# HES1 mRNA expression is associated with survival in sinonasal squamous cell carcinoma

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**Objective.** In squamous cell carcinoma of the pharynx and larynx, NOTCH1 downstream signaling has been shown to be activated. The NOTCH1 signaling pathway has not been examined in detail for sinonasal squamous cell carcinomas (SNSCCs). The aim of this study was to evaluate NOTCH1 signaling by mRNA expression analysis and to examine the occurrence of NOTCH1 mutations in SNSCC.

**Study Design.** In a retrospective study, we analyzed tissues from 44 SNSCCs and 56 head and neck squamous cell carcinomas (HNSCCs) at other locations. Expression of NOTCH1, NOTCH3, HES1, HEY1, and JAG1 mRNA were measured by using quantitative real-time polymerase chain reaction (q-rtPCR). In SNSCC, NOTCH1 mutations were evaluated with sequencing of seven selected exons.

**Results.** Expression of NOTCH1, HEY1, and JAG1 at the mRNA level were significantly higher in tumor tissue compared with normal tissue. In SNSCC, the subgroup of patients with high expression (5th quintile) of HES1 mRNA was associated with better survival ($P = .04$); however these patients with high expression of HES1 mRNA had also a more favorable tumor stage and grade and more unfavorable resections representing potential confounders.

**Conclusions.** Key components of NOTCH1 are upregulated at the mRNA level in HNSCCs. The mechanism, clinical significance, and potential therapeutic options should therefore be further evaluated. (Oral Surg Oral Med Oral Pathol Oral Radiol 2016;122:491-499)

Sinonasal squamous cell carcinomas (SNSCCs) represent a relatively rare entity comprising only 3% of head and neck carcinomas.[1,2] Knowledge of clinical management is based on small studies, and markers predicting the clinical outcome of patients and guiding the therapy are currently limited. Classic genetic markers that are important for SCCs of the pharynx or larynx, such as EGFR, KRAS, PI3KCA, and BRAF, have been shown to be infrequently mutated in this group of SNSCCs and do not correlate with clinical parameters.[3] Only mutations in TP53 were associated with shorter survival in these patients.[3]

NOTCH signaling has several biologic functions in the cell. It plays an important role in cell development and cell fate through control of proliferation, apoptosis, differentiation, and angiogenesis.[4] NOTCH signaling is initiated through binding of the membrane-bound ligands Jagged or Delta-like, leading to proteolytic cleavages and release of NOTCH intracellular fragment (NOTCH-IC).[4-6] NOTCH-IC is translocated to the nucleus, where it interacts with the DNA-binding protein RBPJ to activate transcription of target genes, such as the HES and HEY family, or the MYC transcription factor.[4,5]

NOTCH signaling is altered in several human cancers.[5] Recently, activation of NOTCH signaling via HES1 and HEY1 was demonstrated in head and neck squamous cell carcinoma (HNSCC).[7] The expression pattern of the transcriptionally active NOTCH1 intracellular domain (NICD1) was also associated with clinical high-risk features, human papillomavirus status and NOTCH1 mutations in HNSCC tissue.[8] In addition, it was shown that the NOTCH receptor family and especially the NOTCH1 receptor are frequently mutated in HNSCC.[9,10] The NOTCH1 mutations detected in HNSCC probably result predominantly in loss of function of the receptor.[7] However, the subgroup of SNSCCs was not sufficiently represented in these studies, and only two SNSCC tissues were analyzed without detection of mutations in NOTCH1.[7,9,10] In addition, the NOTCH1 signaling pathway has not been examined in SNSCC so far.

## Statement of Clinical Relevance

Key components of NOTCH1 are upregulated in head and neck squamous cell carcinoma. In sinonasal squamous cell carcinoma, the subgroup of patients with high expression of HES1 mRNA was associated with improved survival. These findings suggest an important role of NOTCH1 signaling in sinonasal squamous cell carcinoma.

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NOTCH pathway alterations can lead to tumor activation or repression, depending on the context.[5] As in HNSCCs, in basal cell carcinoma and hepatocellular carcinoma, tumor suppressor function was discovered for NOTCH1 mutations as well.[7,11]

Recently, we showed in this SNSCC cohort that 34% of tumor probes were positive for EBER (Epstein–Barr virus-encoded small RNAs) and LMP1 (latent membrane protein 1) and that only these progressed to lymph node or distant metastases.[3] Therefore, we came to the conclusion that patients with EBV-positive SNSCC should be more closely monitored and decided to perform neck dissection on EBV-positive patients with doubtful cervical lymph node status.[3] Since about 34% of SNSCCs were EBER/LMP1 positive, it would be clinically important to differentiate between high-risk and low-risk groups in EBV-positive SNSCCs to avoid overtreatment. Promotion of metastasis through NOTCH signaling by modulating epithelial-to-mesenchymal transition or tumor angiogenesis have been described in several malignancies.[12] An association of NOTCH signaling pathway alterations with EBV infection could, therefore, potentially help better stratify patients with SNSCC.

