



# Lectin-like transcript 1 (LLT1) expression is associated with nodal metastasis in patients with head and neck cutaneous squamous cell carcinoma

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## Abstract

The interaction of lectin-like transcript 1 (LLT1) with CD161 inhibits Natural Killer cell activation. Overexpression of LLT1 contributes to the immunosuppressive properties of tumor cells. However, there are little data about LLT1 expression in human solid tumors. The objective of this paper is to investigate the relationship between LLT1 expression with the clinicopathologic features and its impact on the prognosis of head and neck cutaneous squamous cell carcinoma (cSCC). LLT1 expression was analyzed on paraffin-embedded tissue samples obtained from 100 patients with cSCC by immunohistochemistry. The estimator of Fine and Gray was used to estimate the cumulative incidence curves for relapse. Proportional Hazard models and Hazard ratios (HRs) were used for studying the risk of tumor relapse and mortality. LLT1 strong expression was a significant risk factor for nodal metastasis with crude and adjusted ratios (HRs) of 3.40 (95% CI 1.39–9.28) and 3.25 (95% CI 1.15–9.16); and for cSCC specific death of 6.17 (95% CI 1.79–21.2) and 6.10 (95% CI 1.45–25.7). Strong LLT1 expression is an independent predictor of nodal metastasis and poor disease-specific survival and it might be helpful for risk stratification of patients with cSCC.

**Keywords** Cutaneous squamous cell carcinoma · Lectin-like transcript 1 · LLT1 · Natural killer · CLEC2D · Immunohistochemistry · Checkpoint

## Introduction

Non-melanoma skin cancer (NMSC) is the most frequent malignant neoplasm in Caucasians, with increasing incidence in the last decades [9, 10]. Cutaneous squamous cell

carcinoma (cSCC) represents one third of NMSC, with an estimated annual incidence of 700,000 cases in United States [14]; and 4% of these patients suffer metastases or local recurrence after complete excision of the tumor [5]. There is compelling evidence supporting the significance of

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the immune system in the pathogenesis of skin cancer [22, 27], nevertheless the underlying mechanisms and its role in patient's prognosis have been poorly analyzed.

Lectin-like transcript 1 (LLT1, gene CLEC2D), also known as osteoclast inhibitory lectin OCIL) is a C-type lectin-like protein belonging to the super-family of Natural Killer (NK) cell receptors [6]. LLT1 is mainly expressed on activated immune cells including NK cells, T cells, B cells and antigen presenting cells such as dendritic cells and macrophages. LLT1 is also expressed in virus-infected cells and cytokine-stimulated respiratory epithelial cells [25] and in synovial fluid monocytes and joint macrophages from Rheumatoid Arthritis patients [7]. LLT1 is a ligand for the (NK) cell inhibitory receptor NKR1p1a (or CD161) [17, 23]. Cross-linking of CD161 on NK cells inhibits cytotoxicity and cytokine production [1, 3, 23]. Nevertheless, there is conflicting evidence regarding the consequences of LLT1 binding to CD161 expressing T cells and it has recently shown that LLT1 may have a co-stimulatory activity on activated B cells [17].

The evasion of the immune system is a hallmark of cancer cells. The immune evasion mechanisms have become key targets for anti-cancer immunotherapy. Cancer cells frequently upregulate physiological inhibitory immune pathways to evade the immune response, such as PD1 and CTLA-4. Likewise, it has been described that the upregulation of LLT1 expression in glioblastoma, B cell lymphomas and prostate cancer contributes to the immunosuppressive properties of tumor cells and the evasion of the immune system [17–19, 24].

Nothing is known about the role of LLT1 in non-melanoma skin cancer. The overall goal of our study was to analyze LLT1 expression in a large series of patients with cSCC of the head and neck (cSCCHN), to establish associations with clinic and pathologic features of the patients and to analyze its possible impact on patient's prognosis.

## Methods

### Patients, procedures, histopathologic evaluation, and tissue microarray (TMA) construction

The Department of Pathology electronic database at the Hospital Universitario Central de Asturias searched to locate all patients with cSCC who developed head and neck nodal metastases (cSCCHN) between 1998 and 2008. All the electronic medical records were reviewed to determine whether the outcome of interest occurred. Finally, 50 primary cSCCHN, who developed histologically confirmed lymph node metastasis (McSCCs) were included. 50 control patients were randomly selected among those patients

with cSCCHN who did not develop any metastases and had a minimum follow-up of 4 years.

