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Evaluation of the cancer stem cell marker DCLK1 in patients with lymph node metastases of head and neck cancer

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ABSTRACT

Background: Lymph node metastases are frequently detected in head and neck squamous cell carcinoma (HNSCC) patients. Little is known about biomarkers expressed in lymph node metastases or their influence on clinical outcome. Doublecortin-like kinase 1 (DCLK1) is one marker that might be associated with outcome, owing to its correlation with stem cell-like characteristics.

Methods: We assessed the expression of DCLK1 in 74 postoperatively irradiated patients in histologically confirmed HNSCC lymph node metastases. Statistical analysis of the association with DCLK1 on clinical outcomes was performed.

Results: DCLK1 was expressed in 63.5% of our patient cohort. DCLK1(+) HNSCC patients, compared with those without DCLK1 expression, showed a significantly poorer time to recurrence. Moreover, we observed a significantly poorer time to recurrence in HPV(-) HNSCC patients, and significantly shorter overall and disease-free survival rates in HPV(-) oropharyngeal cancer patients, compared with HPV(+) patients with these cancers. HPV (+) patients showed no significant differences in survival time according to DCLK1 expression. However, recurrent disease occurred in only DCLK1(+) patients. Mulitivariate analysis showed that DCLK1 expression in lymph node metastases is an independent marker for recurrence.

Conclusion: DCLK1 expression might be associated with poorer clinical outcomes in HNSCC patients, specifically in HPV(-) move patients. However, larger studies are required to verify our results.

1. Introduction

In total, 550,000 new cases of head and neck malignancies are diagnosed annually. Head and neck squamous cell carcinomas (HNSCC) are the 6th most frequent malignant disease worldwide [1]. Lifestyle factors, such as tobacco and alcohol consumption, are known causative risk factors for HNSCC [2]. Additionally, infection with human papilloma virus (HPV) has been found to be a key factor in HNSCC carcinogenesis in the past decade [3]. HPV infections are associated with an increasing incidence rate of oropharyngeal squamous cell carcinoma (OPSCC), for which approximately 130,000 cases are newly diagnosed every year [4].

The prognosis and treatment of HNSCC partly depend on the presence of lymph node metastasis. More than 50% of all patients with HNSCC present with lymph node metastases at the first clinical examination. Patients with AJCC stage III/IV HNSCC are usually treated with either surgical resection followed by radiotherapy or radio (chemo/immuno)therapy alone. Postoperative radiotherapy is associated with improved locoregional control and survival in stage III and IV disease [5,6].

Despite improved treatment methods, recurrent disease remains a major problem in HNSCC patients, particularly in late-stage and locoregionally advanced cases. Recurrence is one of the major factors impairing quality of life and reducing survival time. One known risk factor for relapse in HNSCC is lymph node involvement, because lymph node metastasis increases the likelihood of locoregional recurrence [7].

Currently, opinions differ regarding the genesis of recurrent disease. One hypothesis involves the presence of cancer stem cells (CSC). The

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theory of CSC may conceptually explain the recurrence of malignant diseases. Generally, CSCs can repopulate a tumor after treatment with irradiation or chemotherapeutical agents. In HNSCC, CSCs may be involved in failed responses to radiotherapy [8].

To date, several different biomarkers expressed in neoplastic cells have been found to be are associated with CSCs. In particular, expression of CD10, CD133, ALDH1 and ABCG2 are associated with HNSCC cells' stem cell like attributes [9–12]. Interestingly, few data are available on biomarkers expressed in lymph node metastases. Recently, another novel CSC marker, doublecortin-like kinase 1 (DCLK1), has gained attention as a prognostic marker and potential therapeutic target. DCLK1 is a member of the family of calmodulin-dependent serine/threonine kinases that is involved in the polymerization of microtubules. Previously reported data indicate that DCLK1 is overexpressed in various cancer types. Most studies investigating DCLK1 in cancerous tissue have found it expressed within the cytoplasm. DCLK1(+) cells display stem cell-like characteristics in preinvasive pancreatic cancer [13]. In addition, expression of DCLK1 has been reported in clear renal cell carcinoma and colon cancer [14,15]. Interestingly, DCLK1 is measurably elevated in the plasma of patients with esophageal adenocarcinoma and pancreatic cancer [16,17]. Our group has demonstrated that DCLK1 expression in HNSCC primary tumors is associated with poorer survival, and thus DCLK1 might serve as a potential therapeutic target in HNSCC, particularly in oropharyngeal SCC (OPSCC) [18]. Moreover, higher recurrence rates have been observed in major salivary gland carcinoma patients with DCLK1 expression, whereas no survival differences have been found in minor salivary gland carcinoma patients [19].