To date, there is insufficient knowledge about NOTCH1 signaling pathway in SNSCC. Therefore, the goal of this study was to evaluate alterations in NOTCH1 pathway in SNSCC. To achieve this objective, we examined the expression of key components of the NOTCH1 pathway with quantitative real-time polymerase chain reaction (qRT-PCR) of NOTCH1, NOTCH3, HES1, HEY1, and JAG1 in the subgroup of SNSCCs and compared with HNSCCs at other locations and with normal tissue. An association with clinical and pathologic parameters and EBV positivity was examined. In SNSCCs, in addition, we screened for mutations in NOTCH1 by sequencing seven selected exons.

## MATERIALS AND METHODS

### Patient tissue samples

Tumor tissue samples were obtained from 44 patients with SNSCC (median age: 61 years, range 37-84 years; 33 males and 11 females) and 56 patients with HNSCC (median age: 58, range 42-94 years; 47 males and 9 females) treated in the Department of Otorhinolaryngology at the Klinikum rechts der Isar, Technical University Munich, Munich, Germany. As control tissue for SNSCC, we used mucosal tissue from the inferior turbinates of patients with nasal obstruction treated with conchotomy (n = 21; median age 27 years, range 18-74 years; 13 males and 7 females). For HNSCC, we used as control tissue normal mucosal tissue from the hypopharynx, which had been excised during pan-endoscopy (n = 22; median age: 55, range 25-87 years; 11 males and 11 females). The SNSCC cohort had been used for a previous study.[3] All specimens were formalin fixed and paraffin embedded (FFPE). The study was approved by the independent ethics committee of the Technical University of Munich (project number 1420/05).

### Clinical data

Clinical data were retrieved manually from electronic medical records and filed medical records. Patients with SNSCC had been diagnosed between April 1994 and April 2013 and patients with HNSCC between January 2002 and December 2004. The study patients were routinely followed up in the tumor consultation hour of the Department of Otolaryngology. The follow-up was performed every 3 months in the first and second years after diagnosis, thereafter every 6 months until the fifth year. From the fifth year on, patients were seen every year. The overall 5-year survival rate was 69.2% for SNSCC and 49.1% for HNSCC. The median survival was 7.26 years (follow-up period, 0.03-14.83 years) for SNSCC and 4.24 years (follow-up period, 0.00-12.98 years) for HNSCC and was calculated by using reverse Kaplan-Meier analysis as previously described.[3]

Of the patients, 68% of those with SNSCC and 27% of those with HNSCC underwent only surgical resection, 30% of those with SNSCC and 57% of those with HNSCC received surgery followed by radiation (SNSCC: 25%, HNSCC: 41%) or radiochemotherapy (SNSCC: 5%, HNSCC: 11%). One (2%) patient with SNSCC and 9 (16%) patients with HNSCC were treated with primary radiotherapy or radiochemotherapy. Additional clinical and pathologic characteristics, such as tumor–node–metastasis (TNM) status and grading, are summarized in Table I.

### DNA and RNA extraction and cDNA synthesis

For DNA extraction, after dewaxing, the FFPE samples were digested with 80 μL of Proteinase K (Roche Diagnostics GmbH, Unterhaching, Germany) in 200 μL of PK buffer (50 mM Tris and 1 mM EDTA diluted in water) overnight at 55°C. The next day, another 50 μL of Proteinase K was added, and the probes were incubated for an additional 24 hours. The enzyme was inactivated by heating at 95°C for 10 minutes. Then, the DNA concentration of the samples was measured with the NanoDrop 1000 system (PEQLAB, Erlangen, Germany), and the probes were stored at 4°C until further processing. For RNA extraction, 32 μL of SDS (16%) was added to the above-described digestion mix, and the samples were further processed by using the InviTrap RNA Mini Kit (Stratec, Birkenfeld, Germany), according to the manufacturer's protocol. After

**Table I.** Depiction of the clinical and pathologic characteristics of the 44 patients with sinonasal squamous cell carcinoma (SNSCC) and 56 patients with head and neck SCC (HNSCC) included in this study

