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Role of cancer stem-cell marker doublecortin-like kinase 1 in head and neck squamous cell carcinoma

Lorenz Kadletz [a], Dietmar Thurnher [b], Robert Wiebringhaus [c], Boban M. Erovic [a], Ulana Kotowski [a], Sven Schneider [a], Rainer Schmid [d], Lukas Kenner [c,e,f], Gregor Heiduschka [a,*]

[a] Department of Otorhinolaryngology, Head and Neck Surgery, Medical University of Vienna, Vienna, Austria
[b] Department of Otorhinolaryngology, Head and Neck Surgery, Medical University of Graz, Graz, Austria
[c] Department of Pathology, Medical University of Vienna, Vienna, Austria
[d] Department of Radiotherapy and – Oncology, Medical University of Vienna, Austria
[e] Ludwig Boltzmann Institute for Cancer Research, Vienna, Austria
[f] Institute of Laboratory Animal Pathology, University of Veterinary Medicine Vienna, Vienna, Austria

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ABSTRACT

Background: So far, no data is available on the role of the tumor stem cell marker doublecortin-like kinase 1 (DCLK1) in head and neck squamous cell carcinoma (HNSCC). The purpose of this study was to evaluate DCLK1 expression in HNSCC patients that underwent surgery and postoperative radiotherapy, and to assess its potential as a therapeutic target in vitro.

Methods: We immunohistochemically stained for DCLK1 in 127 sections of HNSCC samples obtained during surgery of HNSCC patients and correlated the expression to patients' overall- and disease-free survival, as well as human papilloma virus (HPV) status. Additionally, we compared our survival data with data obtained from The Cancer Genome Atlas (TCGA). The effects of the DCLK1 inhibitor LRRK-2-in-1 on HNSCC cell lines alone and in combination with irradiation.

Results: Expression of DCLK1 in 127 patients was associated with poor survival. In particular, DCLK1 expression had a significant impact on survival of oropharyngeal carcinoma patients. Specifically, $DCLK1^{+}/HPV^{-}$ patients had the worst prognosis after simultaneous assessment of DCLK1 and HPV status in comparison to the other three possible DCLK1/HPV constellations. Higher levels of DCLK1 mRNA were also associated with poor clinical outcome. Inhibition of DCLK1 in our HNSCC cell lines led to growth arrest and induction of apoptosis. The combination of DCLK1 inhibition with irradiation had a synergistic effect.

Conclusion: Firstly, DCLK1 is a prognostic biomarker for shortened survival. Secondly, through inhibition of DCLK1, it may serve as a therapeutic target as well.

Introduction

Current global cancer statistics place HNSCC as the sixth most common malignant disease worldwide [1]. Indeed, approximately 500,000 patients are diagnosed annually [1]. Generally, there are two different treatment options for advanced stage HNSCC: either surgical resection followed by irradiation or primary chemoradiotherapy. Despite the plethora of clinical studies in HNSCC, the prognosis has not improved substantially enough over the last decades [2,3]. To improve the prognosis, there is an urgent need to find novel markers to stratify patients, and to thereby identify high-risk patients requiring more specialized treatment. In recent years, the HPV has emerged as a clinically used marker in HNSCC [4].

There is an increasing number of studies that substantiate the concept of cancer stem cells and their influence on the resistance to irradiation and chemotherapy [5,6]. Unlike mature cancer cells, of which a tumor mostly consists of, the comparably small number of CSCs have an unlimited proliferative potential but also lower cell division rates [7]. CSCs are able to repopulate tumors and by reducing reactive oxygen species, CSCs seem to limit the effect of

Abbreviations: CSC, cancer stem cells; DCLK1, doublecortin-like kinase 1; DMSO, dimethyl sulfoxide; DFS, disease-free survival; HNSCC, head and neck squamous cell carcinoma; HPSCC, hypopharyngeal squamous cell carcinoma; HPV, human papilloma virus; LRRK-2-in-1, leucine rich repeat kinase inhibitor 1; LSCC, laryngeal squamous cell carcinoma; OSCC, oral squamous cell carcinoma; OPSCC, oropharyngeal squamous cell carcinoma; OS, overall survival.

* Corresponding author at: Waehringer Guertel 18-20, A-1090 Vienna, Austria.
E-mail address: gregor.heiduschka@meduniwien.ac.at (G. Heiduschka).

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radiotherapy itself [8]. In HNSCC, CSC markers such as CD44, CD10, CD133, ALDH1 and ABCG2 have been studied and found to indicate poor prognosis [9,10].

DCLK1 belongs to the family of calmodulin-dependent kinases [11]. It binds to microtubules and is involved in the regulation of microtubule polymerization [12]. DCLK1 was first described in the field of neurobiology [12]. Lately, DCLK1 has gathered attention in oncologic research as a CSC marker. In preinvasive pancreatic cancer, cells with stem cell-like characteristics were also positive for DCLK1 [13]. An expression of DCLK1 is observed in pancreatic cancer, clear renal-cell carcinoma and colorectal carcinoma [13–15]. Moreover, in patients with esophageal and pancreatic adenocarcinoma, DCLK1 is detectable in the plasma [16,17].

