362	1.813	2.385	0.453	0.430	0.540
23	CTX-RT	4.16*	4.16 [†]	CR	1.173	8.468	2.136	0.149	0.107	0.079	31.801	2.498	1.485	0.181	1.489	0.617
24	CTX-RT	16.36*	16.36 [†]	CR	0.518	3.352	2.045	0.210	0.185	0.514	9.626	1.405	0.583	0.117	3.297	0.267
25	CTX-RT	4.43*	4.43 [†]	CR	1.410	1.619	3.880	0.192	0.212	0.412	83.801	2.693	2.934	0.953	5.318	0.703
26	CTX-RT	9.51	8.95	PD	0.984	8.185	4.820	0.185	0.178	0.375	1.124	0.217	0.378	0.595	6.316	1.230
27	CTX-RT	6.1*	3.80	PD	0.726	9.540	1.876	0.190	0.151	0.176	4.817	0.262	0.511	0.146	0.360	0.412
28	CTX-RT	8.03	6.03	PD	1.967	11.951	1.863	0.286	0.159	0.073	25.142	2.774	1.244	0.138	1.215	0.532
29	CTX-RT	9.28*	9.28 [†]	PR	0.469	12.790	0.832	0.130	0.082	0.062	6.807	0.586	0.418	0.216	0.047	0.513
30	CTX-RT	7.9*	7.9 [†]	PR	1.068	4.132	2.592	0.120	0.200	0.340	12.034	1.100	0.874	0.136	1.785	1.021
31	CTX-RT	5.05*	5.05 [†]	PR	0.611	2.063	1.307	0.047	0.128	0.205	14.908	0.320	0.193	0.102	11.737	0.162

Relative quantification values were calculated with the 2^{-ΔCT} method for target versus *GUSB*.

CCRT indicates concomitant chemoradiotherapy; CR, complete response; CTX-RT, cetuximab + radiotherapy; ED, early death; mo, months; PD, progressive disease; PR, partial response; SD, stable disease.

*Patients still alive.

[†]Patients without events during the follow-up period.

with available RNA data (15 complete responders, 16 non-complete responders), ratios of the four gene transcripts compatible with the described dasatinib response signature, i.e., high *ANXA1/CAV1/EPHA2* - low *IGFBP2*, were obtained 1) for two responders, when assessing RQs

with the upper quartile cutoff; 2) for three responders with the median cutoff; and 3) in four tumors with the low quartile cutoff. The latter four tumors corresponded to one complete responder, one patient with partial response and two patients with progressive disease. Except for

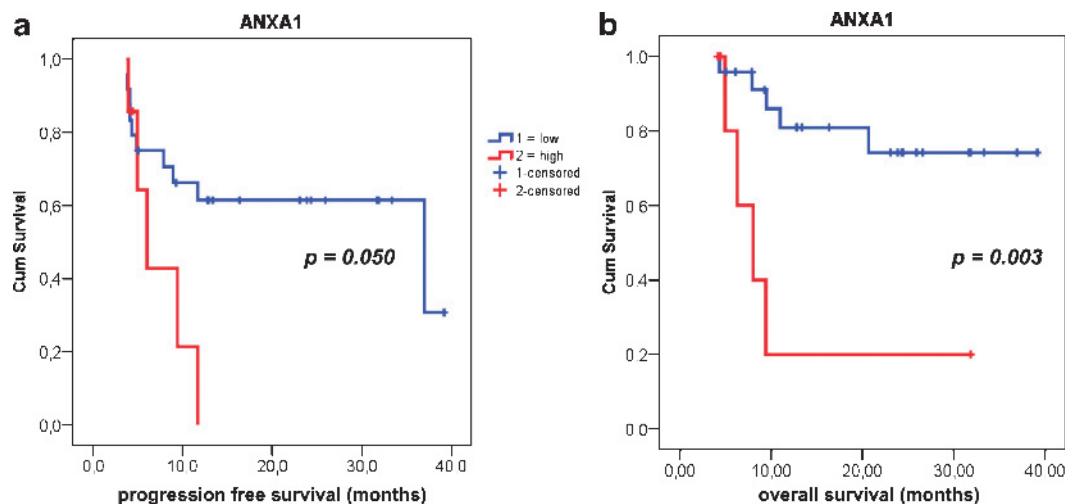


Figure 4. Association of low *ANXA1* expression with favorable LA-SCCHN patient outcome. Although relative *ANXA1* expression was only marginally related to progression free survival (PFS, shown in A), low *ANXA1* RQ values were observed in tumors from patients with longer overall survival (B) (HR = 8.03, 95% CI = 4.32-11.74).