| *Clinical characteristics* | *SNSCC (n = 44)* | *HNSCC (n = 56)* |
|---|---|---|
| Primary site | | |
| Nasal cavity (NC) | 34 (77.3%) | |
| Paranasal sinus (PS) | 5 (11.3%) | |
| NC and PS | 5 (11.3%) | |
| Oral cavity | | 7 (12.5%) |
| Oropharynx | | 28 (50%) |
| Larynx | | 14 (25%) |
| Hypopharynx | | 7 (12.5%) |
| Alcohol consumption | | |
| Daily | 11 (25%) | 25 (44.6%) |
| Occasional/never | 20 (45.5%) | 9 (16.1%) |
| Unknown | 13 (29.5%) | 22 (39.3%) |
| Tobacco exposure | | |
| Smoker | 14 (31.8%) | 34 (60.7%) |
| Nonsmoker | 17 (38.6%) | 1 (1.8%) |
| Unknown | 13 (29.5%) | 21 (37.5%) |
| Staging and grading | | |
| Tumor stage (pathologic) | | |
| T1 | 22 (50%) | 17 (30.9%) |
| T2 | 11 (25%) | 18 (32.7%) |
| T3 | 6 (13.6%) | 12 (21.8%) |
| T4 | 5 (11.4%) | 8 (14.5%) |
| Nodal stage (pathologic) | | |
| N0 | 42 (95.5%) | 15 (26.8%) |
| N1-3 | 2 (4.5%) | 41 (73.2%) |
| Metastasis (initial stage) | | |
| M0 | 44 (100%) | 54 (96.4%) |
| M1 | 0 (0%) | 2 (3.6%) |
| Grading | | |
| G1 | 2 (4.5%) | 3 (5.4%) |
| G2 | 31 (70.5%) | 20 (35.7%) |
| G3/G4 | 11 (25%) | 33 (58.9%) |
| p16 Status | | |
| p16 + | 13 (29.5%) | 30 (53.6%) |

measuring the RNA concentration by using the NanoDrop 1000 system, probes were diluted to a final concentration of 25 ng RNA/μL. Only RNA probes with absorbance$_{260/280}$ between 1.8 and 2.1 and a minimal RNA concentration of 10 ng/μL were used. cDNA was then synthesized by using the Maxima reverse transcriptase (Fermentas, Waltham, MA) according to the manufacturer's protocol.

## Mutational analysis of NOTCH1 in SNSCC

Amplification of seven exons of NOTCH1 was done by using classic PCR. Primer sequences were used, as previously described by Willander et al.[13] The specific annealing temperatures for the different exons were tested by using gradient PCRs with different repeat cycle numbers followed by gel electrophoresis as quality control (annealing temperatures: 65°C for exons 6, 8, 12, 26a, 26b, and 27 and 55.7°C for exons 7 and 13). Exon 26 was covered by two PCR fragments because of its size. All probes with discovered mutations were sequenced twice.

The PCR mix for each probe contained 12.5 μL of KAPA-SYBR Fast Universal (PeqLab, Erlangen, Germany), 5 μL of DMSO, 0.8 pmol of forward primer, 0.8 pmol of reverse primer, and 50 ng of DNA, as well as 3.5 μL of water. The PCR program started with initial heating for 10 minutes at 95°C. Then, the PCR cycle started with heating for 10 seconds, followed by application of the specific annealing temperature for 30 seconds, and ended with elongation at 72°C for 30 seconds. Quality control was performed with electrophoresis by using 12 μL of PCR product and 4 μL of FluoDNA Gel Stain (PromoKine, Heidelberg, Germany) in a 2% TBE agarose gel. Sequencing was done through MWG Eurofins (Ebersberg, Germany) with dideoxy chain termination/cycle sequencing with ABI 3730 XL-sequencer, and the sequencing results were compared with the corresponding NOTCH1 sequence (NM_017617.3) by using SnapGene software (GSL Biotech; snapgene.com). Sequencing was not possible in one tumor probe and in 2% of exons after renewed sequencing.

## Quantitative real time PCR

Expression of NOTCH1, HES1, HEY1, and JAG1 mRNA was quantified by quantitative real time PCR (q-rtPCR) using the Bio-Rad CFX96 cycler for 100 tumor tissue samples and 43 normal tissue samples. The q-rtPCR mix contained 12.5 μL of KAPA-SYBR Fast Universal (PeqLab, Erlangen, Germany), 0.8 pmol of primers, and 2 μL (50 ng) of cDNA template. For HES1 and HEY1, 5% DMSO was added to the mix. Specific annealing temperatures were 56.3°C for NOTCH1, 59.4°C for NOTCH3, 57°C for HES1, 61.4°C for HEY1, and 56.3°C for JAG1. Primer sequences were used as previously described by Man et al.[4] Expression levels were normalized to GAPDH per sample and relative expression compared with ΔΔCT method.