All tumors were excised by conventional surgery and patients with positive margins were excluded. None of the patients received adjuvant therapy after surgery. Ethics approval was obtained from the Hospital Universitario Central de Asturias Committee. The Study was conducted, and the results were reported according to the Strengthening the Reporting of Observational Studies in Epidemiology guidelines (STROBE) for case–control studies [26]. Clinical data were collected retrospectively. Patient age was defined as the age at the time of resection. Pathologic tumor staging based on the 7th, 8th AJCC and the Brigham and Women's classification was also registered [2, 8, 15]. Outcome data were based on one tumor per patient.

### Histopathologic evaluation

Each sample was analyzed using hematoxylin–eosin (H&E) stained slides and the following histopathologic features were registered: Maximum diameter, tumor thickness (dichotomized as  $\leq 6$  mm or  $> 6$  mm; none lower than 2 mm), anatomic level (Clark's level), histopathologic degree of differentiation, presence of desmoplasia, perineural or perivascular invasion, presence of inflammatory infiltrate in the tumor was defined as none (0), mild (1), and strong (2), and the presence and number of tumor budding [13].

### Tissue microarray (TMA) construction

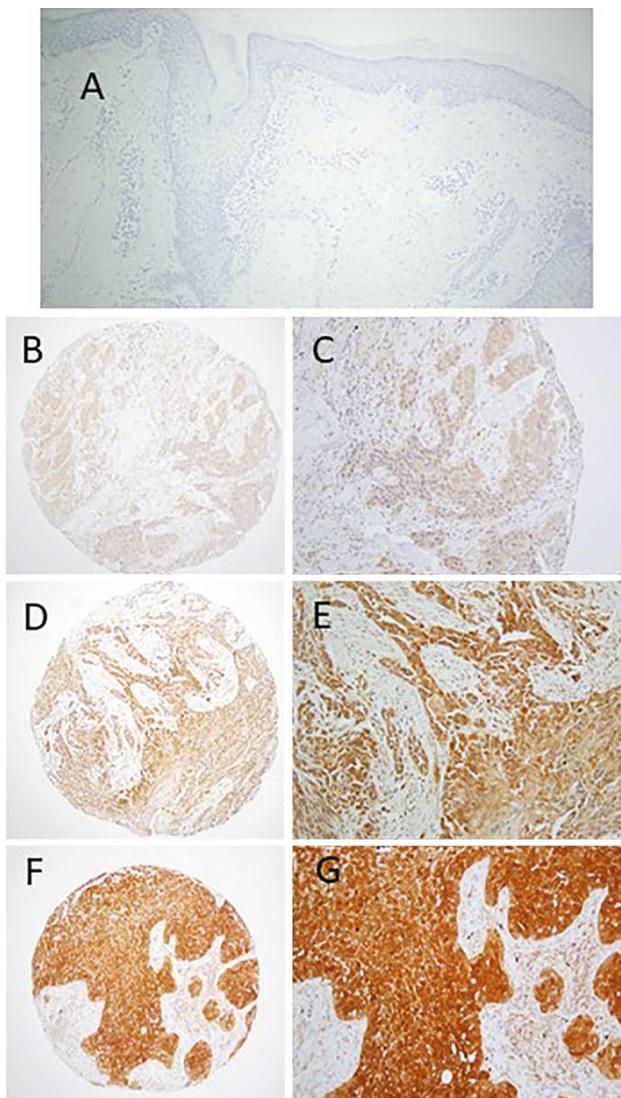
Morphologically representative areas were selected from each individual tumor paraffin block for the construction of TMA. Three 1 mm cylinders were taken to construct TMA blocks, as described previously [21]. A total of four TMAs were created, containing three tissue cores from each of the 100 cSCCHN. In addition, each TMA included three cores of normal skin as an internal control.

### Immunohistochemistry

The TMAs were cut into 3- $\mu$ m sections and dried on Flex IHC microscope slides (DakoCytomation, Glostrup, Denmark). The sections were deparaffinized with standard xylene and hydrated through graded alcohols into water. Antigen retrieval was performed by heating the sections using Envision Flex Target Retrieval solution, high pH (Dako). Staining was done at room temperature on an automatic staining workstation (Dako Autostainer Plus) with mouse monoclonal anti-LLT1 Clone 30 (BD Biosciences Pharmingen, San Diego, CA) at 1:200 dilution, Counterstaining with hematoxylin was the final step.