Hence, we sought to gain insight into the role of DCLK1 in lymph node metastases in HNSCC patients, given that the expression of this marker in lymph node metastases is unknown. The purpose of this study was to evaluate the expression profile of DCLK1 in histologically confirmed positive lymph nodes of HNSCC patients. Moreover, we aimed to assess whether DCLK1 expression might influence survival and recurrence.

2. Materials and methods

2.1. Patients

After multidisciplinary discussion at the institutional Head and Neck Tumor Board, we included all patients with histologically established lymph node metastases in HNSCC who were treated at the Medical University of Vienna between the years 2002 and 2012 and received surgical resection of the primary tumor together with neck dissection and postoperative radiotherapy. Patients were excluded if any other malignant diseases were present in their medical history or insufficient data were available. According to the board's guidelines, some patients (with extracapsular spread or R1 resections) were additionally treated with chemotherapy. HPV status was assessed via in-situ hybridization, as described previously [18]. The classification of nodal metastases into conventional macrometastases (≥ 2 mm) and micrometastases (< 2 mm) was performed according to the Hermanek Classification. Additionally, the presence of cystic metastases was described.

2.2. Tissue microarray analysis and immunohistochemistry

Samples of HNSCC lymph node metastases obtained during neck dissection and before radiotherapy were collected via a manual tissue punch (Estigen, Tartu, Estonia). A total of three cores with a diameter of 2 mm were taken from each patient's metastatic lesions. HE staining had been performed earlier to verify the histology. The immunohistochemical staining was performed on 3 µm sections. Initially, the adequate antibody dilution was determined by using gastric cancer and clear renal cell carcinoma samples. After sample deparraffinization with xylol and rehydration with ethanol and water, endogenous peroxidase activity was inhibited with 3% H_2O_2. Thereafter, antigen retrieval was performed with a 600 W microwave oven and EDTA buffer. Samples were then incubated with Ultra V Block (Thermo Scientific, Fremont, CA, USA). Then, the primary antibody against DCLK1 (1:400, Abcam, Cambridge, UK) was applied. Primary Antibody Enhancer (Thermo Scientific, Fremont, CA, USA) was added for 10 min, and HRP polymer was added for another 15 min. DCLK1 staining was visualized with an UltraVision Plus Detection System DAB Plus Substrate System (Thermo Scientific, Fremont, CA, USA). Subsequently, samples were counterstained with Hematoxylin Gill III (Merck, Darmstadt, Germany). Samples were evaluated with an Olympus BH-2 microscope (Olympus, Tokyo, Japan). The samples were categorized into two groups without or with expression of DCLK1. Two independent investigators (RW and LK) examined each micro-section while blinded to patient clinical data. DCLK1 expression in the primary tumor was described [18].

2.3. Statistical analysis

Overall survival (OS, initial diagnosis to death), time to recurrence (TTR, initial diagnosis to recurrence) and disease free survival rates (DFS, initial diagnosis to death or recurrence) were calculated with the Kaplan-Meier method.

Log-rank test (LRT), which gives equal weight to all time points, was used to statistically analyze differences in survival time between both groups. Additionally, Gehan-Breslow test (GBT) was used to give more weight to events at early time points. In addition, univariate and multivariate cox proportional hazard models were calculated, and Fisher's exact test or chi-square test was applied to compare proportions of a categorical outcome in two or more groups, respectively. McNemar's test, a statistical test used on paired nominal data, was used to analyze DCLK1 expression in the primary tumors and lymph node metastases. Hazard ratios were also computed. SPSS software (version 23.0; SPSS, Inc., Chicago, IL) and Prism GraphPad Software (GraphPad Software, Inc., La Jolla, CA) were used to analyze data.

3. Results

3.1. Patient characteristics

A total of 74 patients with nodal metastases were evaluated. The mean age was 59.2 years at the time of initial diagnosis (range 38.3–80.2 years). The median follow-up time was 121 months (range 14–175 months). All patients received postoperative radiotherapy with a mean dosage of 57.7 Gy (range 40–60 Gy). Moreover, eight patients received chemotherapy as an additional treatment. Data on cigarette consumption was available in 42 of 74 cases. The group of non-smokers included 17 patients (40.5%), whereas 25 patients (59.5%) were smokers.