## Immunohistochemistry

Fresh 1.5 μm sections from TMA blocks were transferred to glass slides, deparaffinized, and rehydrated. Antigen retrieval method (microwave oven heating in citrate buffered saline) was applied, according to the instructions provided by the manufacturer. The TMA slides were cooled down and incubated with the following antibody: cleaved NOTCH1 (rabbit) 1:250 (Biomol, Hamburg, Germany). The reaction was developed with the labeled streptavidin–biotin–peroxidase system. DAB was used as the reaction indicator. After counterstaining with hematoxylin, the slides were dehydrated in ascending concentrations

**Table II.** Depiction of relative mRNA expression in tumor tissue versus normal tissue

| | *Relative mRNA expression in tumor tissue versus normal tissue* | | | | |
|---|---|---|---|---|---|
| | *SN normal tissue* | *SNSCC* | *HN normal tissue* | *HNSCC* | |
| *Target* | *Median* | *Median* | *Median* | *Median* | *P value* |
| NOTCH1 | 0.99 | 2.37 | 0.81 | 1.27 | .003 |
| NOTCH3 | 1.07 | 1.46 | 1.15 | 0.98 | .275 |
| HES1 | 1.00 | 0.64 | 1.02 | 6.98 | <.001 |
| HEY1 | 0.90 | 1.63 | 1.04 | 1.50 | .040 |
| JAG1 | 0.83 | 2.94 | 0.77 | 3.16 | <.001 |

*HN,* head and neck; *HN normal tissue,* hypopharynx tissue; *SN,* sinonasal; *SN normal tissue,* inferior nasal turbinate.
Relative expression was compared with the ΔΔCT method and *P* value calculated with the Kruskal-Wallis test.

with ethanol and mounted. For positive control, tissue with known expression of the respective antigen was used. For negative control, irrelevant antibodies with the immunoglobulin isotype were used.

### Analysis of EBV status

The presence of EBV-encoded RNA (EBER) and LMP1 indicating activity was determined using in situ hybridization and immunohistochemistry, respectively, in a previous examination of this cohort and has been described in detail.[3]

### Statistical analysis

All statistical tests were two sided and significance was determined at a level of 5%. For comparison of mRNA expression in normal tissue versus tumor tissue, Mann-Whitney U test or Kruskal-Wallis test were used to calculate *P* values.

To examine the impact of high mRNA expression on clinical parameters, we categorized patients into a high-expression group (5th quintile) and an intermediate- to low-expression group (1st-4th quintiles). Furthermore, we evaluated the impact of low mRNA expression (comparison of patients in the 1st quintile with patients in the 2nd to 5th quintiles). The impact of expression levels on survival were analyzed with Kaplan-Meier curves, and significance was calculated by using the log rank (Mantel-Cox) test. To examine the association of expression levels with clinical data, contingency tables were created and compared with Fisher's exact test. Statistical calculations were done in SPSS version 22 (IBM, Ehningen, Germany).

## RESULTS

The clinical and pathologic characteristics of this cohort are depicted in Table I.

**Table III.** Depiction *P* values of survival analysis with Kaplan-Meier method and log-rank test for different targets

| | *P value* | | |
|---|---|---|---|
| *Comparison of relative mRNA expression*[*] | *SNSCC* | *HNSCC excl. SNSCC* | *HNSCC incl. SNSCC* |
| NOTCH1: 5th quintile vs 1st-4th quintiles | .356 | .773 | .705 |
| NOTCH1: 1st quintile vs 2nd-5th quintiles | .055 | .990 | .412 |
| NOTCH3: 5th quintile vs 1st-4th quintiles | .306 | .948 | .487 |
| NOTCH3: 1st quintile vs 2nd-5th quintiles | .407 | .660 | .474 |
| HES1: 5th quintile vs 1st-4th quintiles | .042 | .460 | .075 |
| HES1: 1st quintile vs 2nd-5th quintiles | .082 | .273 | .814 |
| HEY1: 5th quintile vs 1st-4th quintiles | .226 | .213 | .936 |
| HEY1: 1st quintile vs 2nd-5th quintiles | .716 | .385 | .593 |
| JAG1: 5th quintile vs 1st-4th quintiles | .452 | .559 | .766 |
| JAG1: 1st quintile vs 2nd-5th quintiles | .388 | .179 | .416 |

| | *P value* | |
|---|---|---|
| *IHC staining*[†] | *SNSCC* | *HNSCC* |
| Cleaved NOTCH1: Positive vs negative | .397 | .132 |

*Association of relative mRNA expression and overall survival. For each target, two comparisons were made: (1) high mRNA expression (5th quintile) versus intermediate and low expression (1st-4th quintiles); (2) relative low mRNA expression (1st quintile) versus intermediate and high expression (2nd-5th quintile).
[†]Association of immunohistochemistry staining for cleaved NOTCH1 and overall survival.

### NOTCH1, NOTCH3, and JAG1 expression

First, we compared NOTCH1 and NOTCH3 mRNA expression in tumor tissue and normal tissue. NOTCH1 mRNA expression was higher in the HNSCC and SNSCC tumor probes compared with normal nasal tissue ($P = .003$; Table II). No significant difference between tumor tissue and normal tissue was detected for NOTCH3 mRNA expression ($P = .275$; Table II).