LLT1 staining showed a homogeneous distribution within tumor samples. Immunostaining was preferentially

detected in the cytoplasm of tumor cells, although some cases showed protein enrichment at the cell membrane. Expression levels were quantitated, independently, by two investigators. Since LLT1 expression was homogeneous within tumor samples, a semiquantitative scoring system based on staining intensity was applied: LLT1 expression was classified as none, weak, moderate, or strong (Fig. 1). For statistical purposes, and based on preliminary analyses, the score was simplified as none-weak expression, moderate expression, and strong expression (Fig. 1).



**Fig. 1** Immunohistochemical analysis of LLT1 expression in cSCC tissue specimens. **a** Normal skin ( $\times 20$ ), **b** none-weak ( $\times 100$ ), **c** none-weak ( $\times 200$ ), **d** moderate ( $\times 100$ ), **e** moderate ( $\times 200$ ), **f** strong ( $\times 100$ ), **g** strong ( $\times 200$ )

## Statistical analysis

Baseline demographic and clinical characteristics of the patients and pathological data are summarized with the standard descriptive statistics. The primary endpoints were time to lymph-node metastasis and time to death, defined as the time from the date of diagnosis of the primary tumor to the date of diagnosis of metastasis or death, respectively.

Conventionally, variables are described using mean  $\pm$  standard deviation, medians with 25th and 75th percentiles or relative and absolute frequencies depending of their symmetry and nature.

Since the main studied outcome (i.e. presence of relapse) is not a final event, and therefore, potentially affected by competing risk-death without relapse is its competing event—the cumulative incidence curves for relapsing were computed. The Gray estimator [12] was used to estimate them and were compared from the procedure described therein. Then, the proportional hazard regression model proposed by Fine and Gray [11] was used to compute the hazard ratios (HR)—used as average effect size measure—crude and adjusted by potential confounders. The 95% confidence intervals for the HRs are also reported.

The influence of factors on mortality was analyzed using standard proportional hazard Cox regression model. Crude and adjusted HRs and 95% confidence intervals are provided. Adjusted models include variables related with severity of disease. Relapse was included as a time-dependent covariate. Probabilities of disease-free survival (nodal metastasis or death) and overall survival were estimated by Kaplan–Meier.

Differences reporting  $p$  values below 0.05 were considered statistically significant. All analyses were made using the free environment R ([www.r-project.org](http://www.r-project.org)). Particularly, package survival and cmprsk were used.

## Results

100 Caucasians patients with cSCCHN were analyzed in this study (Table 1). 78% were male and the mean age was 78.9 years. 50 patients developed lymph node metastasis, and 50 patients did not develop any metastasis with a minimum follow-up of 4 years.

Analysis of LLT1 expression (Table 1) showed that 25% of tumors had none-weak staining, 38% moderate staining, and 37% strong staining. Regarding the relationship of LLT1 expression to the clinical and pathological features of cSCC patients, strong LLT1 expression was significantly associated with tumor thickness  $> 6$  mm, anatomic level  $\geq 4$ , presence of desmoplastic growth, and tumors classified as pT2 or higher following the seventh edition of the TNM classification (Table 1). No other associations with the clinical or pathological parameters were found.