Furthermore, we assessed the anatomic sites of the primary tumors. The primary tumors comprised 12 in the oral cavity (OSCC), 44 in the oropharynx (OPSCC), 13 in the hypopharynx (HPSCC) and 5 in the larynx (LSCC) (Table 1).

The mean number of positive lymph nodes in the neck dissection specimen was 3.4 (range 1–21). In most patients, the histopathologic report described conventional lymph node metastases (n = 65). Additionally, three patients with a pN1 neck had micrometastases, six patients (all HPV(+)) had cystic metastases, and two patients exhibited extracapsular spread.

We were able to analyze 70 patients for HPV. We detected the presence of HPV DNA in 19 patients through in-situ hybridization. All HPV(+) patients had a primary tumor located in the oropharynx.

Table 1
Patient's baseline clinical characteristics in dependence of DCLK1 expression.

	DCLK1 $^{-}$	DCLK1 $^{+}$	Total	*p* - value
Number of patients	n = 27	n = 47	n = 74	
Age (median)	59.5	60.0	59.8	0.7941
Range	38-78	43-80	38-80	
pT1	n = 10	n = 8	n = 18	0.0505
pT2	n = 8	n = 29	n = 37	
pT3	n = 6	n = 8	n = 14	
pT4	n = 3	n = 2	n = 5	
Locally advanced Disease	n = 9	n = 10	n = 19	0.2796
pN0	n = 0	n = 0	n = 0	0.6463
pN1	n = 8	n = 12	n = 20	
pN2a	n = 3	n = 3	n = 6	
pN2b	n = 11	n = 26	n = 37	
pN2c	n = 3	n = 5	n = 8	
pN3	n = 2	n = 1	n = 3	
No. positive Lymph Nodes (mean +- SEM)	2.9 +-0.5	3.7 +-0.6	3.4 +-0.4	0.3808
Oral Cavity	n = 3	n = 9	n = 12	0.4628
Oropharynx	n = 19	n = 25	n = 44	
Hypopharynx	n = 3	n = 10	n = 13	
Larynx	n = 2	n = 3	n = 5	
HPV positive	n = 7/25	n = 12/45	n = 19/70	0.5421

DCLK1…double cortin like kinase 1, SEM…standard error of mean, HPV… human papilloma virus.

3.2. Immunohistochemical staining of DCLK1 in lymph node metastases of HNSCC patients

We were able to analyze samples of 74 lymph node metastases in HNSCC patients treated with postoperative radiotherapy (Fig. 1). Most of the patients (63.5%; n = 47) showed cytoplasmic expression of DCLK1 in the affected lymph nodes, whereas DCLK1 expression was undetectable in 36.5% (n = 27) of all lymph node metastases. (Table 1) DCLK1(-) was defined as an absence of expression or weak expression in < 5% of all cells. Only tumor cells were counted for DCLK1 analysis. Samples that showed only peritumoral staining, which is associated with lymphocytes, were also counted as DCLK1(-) (Fig. 1A). DCLK1 expression was always detected in the cytoplasm in SCC cells. In DCLK1(+) samples, focal expression in single cells or cell nests (> 5% of all cells) was found in ten specimens. Widespread expression was found in most DCLK1(+) patients (n = 37). Weak or moderate expression intensity was detected in 9 and 11 patients, in 5–50% and > 50% of all tumor cells, respectively. Strong intensity was found in 10 patients in 5–50% of all tumor cells and 7 patients in > 50% of all tumor cells.

OPSCC represented the largest anatomic subunit, and DCLK1(+) regional metastases occurred in 56.8% (n = 25) of all cases.

We did not find any significant differences in comparing clinical characteristics such as pT and pN classification. However, analysis of T classification with a chi-square test indicated a nearly significant difference, owing to the large number of T2 primary tumors in DCLK1(+) patients (p = 0.0505). Thus, we also compared early pT classifications (pT1&2) with locally advanced cases (pT3&4). Fisher's exact test did