Expression of JAG1 mRNA was significantly higher in tumor tissue compared with normal tissue ($P < .001$). No significant association was detected between survival and NOTCH1, NOTCH3, and JAG1 mRNA (Table III).

### NOTCH1 downstream signaling

Relative mRNA expression of HEY1 mRNA was higher in tumor tissue compared with normal tissue ($P = .040$; Figure 1; Table II). HES1 mRNA expression

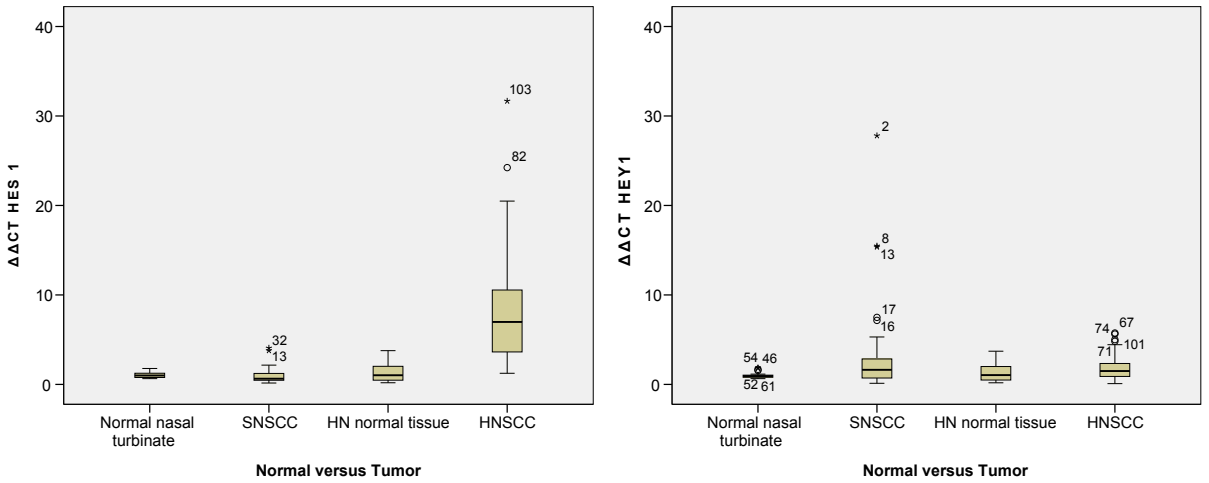


Fig. 1. Depiction of relative mRNA expression in tumor tissue versus normal tissue. Qualitative polymerase chain reaction (q-rtPCR) was performed on Biorad CFX-96 cycler, and data were normalized to GAPDH per sample. Relative expression was compared with ΔΔCT method and *P* value calculated with Kruskal-Wallis test. HES1 mRNA expression was higher in HNSCC and decreased in SNSCC ($P < .001$). HEY1 mRNA was upregulated in SNSCC and HNSCC ($P = .040$). Outliers are depicted with * and extreme values with ° in each case in addition to respective sample identifier.

was decreased in SNSCC and higher in HNSCC, excluding SNSCC compared with normal tissue ($P < .001$; Figure 1; Table II).

In SNSCC, high mRNA expression of HES1 was associated with better overall survival ($P = .042$), as illustrated in Figure 2. Low mRNA expression of HES1 (1st quintile), compared with intermediate and high expression (2nd to 5th quintiles), seems to be associated with worse survival in SNSCC (not significant, $P = .082$). Expression levels in both groups were correlated with clinical factors by using contingency tables. Age, tumor stage, grade, smoking, or alcohol abuse were not significantly different in both groups (Table IV). However, patients with high HES1 mRNA expression had lower tumor stages and grades and more unfavorable resections in comparison with patients with lower expression of HES1 (Table IV). In HNSCC, excluding SNSCC, no significant association was found between HES1 mRNA and survival (Table III). There was no significant association between high or low HEY1 mRNA expression and overall survival (Table III).

Cleaved NOTCH1 staining was positive in 62% (34 of 55) of HNSCC and 75% (30 of 40) of SNSCC. Cleaved Notch1 expression was not associated with survival ($P = .397$ for SNSCC, $P = .132$ for HNSCC; Table III).

## NOTCH1 mutations in SNSCC

In addition, screening for mutations in seven selected exons of NOTCH1 (exons 6, 7, 8, 12, 13, 26, and 27) in 44 SNSCC cases was performed. Sequencing was not possible in one tumor probe and in 2% of exons after renewed sequencing. Exon 6, 7, 8, 12, and 13 encode for NOTCH1 extracellular region and exons 26 and 27 for the heterodimerization domain (HD). We chose to primarily examine exons coding for the extracellular region, since most mutations were found in this region in previous studies on HNSCC tumor tissue.[9,10] One heterozygous nonsense mutation in the extracellular region in exon 8 in one patient probe was detected (amino acid: p.Q475 Stop; base exchange: C→T). This patient was the only one who developed a distant metastasis during the follow-up period.