**Table 1** Association between LLT1 expression and clinicopathologic features of patients with primary cSCC

	Global	LLT1			<i>p</i> value
		Negative-weak ( <i>n</i> = 25)	Moderate ( <i>n</i> = 38)	Strong ( <i>n</i> = 37)	
Gender, male	78	19 (76%)	31 (81.6%)	28 (75.7)	0.795
Age, mean ± SD	78.97 ± 8.06	78.7 ± 7.7	79.6 ± 7.8	78.5 ± 8.7	0.854
Tumor thickness (mm)					
Mean ± SD	8.95 ± 6.56	8.2 ± 7.3	8.4 ± 7.0	10.1 ± 5.5	0.245
> 6	50	11 (44%)	14 (37%)	25 (68%)	<b>0.023</b>
Anatomic level, ≥ 4	75	15 (60%)	25 (66%)	35 (95%)	<b>0.002</b>
Tumor horizontal size (mm)					
Mean ± SD	22.64 ± 14.20	20.4 ± 14.6	23.4 ± 16.4	23.3 ± 11.4	0.470
> 20	35	7 (28%)	12 (32%)	16 (43%)	0.399
Tumor differ, 3/4	96	25 (100%)	36 (95%)	35 (95%)	0.499
Desmoplastic growth	14	0 (0%)	5 (13%)	9 (24%)	<b>0.025</b>
Tumor site, ear	36	8 (32%)	13 (34%)	15 (41%)	0.757
TNM, pT2 = 2	86	21 (84%)	29 (76%)	36 (97%)	<b>0.031</b>
TNM					
T1	37	13 (52%)	15 (39%)	9 (24%)	0.079
T2	6	0 (0%)	3 (8%)	3 (8%)	0.345
T3	57	12 (48%)	20 (57%)	25 (68%)	0.246
BWH					
T1	43	14 (56%)	15 (39%)	14 (38%)	0.314
T2	26	5 (20%)	12 (32%)	9 (24%)	0.566
T3	31	6 (24%)	11 (29%)	14 (38%)	0.483
Inflammation					
0	25	7 (28%)	9 (24%)	9 (24%)	0.921
1	61	14 (56%)	24(63%)	23(62%)	0.836
2	14	4 (16%)	5 (13%)	5 (14%)	0.945
Another SCC	29	6 (24.0%)	11 (29%)	12 (32%)	0.773
Immunosuppression	11	1 (4%)	6 (16%)	4 (11%)	0.343
Perineural invasion	14	1 (4%)	7 (18%)	6 (16%)	0.241
Lymph-vasc. invas.	4	1 (4%)	2 (5%)	1 (3%)	0.852
Tumor buds					
> 0	45	7 (28%)	20 (53%)	17 (46%)	0.121
> 5	20	2 (8%)	8 (5%)	10 (27%)	0.181

Bold values indicate  $p < 0.05$

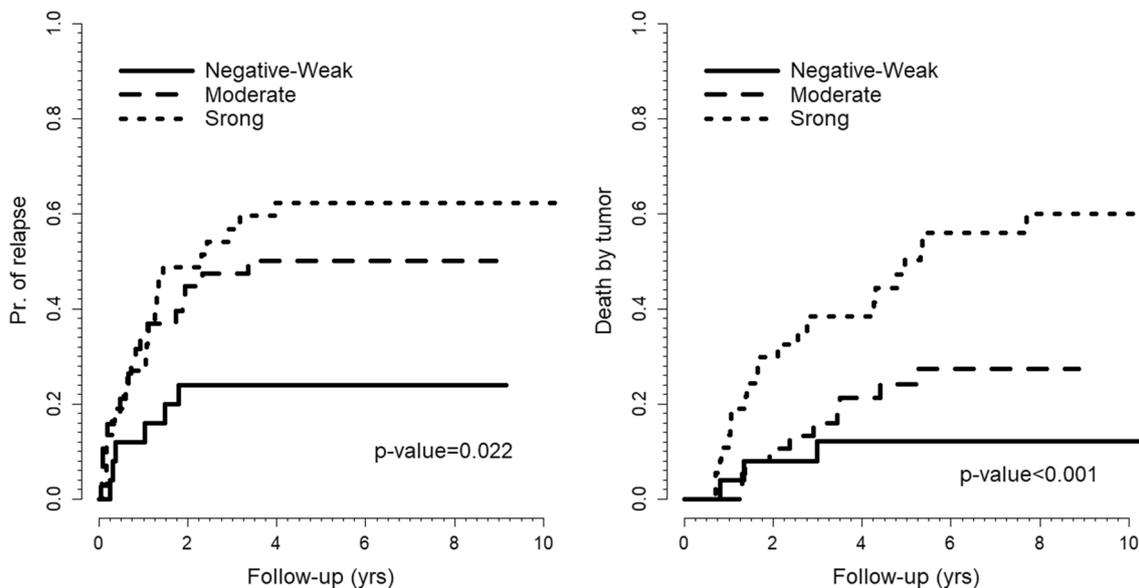
When analyzing the prognostic significance of LLT1 expression in a univariate model, we found that moderate and strong LLT1 expression were significantly associated with an increased risk of nodal metastasis (Table 2). Further, LLT1 expression was found to be also a significant independent predictor of nodal metastasis in multivariate model (Table 2). Strong LLT1 expression was also associated with an increased risk of death by the tumor on univariate and multivariate analysis (Table 2). However, no associations were observed between LLT1 expression and disease-free survival or overall survival (Table 2).