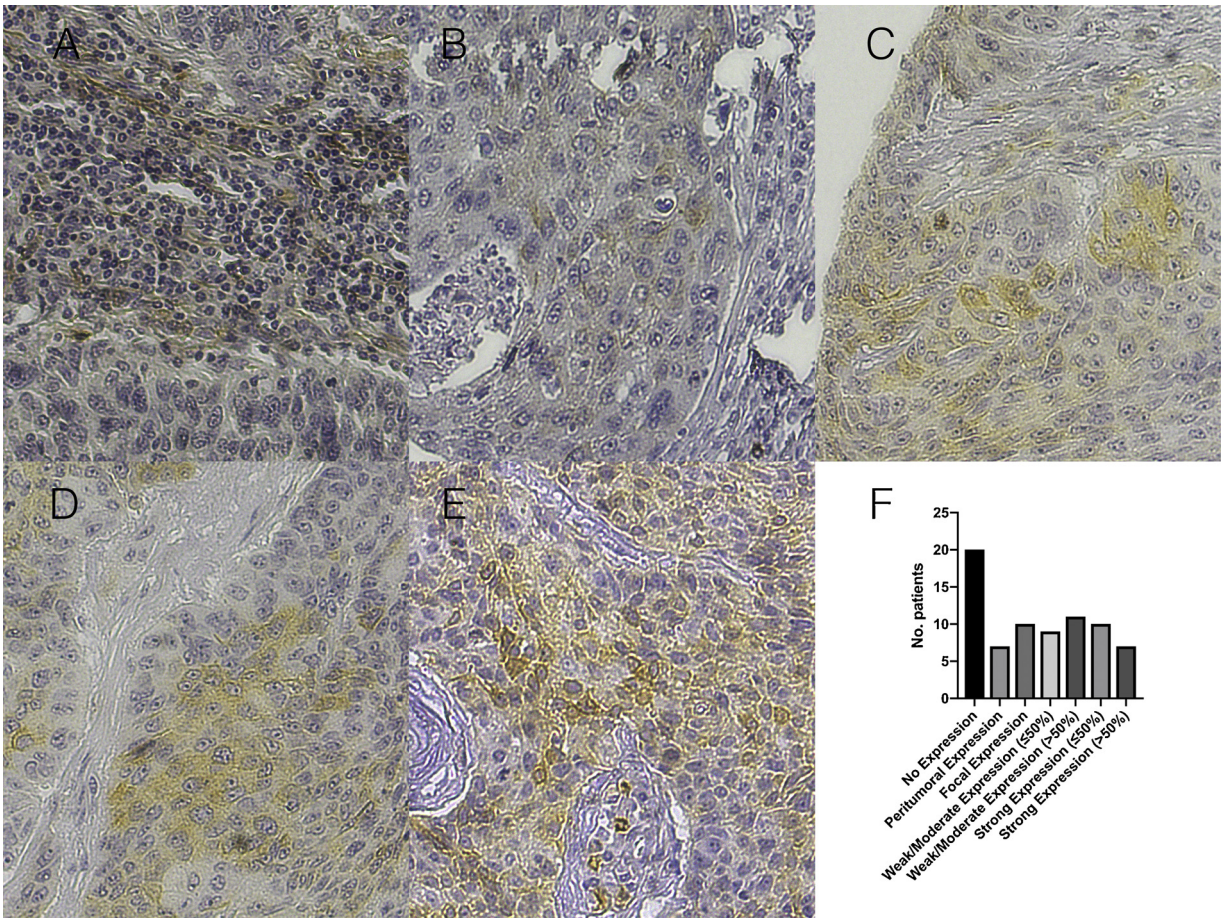

Fig. 1. Paraffin-embedded samples of head and neck squamous cell carcinoma lymph node metastases. (A) shows a section of peritumoral expression of DCLK1 but no expression within the metastatic tumor cells. In figures (C–E) the different expression patterns are demonstrated that were classified as DCLK1 positive. (F) shows the distribution pattern of different staining qualities.

not reveal a significant difference in this case (p = 0.2796). Next, we assessed the number of infiltrated lymph nodes in the neck dissection specimens. Both cohorts showed a median number of two nodal metastases, and the mean of 3.7 and 2.9 in DCLK1(+) and DCLK1(-) patients, respectively. These results were not significantly different between cohorts (p = 0.3808).

Additionally, we evaluated whether DCLK1 expression in affected lymph nodes might be associated with HPV infection. DCLK1(+) lymph nodes showed a similar rate of HPV positivity. A total of 12 (26.7%) DCLK1(+) patients tested positive for HPV infection (26.7%), whereas 7 patients (28.0%) with DCLK1(-) lymph nodes tested positive for HPV. Fisher's exact test indicated that the results were not statistically significant (p = 0.5421).

Moreover, we sought to determine whether there was a correlation between expression of DCLK1 in the primary tumor and corresponding lymph node metastases, because the expression of DCLK1 was assessed previously [18]. As mentioned above, most lymph node metastases were positive for DCLK1 (n = 47/74, 63.5%). Although we observed the same expression status of DCLK1 in primary tumors and lymph node metastases in most cases (65.7%, n = 28 for double positive and n = 18 for double negative), McNemar's test did not indicate a statistically significant correlation (p = 0.1530).