## Association of HES1 expression in SNSCC with EBV and development of metastases

Relative HES1 expression in the EBER/LMP1 positive group was 0.833 versus 1.05 in the negative group ($P = .892$). In patients with metastases, HES1 expression was 0.76 versus 1.01 in patients without metastases ($P = .777$). In the group with high HES1 expression no patient out of nine developed metastases (0%) while two of 26 patients in the intermediate-expression group (8%) and two of nine patients (one patient with lymph node metastasis and one patient with distant metastasis) in the low-expression group (22%) showed metastases (Fisher's exact test, $P = .351$). In the high-expression group, one patient was EBER/LMP1 positive. Exclusion of this patient with high HES1 expression from the EBER/LMP1 positive cohort improved sensitivity slightly from 40% to 43% without affecting specificity (100%). No significant pattern was seen for the expression of NOTCH1, NOTCH3, HEY1, or JAG1.

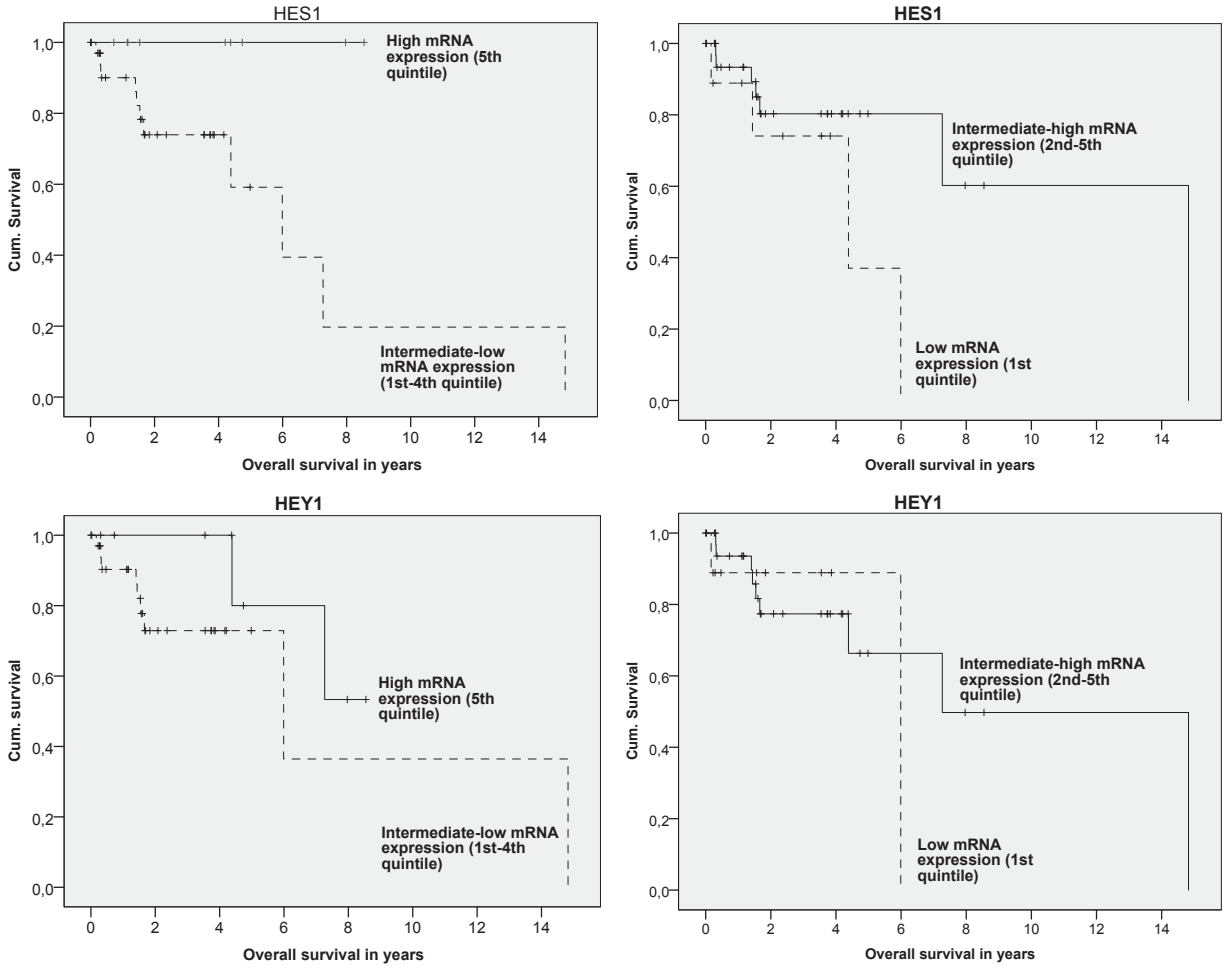


Fig. 2. Association of relative mRNA expression and overall survival in SNSCC was analyzed by using the Kaplan-Meier method, followed by the log-rank test. The patients were grouped into a relative high expression (5th quintile) group and an intermediate-low mRNA expression group (1st-4th quintiles). A significantly longer survival was found for patients with relatively high mRNA expression of HES1 compared with those with intermediate or low expression (*top left*) ($P = .042$). Relatively low expression of HES1 mRNA (1st quintile) was associated with shorter survival in comparison with intermediate and high expression (*top right*) (not significant, $P = .082$). No significant patterns were seen in the high or low HEY1 expression group ($P = .226$ and $P = .716$).

## DISCUSSION

The NOTCH pathway is involved in the tumorigenesis of several human malignancies, including HNSCC.[5] In some tumor subgroups, the NOTCH pathway exhibits tumor suppressive effects but also has tumor-promoting properties in others.[7] In HNSCC, a bimodal pattern of NOTCH pathway alterations has been recently revealed.[7] However, the role of the NOTCH signaling pathway in the subgroup of SNSCC has not been evaluated so far. We therefore examined the mRNA expression of key proteins in NOTCH1 signaling in an SNSCC cohort and an HNSCC cohort in comparison with tissues from a normal control group and correlated the expression with clinical outcome. In addition, screening for mutations in NOTCH1 was performed in SNSCC tissue.