As illustrated in Fig. 2 (left panel), a striking difference in nodal metastasis-free survival rates was observed between patients with LLT1 negative/weak expression, LLT1 moderate expression, and LLT1 strong expression ( $p = 0.022$ ). In addition, patients with tumors with strong LLT1 expression exhibited an increased risk of death compared to patients with LLT1-negative-weak tumors ( $p < 0.001$ ) (Fig. 2, right panel). No significant differences were observed neither in free-disease survival rates ( $p = 0.204$ ; Fig. 3, left panel) nor in overall survival (Fig. 3, right panel;  $p = 0.234$ ).

**Table 2** Univariate and multivariate model for the effect of LLT1 expression on nodal metastasis and specific death

	Nodal metastasis		Specific death	
	HR (95% CI)	<i>p</i> value	HR (95% CI)	<i>p</i> value
<b>Model 1 (crude)</b>				
None-weak	1 (referent)		1 (referent)	
Moderate	2.57 (1.01–6.54)	0.047	2.28 (0.62–8.43)	0.220
Strong	3.40 (1.39–8.28)	0.007	6.17 (1.79–21.2)	0.004
<b>Model 2 multivariate (adjusted)</b>				
None-weak	1 (referent)		1 (referent)	
Moderate	4.68 (1.63–13.4)	0.004	2.71 (0.59–12.5)	0.200
Strong	3.25 (1.15–9.16)	0.026	6.10 (1.45–25.7)	0.014
<b>Free-disease survival</b>				
<b>Model 1 (crude)</b>				
None-weak	1 (referent)		1 (referent)	
Moderate	1.66 (0.92–2.96)	0.090	1.36 (0.73–2.52)	0.334
Strong	1.55 (0.86–2.77)	0.144	1.68 (0.91–3.09)	0.095
<b>Model 2 multivariate (adjusted)</b>				
None-weak	1 (referent)		1 (referent)	
Moderate	2.60 (1.40–4.83)	0.002	1.43 (0.76–2.68)	0.266
Strong	1.56 (0.86–2.83)	0.145	1.55 (0.84–2.87)	0.161

Multivariate model: adjusted by age, gender, thickness, tumor horizontal size, and tumor site

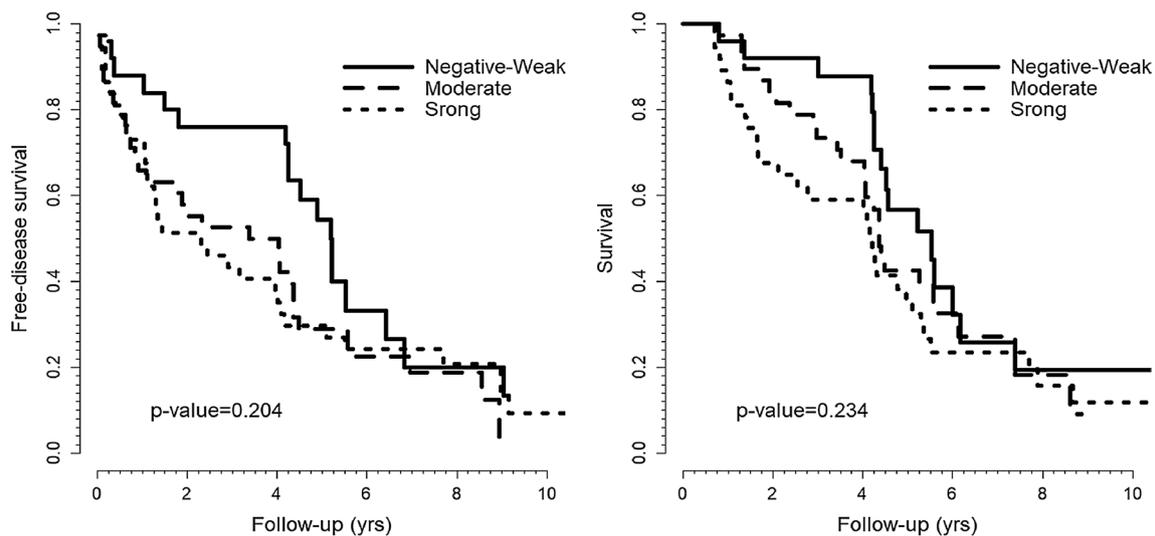


**Fig. 2** Fine Gray estimates of nodal metastasis after resection of the cSCC on LLT1 negative-weak versus LLT1-moderate *versus* LLT1-strong subgroups (left), and death by tumor (right)