3.3. Effects of DCLK1 expression on survival and recurrence

A total of 74 HNSCC patients were treated with primary resection and neck dissection followed by radiotherapy. Subsequently, these patients were stratified into two groups as described above: DCLK1(-) and DCLK1(+) lymph node samples. Among the DCLK1(-) patients, 25.9% (n = 7) were reported dead (Table 2). DCLK1(+) patients showed a higher mortality rate of 42.6% (n = 20). Moreover, recurrent disease occurred in 36.2% (n = 17) of patients with DCLK1(+) lymph nodes. Interestingly, only a single patient (3.7%) with DCLK1(-) lymph node metastases developed recurrent disease.

Next, we computed Kaplan-Meier estimates to assess the effects of DCLK1 expression on survival time. We observed no significant differences in OS between patients with positive or negative DCLK1 expression (p = 0.2174 LRT; 0.2463 GBT). However, patients with DCLK1(+) lymph nodes showed a significantly poorer TTR (p = 0.0030 LRT; p = 0.0037 GBT). The p values for DFS measured just reached significance (p = 0.0555 LRT; p = 0.0563 GBT) (Fig. 2 A–C).

Because HPV has high value as a prognostic marker in HNSCC and OPSCC patients in particular, we aimed to gain insight into the dependence of the effects of DCLK1 on HPV status (Figs. 2D–F & 3A–F).

First, we analyzed survival times for all HPV(-) HNSCC patients in the context of DCLK1 expression. Again, OS and DFS were not significantly associated with DCLK1 expression. However, recurrent disease was detected significantly more often (p = 0.0195), and TTR was also significantly shorter, in the presence of DCLK1 expression in the cohort of HPV(-) HNSCC patients (p = 0.0212 LRT; p = 0.0241 GBT).

In addition, we stratified patients with a primary tumor of the oropharynx into HPV(+) and HPV(-) groups and evaluated them in the context of DCLK1 expression. DCLK1 expression was associated with a significantly shorter OS (p = 0.0303 LRT; 0.0369 GBT) and DFS (p = 0.0282 LRT; 0.0342 GBT) in HPV(-) OPSCC patients. HPV(+) OPSCC patients showed no significant differences in OS, DFS or TTR according to DCLK1 expression. However, recurrent disease occurred in only DCLK1(+) patients.

Additionally, Cox regression analysis was performed, including DCLK1 status in lymph nodes and primary tumors, HPV status, localization, and T and N classification. DCLK1 in primary tumors was the only independent marker (p = 0.038) after multivariate analysis for reduced OS. However, DCLK1 expression in lymph nodes was a statistically independent marker for recurrent disease (p = 0.014).

4. Discussion

Biomarkers and their prognostic relevance in primary HNSCC tumors have been extensively studied. HPV and p16 are particularly important in everyday clinical use. We previously demonstrated that DCLK1 expression in primary tumors of HNSCC and OPSCC patients is associated with poor survival. [18] However, especially in pathologically invaded lymph nodes in HNSCC patients, knowledge of the expression of biomarkers and their effects on recurrence and survival is lacking. We therefore sought to examine the role of DCLK1 in the lymph nodes of postoperatively irradiated HNSCC patients.

Table 2
Statistical analysis of survival times in dependence of DCLK1 expression.

Group	Parameter	DCLK1$^+$	DCLK1$^-$	*p* - value	*HR (95%CI)*
HNSCC	*No. patients*	n = 47	n = 27		
(n = 74)	*Mortality*	n = 20	n = 7	0.2110	
	Recurrence	n = 17	n = 1	**0.0016**	
	5-year OS rate	53.8%	69.4%	0.2174	1.6 (0.8-3.6)
	5-year DFS rate	46.9%	69.4%	0.0555	2.0 (1.0-4.2)
	5-year TTR rate	58.1%	95.7%	**0.0030**	4.2 (1.6-10.7)
HNSCC HPV(-)	*No. patients*	n = 35	n = 20		
(n = 55)	*Mortality*	n = 18	n = 6	0.1621	
	Recurrence	n = 12	n = 1	**0.0195**	
	5-year OS rate	46.7%	67.5%	0.2398	1.7 (0.7-3.8)
	5-year DFS rate	43.8%	67.5%	0.1278	1.9 (0.8-4.2)
	5-year TTR rate	59.1%	93.8%	**0.0212**	3.7 (1.2-11.3)
OPSCC HPV(-)	*No. patients*	n = 12	n = 11		
(n = 23)	*Mortality*	n = 6	n = 1	0.0686	
	Recurrence	n = 3	n = 0	0.2174	
	5-year OS rate	44.4%	90.9%	**0.0303**	5.2 (1.2-23.3)
	5-year DFS rate	44.4%	90.9%	**0.0282**	5.3 (1.2-24.0)
	5-year TTR rate	64.6%	100%	0.0519	9.7 (1.0-95.2)
OPSCC HPV(+)	*No. patients*	n = 12	n = 7		
(n = 19)	*Mortality*	n = 2	n = 1	1.0000	
	Recurrence	n = 5	n = 0	0.1060	
	5-year OS rate	78.8%	75.0%	0.7447	1.5 (0.2-14.5)
	5-year DFS rate	55.6%	75.0%	0.2213	2.8 (0.5-14.1)
	5-year TTR rate	55.6%	100%	0.0641	5.4 (0.9-32.6)