### Expression analysis

We detected an upregulation of NOTCH1, HEY1, and JAG1 mRNA in comparison with normal tissue. Expression of HES1 mRNA was decreased in SNSCC tumor tissue and higher in HNSCC of other locations in comparison with normal tissue. Patients in the control group for normal sinonasal tissue were younger than those in the tumor group. We therefore analyzed whether mRNA expression levels are associated with age, and no significant difference was seen. Previously, in HNSCC of the pharynx and larynx also an upregulation of NOTCH3, JAG1, and JAG2 had been demonstrated, and in 32%, an activation of HES1/HEY1 was found.[7] In this examination, an upregulation of NOTCH1 in HNSCC but not NOTCH3 was detected. Furthermore, a bimodal pattern of NOTCH1

**Table IV.** Depiction of the clinical and pathologic characteristics of the 44 patients with sinonasal squamous cell carcinoma (SNSCC) included in this study in relation to relative HES1 mRNA expression

| | *HES1 expression* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *1st-4th quintile* | | *5th quintile* | | *Total* | | | |
| *Clinical and pathologic characteristic* | *n* | *%* | *n* | *%* | *n* | *%* | *P value* | *OR* |
| No. of Patients | 35 | | 9 | | 44 | | | |
| Gender | | | | | | | .67 | 1.69 |
| Male | 27 | 77 | 6 | 67 | 33 | 75 | | |
| Female | 8 | 23 | 3 | 33 | 11 | 25 | | |
| Age (years) | | | | | | | 1.00 | 0.94 |
| <55 | 15 | 43 | 4 | 44 | 19 | 43 | | |
| >55 | 20 | 57 | 5 | 56 | 25 | 57 | | |
| Tobacco | | | | | | | .41 | 2.53 |
| No | 14 | 61 | 3 | 38 | 17 | 55 | | |
| Yes | 9 | 39 | 5 | 63 | 14 | 45 | | |
| Alcohol | | | | | | | .67 | 0.52 |
| No | 14 | 61 | 6 | 75 | 20 | 65 | | |
| Yes | 9 | 39 | 2 | 25 | 11 | 35 | | |
| T stage | | | | | | | 1.00 | 0.83 |
| T1-2 | 26 | 74 | 7 | 78 | 33 | 75 | | |
| T3-4 | 9 | 26 | 2 | 22 | 11 | 25 | | |
| N stage | | | | | | | 1.00 | * |
| N0 | 33 | 94 | 9 | 100 | 42 | 96 | | |
| N1-N2 | 2 | 6 | 0 | 0 | 2 | 4 | | |
| Overall stage | | | | | | | .32 | * |
| Stage <4 | 29 | 83 | 9 | 100 | 38 | 86 | | |
| Stage >4 | 6 | 17 | 0 | 0 | 6 | 14 | | |
| Differentiation | | | | | | | .41 | 0.31 |
| Well to moderate | 25 | 71 | 8 | 89 | 33 | 75 | | |
| Poor | 10 | 29 | 1 | 11 | 11 | 25 | | |
| M stage | | | | | | | 1.00 | * |
| M0 | 35 | 100 | 9 | 100 | 44 | 100 | | |
| M1 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| Resection status | | | | | | | 1.00 | 0.50 |
| R0 | 28 | 80 | 8 | 89 | 36 | 82 | | |
| RX-R2 | 7 | 20 | 1 | 11 | 8 | 18 | | |
| Recurrence | | | | | | | 1.00 | 0.83 |
| No | 26 | 74 | 7 | 78 | 33 | 75 | | |
| Yes | 9 | 26 | 2 | 22 | 11 | 25 | | |