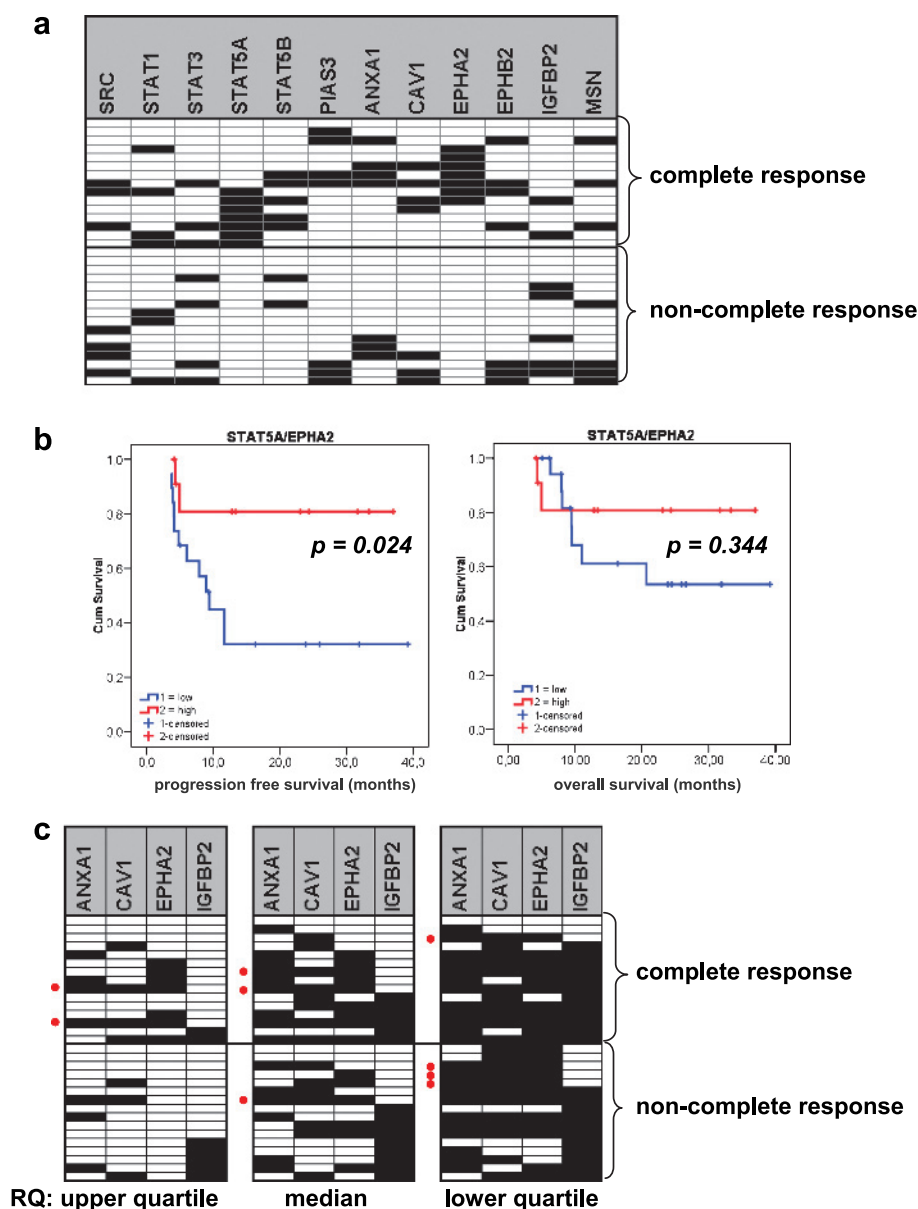


Figure 5. *STAT/SRC*-related mRNA expression profiles were associated with response to treatment in LA-SCCHN. In A, a high expression of *STAT5A* and/or *EPHA2* was associated with complete response to CTX-based treatments. Most tumors from patients who performed best on CTX-based treatments (12/15 [80%]) expressed high levels of *STAT5A* and/or high levels of *EPHA2* ($P < .0001$; odds ratio = 58.67; 95% CI = 5.4–64.5; positive predictive value = 1, negative predictive value = 0.8) as assessed by using the upper RQ value quartile (75% percentile). This high *STAT5A/EPHA2* pattern was further associated with prolonged PFS (HR = 9.44, 95% CI = 6.48–12.40) but not with patient overall survival, as shown in B. In C, the proposed dasatinib response–predictive signature with high *ANXA1/CAV1/EPHA2* and low *IGFBP2* was seldom encountered in LA-SCCHN (red dots). Cases are listed in the same order in the three panels. According to the definition of high and low (upper quartile, median, and lower quartile cutoffs for RQ values), different tumors seemed positive for this profile, some of which responded completely to CTX-based treatments. Panels in A and C: ■ = high; □ = low RQ values.

one tumor that was identified as positive for this dasatinib response predictive signature with both the upper quartile and median cutoffs, all other tumors positive for this signature did not overlap among the three different cutoff result groups.