DCLK1…double cortin-like kinase 1, HR…hazard ratio, CI…confidence interval, HNSCC…head and neck squamous cell carcinoma, HPV…human papilloma virus, OPSCC…oropharyngeal squamous cell carcinoma, OS…overall survival, DFS…disease free survival, TTR…time to recurrence.

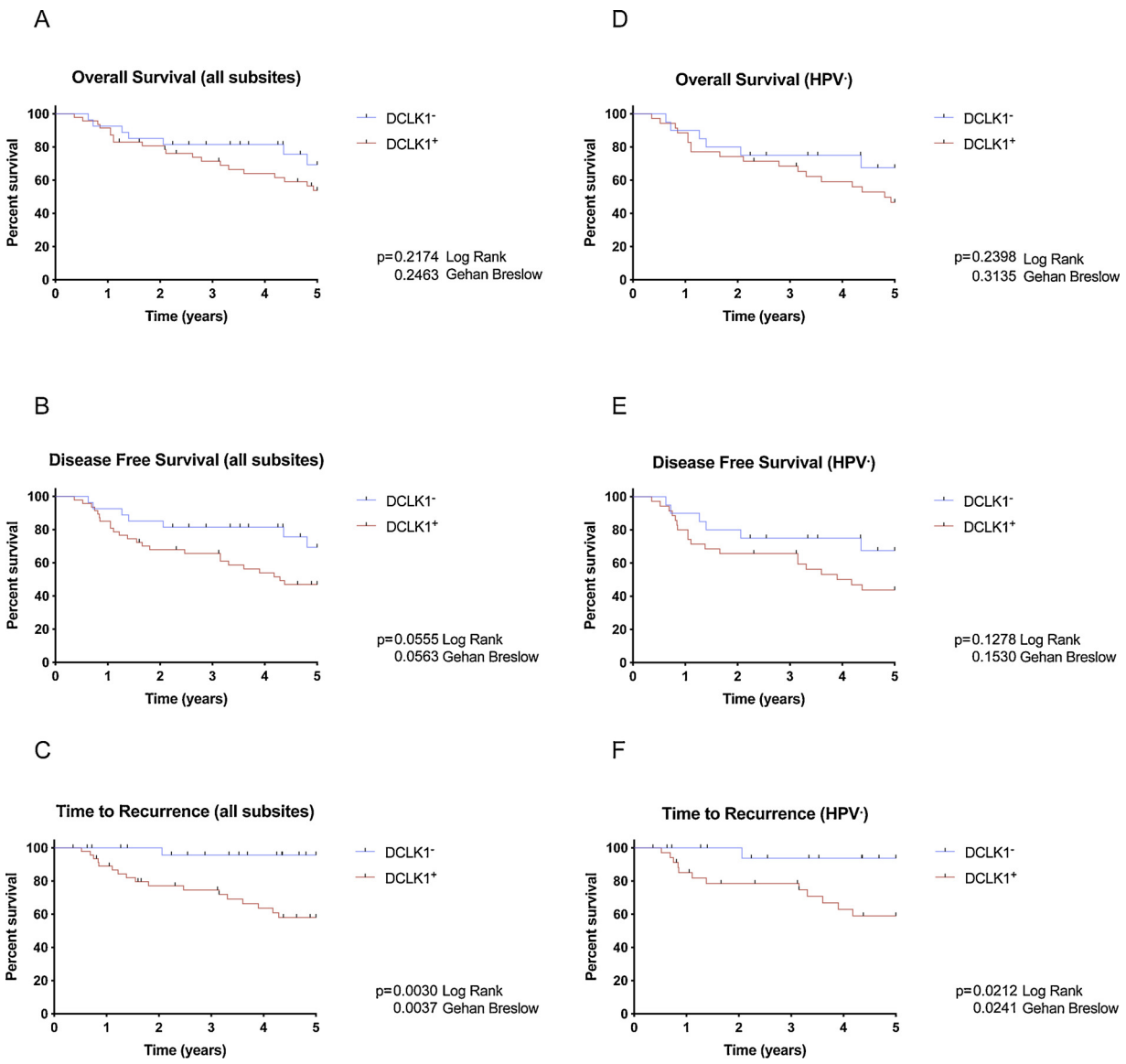

Fig. 2. Overall (OS), disease-free survival (DFS) and time-to recurrence (TTR) in dependence of DCLK1 expression. (A–C) show the 5-year Kaplan-Meier estimates for all subsites. (D–F) show survival curves for HPV(-) and HPV(+) oropharyngeal tumours.

To date, DCLK1 expression has been described in several primary tumors of different entities [14,15]. Moreover, Gao and colleagues have identified DCLK1 expression in primary tumors as an important contributor to the formation of lymph node metastases in colorectal cancer [20]. In our study, we detected expression of DCLK1 in lymph node metastases of HNSCC. In our cohort, DCLK1 was expressed in lymph node metastases in nearly two-thirds of all cases. Mannelli and coworkers have detected CD133 and CD44, both cancer stem cell markers, in HNSCC lymph node metastases [21]. In their cohort, CD44 was widely expressed, in contrast to CD133.

Expression of CSC markers such as MAGEA3/6 [22], SOX2 [23], SDF-1 [24], Krüppel-like factor 4 [25] ALDH 1 [26] and CD166 [27] in primary HNSCC is often associated with poor outcomes. In comparison, little is known about the prognostic effects of CSC in lymph node metastases. As already mentioned, only a single study has evaluated CSC in HNSCC lymph node metastases [21]. However, no correlation analysis with survival data was performed. Our