*OR,* odds ratio.
Cases with missing data (e.g., smoking status) were excluded in calculation in category.
*Calculation of odds ratio was not possible.

with separate inactivation through mutations has been detected in HNSCC.[7]

In addition, an association with the clinical outcome of the differential mRNA expression was examined in this study. To best identify differentiating ranges of mRNA expression to stratify patients, we examined all quintiles of expression for best discrimination. The best differentiating quintiles of expression were the 1st and 5th quintiles. We did not perform computerized exact crossing point calculation because of the small sample size. The survival of the subgroup of patients with high expression (5th quintile of mRNA expression) was compared with the subgroup with intermediate and low expression. In SNSCC, the subgroup with high mRNA expression of HES1 was associated with a significant better overall survival. However, patients with intermediate and low expression of HES1 mRNA showed a more advanced tumor stage, higher grade, and more unfavorable resections as potential confounders. In HNSCC other than SNSCC, no significant pattern between HES1 mRNA and survival was detected. To evaluate if protein expression correlates with mRNA as surrogate for HES1/HEY1 expression, staining for cleaved NOTCH1 was performed and did not reveal a significant difference in survival in SNSCC or HNSCC. Considering the small number of cases and potential confounders, these findings need to be further validated in a bigger patient group. In addition, the impact of NOTCH signaling and HES1 expression could be examined in vitro (e.g., with small molecule inhibition of NOTCH signaling or knockdown of HES1). Inhibition of NOTCH signaling with a gamma

secretase inhibitor attenuated pancreatic cancer growth and reduced proliferation in lung cancer in a preclinical model.[14,15] In esophageal SCC, HES1 mRNA underexpression was recently identified to correlate with tumor invasion.[16] In contrast, in colorectal cancer, high HES1 expression served as an unfavorable prognostic factor, and in lung adenocarcinoma, high HES1 expression correlated with worse survival.[17,18] In HNSCC, the role of HES1 and HEY1 expression on clinical outcome has not been examined so far. However, the expression pattern of the transcriptionally active NOTCH1 Intracellular Domain (NICD1) was correlated with clinical high-risk features and NOTCH1 mutations.[8] HEY1, NOTCH1, and JAG1 mRNA expression, even though upregulated, had no significant impact on survival in this SNSCC cohort.

### Mutational analysis

Since mutation rates of 14% and 15% have been shown for NOTCH1 in HNSCC,[9,10] we also screened for mutations in the subgroup of SNSCCs. Seven selected exons of NOTCH1 were examined, and one nonsense mutation was detected in the 43 examined tumor probes (2%). The lower mutation rate in comparison with the published rates in HNSCC can be partly explained because only seven of 34 exons were sequenced. Another reason could be the limited sample size analyzed. The detected nonsense mutation in exon 8 lies in the extracellular region and probably results in a loss of function of the protein. This is in agreement with the inactivating mutations detected by Agrawal et al.[9] and Stransky et al.[10] in HNSCC.

### EBV infection and NOTCH activation in SNSCC

We could recently demonstrate in the same SNSCC cohort that 34% (15 of 44) of tumors stained positive for EBER/LMP1 and that only these patients had metastases.[3] This finding could help in the decision making on the extent of treatment, especially whether to perform elective neck dissection. However, only 5% (2 of 44) had metastases at initial presentation, and only 9% of patients (4 of 44) developed metastases during the follow-up.[3] Therefore, additional markers to increase the sensitivity of prediction of metastases could help to prevent overtreatment of patients. The impact of NOTCH signaling on metastases by modulating epithelial-to-mesenchymal transition or tumor angiogenesis has been previously described in several malignancies.[12,19,20] We therefore examined the association of mRNA expression of NOTCH1 pathway proteins and EBV positivity. No significant association between NOTCH1 signaling and EBV was detected. Exclusion of patients with high HES1 expression from the group of EBER/LMP1−positive patients increased sensitivity for positive metastases slightly (from 40% to 43%). The only patient who developed distant metastasis also harbored a NOTCH1 mutation and had a low HES1 expression in our cohort. This pattern could potentially help in identifying high-risk patients in the EBER/LMP1−positive group. The clinical relevance of this finding, however, remains unclear because of the small patient collective and low frequency of metastases in SNSCC.

### Limitations

Our study has certain limitations, and results have to be interpreted with caution. First, due to the limited sample size, results are less reliable. Because SNSCC occurs infrequently, a multicenter study should be performed to validate the results. Second, results by chance are possible because of the multiple testing performed, especially to find the best differentiating ranges of mRNA expression for survival analysis.

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