Discussion

This study shows that LA-SCCHN may express high levels of *SRC*, *STAT1*, *STAT3*, and *STAT5A* transcripts and that most of these tumors exhibit activated Stat1, Stat3, and Stat5 proteins. It seems that *STAT3*

and *STAT5* are functionally more closely related to each other than to *STAT1*, perhaps in accordance with previous reports showing that *STAT1* plays different roles than *STAT3* in SCCHN [39], where *STAT1* may function as a tumor suppressor and may be silenced [40]. Indeed, activated Stat1 was present in the best differentiated parts of the tumor, which might support a protective effect of this protein *versus* tumor progression [40,41]. However, *STAT1* expression was the highest among all STATs examined, indicating that this gene is not silenced in SCCHN, in accordance with another previous report [42].

Overall, none of the STAT markers (mRNA or activated protein) was associated with disease outcome in our series; yet, activated Stat3 and Stat5 proteins were associated with *CAVI*, *MSN*, and *IGFBP2* mRNA expression in LA-SCCHN. Although the number of cases examined in this study was small and the data obtained need validation in larger studies, the association of activated Stat5 with *CAVI*, *MSN*, and *IGFBP2* expression is of interest especially because all these molecules represent SRC signaling targets, whereas *SRC* and *STAT5A* mRNA expression correlated significantly with each other. Caveolins participate in the function of the caveolae, structures involved in the internalization of inactive signaling molecules. *CAVI* is generally considered as a tumor suppressor [43] and may play an antimetastatic role in SCCHN; its absence or low expression is associated with metastatic growth, whereas its restoration induces growth arrest and prevents metastatic spread of SCCHN cells in animal models [44]. *Msn*, a cytoskeletal protein, decreases with progression of carcinogenesis in the squamous oral cancer model [45], whereas another member of the same protein family, *ezrin*, has been associated with worse prognosis in SCCHN [46]. Decreased *MSN* expression [45] and altered subcellular localization [47] are also associated with increased metastatic potential of oral squamous cell tumors. Hence, *CAVI* and *MSN* may be regarded as tumor suppressors in SCCHN. In comparison, *IGFBP2* is usually regarded as an oncogene with a well-established role in promoting tumor growth and metastasis in various types of cancers [48–54], whereas *IGFBP2* mRNA and protein expression have been associated with unfavorable tumor characteristics [55,56]. In addition, *IGFBP2* regulates IGF binding to and activation of IGF1R, which is implicated in resistance to CTX and is considered as a therapeutic target in SCCHN [57]. On the basis of these data, because Stat3 and Stat5 phosphorylation was mostly observed in tumors with high *CAVI* and *MSN* but low *IGFBP2* expression, it does not seem likely that activation of these two proteins occurs in the tumor-promoting setting in LA-SCCHN. In line with this view, constitutive activation of Stat3 and Stat5 in nasopharyngeal carcinomas correlates with better prognosis [58]. In addition, when both Stat3 and Stat5 are activated in breast cancer cells, they are also associated with favorable prognostic parameters, such as decreased proliferation and increased chemosensitivity to taxanes and vinorelbine [59]. Individually, Stat5 protein expression has been described as a favorable prognostic factor in breast cancer [35], whereas nuclear Stat3 protein expression seems associated with a favorable outcome in SCCHNs [60]. The above evidence along with the data from this study indicates that the effect of Stat3 and Stat5 activation in SCCHN development, maintenance, and clinical/pharmacological behavior may be different in tissues than in cell culture systems.

Activated Stat3 and Stat5 were not related to CTX-containing treatment response in our series. This finding may seem as contrasting to the previously reported experimental evidence on the role of these Stat proteins in promoting resistance to EGFR-inhibiting agents [19,20]. An explanation for this discrepancy might be that Stats may also be activated in an EGFR-independent paracrine manner in SCCHN [16], which may well be the case at the tissue level. As shown herein, LA-SCCHN expressing the highest levels of *STAT5A* mRNA responded best to CTX-based treatments in this study, whereas SCCHNs were found to express low to very low levels of *STAT5B*. With the IHC antibody available to assess Stat5 phosphorylation, it is impossible to distinguish whether Stat5a or Stat5b or both are phosphorylated in tissue sections; hence, the activation status of each one of these two proteins in our LA-SCCHN series remains unknown. Our finding on the association of *STAT5A* expression with CTX response may be related to the different

actions of the two Stat5 components reported in several systems including SCCHN: Stat5a does not promote SCCHN growth, whereas Stat5b does [61,62], prompting for a distinction between these two molecules in the research setting and in data reporting.