results showed an association between expression of DCLK1 and poor outcomes in HNSCC patients, particularly HPV(-) patients. However, DCLK1 has been described to be associated with poor outcomes in gastrointestinal cancers [28]. Our group previously analyzed DCLK1 expression in the major salivary glands of the head and neck, where DCLK1 positivity was correlated with reduced disease free survival [19].

Nevertheless, the existence of CSC and its effects on loco-regional relapse in HNSCC remain theoretical; and there is a study that has directly demonstrated a connection between CSC and HNSCC. Thus, there might be other explanations other than CSCs for the adverse effects associated with DCLK1 expression. DCLK1 has several characteristics that might explain its association with poor outcomes. Originally, DCLK1 was described in the field of neurobiology, where it has an important role during neuronal migration, owing to its interaction with the microtubules of the cytoskeleton. The cytoskeleton is also highly important for migration of cancer cells, and it ultimately contributes to disease progression. Several studies have shown that DCLK1 expression is linked to increased cell motility and enhanced cellular migration [29,30].

Moreover, we examined the sub-sites of the HNSCC primary tumors. Because the numbers of patients with OSCC, HPSCC and LSCC were too small, we were unable to further analyze these subgroups. Thus, all patients with HPV(-) tumors were analyzed together, and DCLK1 was found to be significantly associated with the development of recurrent disease. Understandably, better-powered follow-up studies are needed