## Discussion

Immune system plays a key role in the pathogenesis of cancer, and particularly, in the development of skin cancer [22,

27]. The evasion of the immune response is a hallmark of the cancer cells. NK cells play a critical role in elimination of cancer cells [18], and to evade their anti-tumor capabilities, cancer cells frequently up-regulate ligands of NK



**Fig. 3** Kaplan–Meier estimates of disease-free survival (left) and overall survival (right) after resection of cSCC for the LLT1

cell inhibitory receptors. Thus, LLT1 is a good candidate to be involved in immune evasion in cancer, since it is a ligand for the inhibitory NK cell receptor CD161, which negatively regulated the lytic activity and cytokine production by NK cells [4, 18]. Further, LLT1 is expressed on a wide variety of tissues, such as the gallbladder, the digestive tract (glandular cells), the kidneys and the lung [4, 16, 18]. Up-regulation of LLT1 expression has been also reported in several B cell-derived and monocyte-derived tumor cell lines protecting them from NK cell activity [18, 19]. Roth et al. also showed that glioblastoma cells over-expressed LLT1 allowing them to escape from NK cell mediated killing [14] and Mathew et al. showed similar results on prostate cancer cells [24]. Moreover, Marrufo et al. have shown that blocking LLT1–NKRPIA interaction enhances NK cell mediated killing of triple negative breast cancer cells [20]. In this study, we assessed the expression of LLT1 in cSCCHN and its potential role as a predictive factor for nodal metastasis, disease-specific survival, disease-free survival and overall survival.

Now, no standardized staining and evaluation scheme for LLT1 expression exists. As in our series the staining of LLT1 in the tumors was homogeneous, we chose a semi-quantitative method to evaluate LLT1 expression in tumor cells based on the intensity of staining. An important advantage of this method is its simplicity and reproducible measurement of the results.

In agreement with the hypothesis that the immunosuppressive capacities of cSCC increase with malignancy, our findings show that the expression of LLT1 in cSCC correlates with the tumor thickness, anatomic level, presence of desmoplastic growth and pT classification following the seventh edition of TNM classification [8]. However,

LLT1 expression was not correlated with the inflammatory response to the tumor, suggesting that LLT1 expression is not a consequence of the infiltration of the tumor by immune cells. Further, we found that the risk of nodal metastasis and death by the tumor in patients exhibiting LLT1 moderate and strong expression increases in both the univariate and multivariate models. Overall, the expression of LLT1 observed may support an immune-mediated selection process favoring the survival of less immunogenic tumor cells. In addition, our results clearly support that LLT1 expression may play a prominent role in cSCC nodal metastasis process. This could be likely to be associated with the robust presence of a variety of CD161-positive immune cells in the lymph node including NK cells, T cells, B cells, dendritic cells and macrophages [19]. These data also delineate a potential target for immunotherapy that should be further explored in this disease.

Nevertheless, we did not find significant associations of LLT1 expression with disease-free survival and overall survival rates; however, this lack of relevant differences may be attributable to the old age of patients. In this group of people, the mortality by other causes rather than tumor is not negligible. Our marker shows to be itself very specific regarding to the death by the cSCC, but the association with mortality dilutes when all-causes are considered. Alternatively, another possible explanation is that the number of patients might be too small to obtain enough statistical power to detect significant associations.

We are aware that there are several limitations in our study. First, there are potential biases due to the retrospective nature of the study. Second, immunohistochemically analysis was carried out using tissue microarrays; however, the expression pattern is quite concordant in the three

representative tissue cores selected from each tumor. Third, no standardized staining and evaluation scheme for LLT1 expression exist.

In conclusion, we address the independent prognostic relevance of tumor LLT1 expression using a large series of cSCCHN. According to the data presented herein, tumor LLT1 expression is a significant predictor of risk for nodal metastasis and disease-specific survival in cSCC patients. These results require confirmation in prospective studies to further support the clinical application of LLT1 expression as a useful biomarker for risk stratification.

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### Compliance with ethical standards

**Conflict of interest** The authors have no conflict of interest to declare.

**Ethical approval** Ethical approval was obtained from the Hospital Universitario Central de Asturias Committee.

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