Except for *STAT5A*, *EPHA2* expression was also associated with best response to CTX-based treatments in our LA-SCCHN. EphA2 is generally considered as a pro-oncogenic molecule, but the function of this receptor and its ligand ephrinA1 in tumorigenesis and tumor progression is complex and seems to be dependent on cell type and micro-environment [63]. EphA2 has been correlated with poor prognosis in esophageal carcinomas [64], glioblastomas [65], and NSCLC [66], whereas EphA2 overexpression might be involved in the early development of SCCHN [67]. Interestingly, *EPHA2* gene transcription may be upregulated by EGFR in squamous cell carcinoma cell lines because of EGF activation [68]. At the tissue level, we could observe a statistical trend for increased *EPHA2* expression in LA-SCCHN with high EGFR protein score, as assessed by IHC [30] (data not shown). Thus, *EPHA2* seems to be an EGFR target gene and may be considered as a surrogate marker for EGFR activation through EGF, which represents the optimal condition for CTX to exert its growth inhibitory properties [69]. Considering *EPHA2* as a marker of EGF/EGFR activation might further explain why tumors expressing high levels of *EPHA2* respond best to CTX-based treatments, as reported herein. Clearly, to use mRNA expression levels and profiles in drug response prediction, solid cutoffs and reference systems need to be established. As shown here, the *STAT5A* and *EPHA2* mRNA profile seems promising as a marker predictive of response to CTX-based treatments in LA-SCCHN, when relative expression values in the upper quartile are considered (very high expression). The threshold for “very high expression” remains to be defined in larger studies with reliable external reference systems.

The same considerations on the evaluation of mRNA profiles apply to the proposed dasatinib response-predicting signature [28], which was tested here to identify LA-SCCHN that would probably benefit from dasatinib treatment on CTX failure. Except for *EPHA2* that has been described as an SRC target in colon carcinogenesis [70] and as a dasatinib target in breast and prostate cancers [28,29], this profile further involves *ANXA1*, three caveolae-related genes, and *IGFBP2*. As shown here, according to the applied cutoff for relative expression values (upper/lower quartiles, median), only 7% to 13% of tumors expressed high *ANXA1/EPHA2/CAVI* and low *IGFBP2*. Whereas it is not surprising that different patients were identified as possibly sensitive to dasatinib according to how the cutoffs were defined, it should be noted that the predictive value of this older one or of the new modified dasatinib predictive signatures remains unknown [71].

ANXA1, an inhibitor of phospholipase A2 and anti-inflammatory protein, is reported as downregulated in most types of cancer [72] and may serve as a differentiating factor in SCCHN development and histopathologic status [72–75]. In our series, however, overall survival of LA-SCCHN patients was adversely affected by high *ANXA1* expression, in line with a recent report on high *ANXA1* mRNA in association with poor survival in colon cancer [76], where this molecule was examined in the frame of inflammatory response in cancer. This unfavorable *ANXA1* association was not related to treatment variations (CCRT vs CTX-RT). Of note, however, all treatment schemes for LA-SCCHN involve local irradiation, although response to this modality is not evaluated separately. In this context, *ANXA1* has been found upregulated in radiation-resistant NSCLC cells [77], whereas it may also protect breast cancer cells from heat-induced growth arrest [78]. Additional preclinical evidence is needed to elucidate whether *ANXA1* affects survival of patients

with LA-SCCHN and possibly other tumors as well, as an inflammatory molecule or by conferring tumor cell survival advantage on irradiation or by both mechanisms.

Finally, we observed that Akt/PKB is commonly activated in SCCHNs, mostly at T308, implying a preferential activation through the phosphoinositide-3-kinase/pyruvate dehydrogenase kinase (PI3K/PDK1) pathway than through the mechanistic target of rapamycin (mTOR) complex [79]. However, other than previously suggested for prediction of response to EGFR inhibitors [80], no clear association was observed between Akt phosphorylation and response to CTX-based treatments.

In summary, this exploratory study provides a global overview of the effect of STAT and SRC-related parameters in the behavior of LA-SCCHN. In this respect, activated Stat proteins are frequently observed in LA-SCCHN, but Stat3 and Stat5 activation seems associated with an antioncogenic gene expression profile. In the same tumors, an mRNA expression profile involving *STAT5A* and *EPHA2* was associated with complete response to CTX-based treatments. If validated in larger cohorts and standardized for the evaluation of individual tumors, the high *STAT5A*/high *EPHA2* profile may be applied for assessing LA-SCCHN patient treatment. The same profile might be worth investigating as a positive marker for predicting response to CTX in tumors lacking the established negative predictors in this context, namely, *KRAS*/*BRAF* mutations.

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