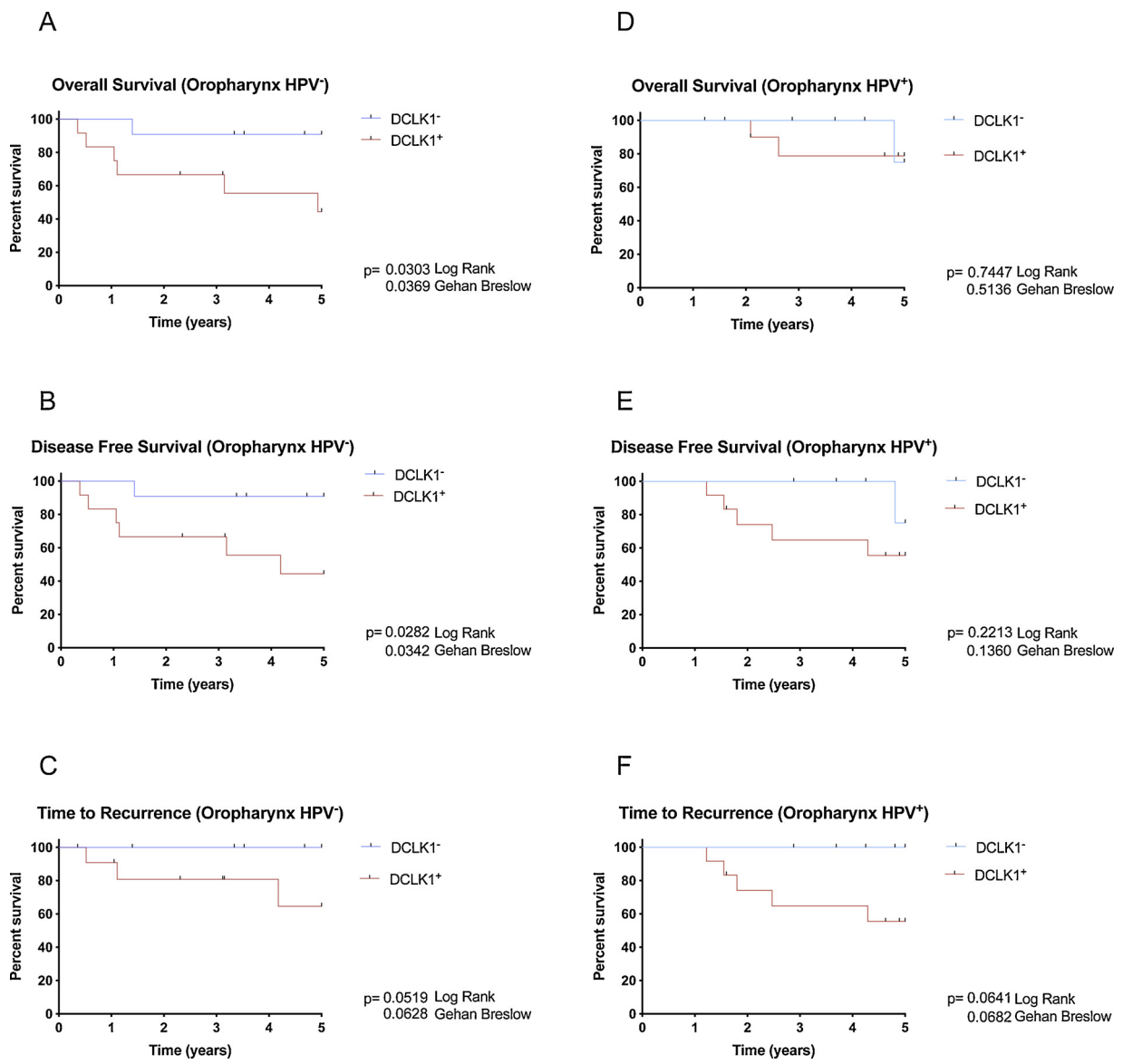

Fig. 3. Evaluation of the effects of DCLK1 in HPV(+) and HPV(-) oropharyngeal carcinoma patients. (A–C) show Kaplan-Meier survival curves for HPV(-) patients and (D–F) represent HPV(+) patients.

to evaluate these patient groups in the context of DCLK1.

However, in patients with HPV(-) OPSCC, we observed significantly shorter OS and DFS. In HPV(+) OPSCC, we did not find significant differences despite shorter DFS and TTR rates, because of the underpowered study population. To date, no study that has analyzed the molecular effects of DCLK1 in the context of HPV in HNSCC cell lines. We hypothesize that DCLK1's effect on survival is less prominent in HPV(+) patients because of the comparably better prognosis in this HNSCC subgroup. Moreover, CSC in HPV(+) OPSCC appeared to show better response rates to radiotherapy. This finding might explain the lesser effects of DCLK1 on outcome in our HPV(+) cohort than our HPV (-) cohort, because all of our patients were postoperatively treated with irradiation.

Because DCLK1 is known as marker for cells with stemness like potential [31], and CSCs are able to repopulate tumors after treatment, we conclude that DCLK1 expression in lymph node metastases might contribute to the development of recurrent disease. However, this conclusion is mainly limited to HPV(-) OPSCC patients, because of the small patient numbers with primary tumors in other sub-sites. We therefore recommend further prospective studies to assess DCLK1 expression in lymph node metastases of HNSCC patients.

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Declaration of Competing Interest

Lukas Kenner is supported by FWF, P26011, the Genome Research-Austria project 'Inflammobiota' grants as well as the MCEU-ITN ALK-ATRAS Network, No 675712. Neither project has a financial or personal relationship with this study. All other authors as listed below state that they have no conflicts of interest. This study obtained no third party

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