The siRNA-mediated knockdown of DCLK1 results in growth inhibition of colon and pancreatic tumor xenografts and in neuroblastoma cells also indicating an oncogenic role of DCLK1 [18–21].

Beside siRNA, LRRK-2-in-1 was initially developed for patients with Parkinson's disease [22]. As described by Weygant and coworkers, LRRK-2-in-1 is an ATP-competitive inhibitor of DCLK1 that shows potent inhibition of cell proliferation and induction of apoptosis in colorectal and pancreatic cancer cell lines [23].

So far, there is no data available on DCLK1 in HNSCC. In this study we examined for the first time the expression of DCLK1 in samples from HNSCC patients using immunohistochemistry. DCLK1 expression and HPV status were assessed and correlated to patients' data to evaluate a possible link to overall and disease-free survival. Finally, we analyzed the effect of the small molecule LRRK-2-in-1 in HNSCC cell lines and investigated a combinatorial treatment with irradiation.

Materials and methods

Patients

In this study we included 127 patients with head and neck cancer who were treated with surgery and adjuvant irradiation at the Medical University of Vienna in the period between 2002 and 2012. External treatment, secondary primary carcinoma, previous irradiation were seen as exclusion criteria for this study. All patients were presented to the Head and Neck Tumor Board of the Medical University of Vienna and underwent multidisciplinary discussion. The Institutional Ethics Committee approved this study (612/2009). Patients with extra capsular spread and R1 resections at the final histopathological work up were additionally treated with chemotherapy. In situ hybridization was performed in order to evaluate a possible HPV infection as described previously [24,25]. In brief, in situ hybridization was carried out on 4 μm sections of representative tumor regions. DAKO GENEPOINT system for HPV DNA 6/11 and 16/18 (DAKO, Carpinteria, CA USA) was used to determine HPV status. Additionally p16 expression was assessed immunohistochemically in accordance to a previously described protocol [24]. These data were compared with HPV in situ hybridization results.

Tissue microarray and immunohistochemistry

Samples were obtained from previously evaluated HNSCC paraffin-embedded specimens that were gained from surgical resection specimens prior to radio- or chemotherapy via a Galileo TMA CK Series-HTS Tissue computer assisted Microarray Platform (Integrated Systems Engineering Srl, Milan, Italy).

The appropriate antibody dilution and retrieval buffer (EDTA) were determined in advance using gastric and clear renal cell-carcinoma sections. These sections served as a positive control as well since both of them are reported to express DCLK1. Moreover, the expression of DCLK1 was analyzed in healthy oral mucosa of 10 patients without HNSCC. In addition, we evaluated samples from healthy tissue of the oropharynx (n = 5). The protocol has been described previously [26].

The samples were categorized into two groups: (i) no expression and (ii) expression of DCLK1. Furthermore, DCLK1-expressing samples were subdivided into three groups (weak, moderate and strong expression). Two independent investigators examined every single microsection (LK and RW). Four different categories were defined based on the percentage of stained cells and staining intensity. The samples were categorized into four groups: (i) no or weak intensity in <10% of neoplastic cells, (ii) weak intensity, focal expression of DCLK1 (in 10–40% of neoplastic cells), (iii) moderate expression of DCLK1 in 40–80% and (iv) strong expression in >80% of neoplastic cells.

Analysis of The Cancer Genome Atlas (TCGA) patient data

In order to evaluate the effects of DCLK1 on survival by mRNA levels of DCLK1 we analyzed the TCGA head and neck dataset (Head and neck squamous cell carcinoma Nature 2015, 279 samples). Therefore, we downloaded data from cBioPortal for Cancer Genomics. [27,28]

Cells and reagents

Three different HNSCC cell lines, SCC25, CAL27 and FaDu, were purchased either from the American Type Culture Collection (SCC25 and CAL27, ATCC, Manassas, VA, USA) or the German Collection of Microorganisms and Cell Cultures (FaDu, DMSZ, Braunschweig, Germany). RPMI Medium and Fetal Calf Serum were obtained from Gibco (Grand Island, NY, USA). Cells were grown as monolayers at 37 °C and 5% CO_2 and 1% of penicillin/streptomycin (PAA, Pasching, Austria) was added. LRRK-2-in-1 (Calbiochem, Billerica, MA, USA) was solved in dimethyl sulfoxide (DMSO), divided into aliquots and stored at −20 °C. Spheroids were grown as described before [29].

Moreover, multicellular spheroids were evaluated. Therefore, after 5 days of growth, multicellular spheroids were fixed in 8% formaldehyde solution for 30 min and consecutively casted with 4% agarose gel and stored in phosphate buffered saline (PBS) at 7 °C until parraffinization. Ultimately, sections of 2–3 μm thicknesses were created and stained according to the aforementioned protocol.

Irradiation

Cell lines were irradiated at a dose of 1.7 Gy/min at a fixed-focus object distance of 52 cm. A 150 kV X-ray machine (Gulmay D3300, Gulmay Medical Ltd., Byfleet, UK) served as a radiation source. Thermoluminescence dosimetry was performed ahead of all experiments to measure the irradiation dose for the following experiments.

Cytotoxicity

In order to assess cell proliferation, 3×10^3 cells were seeded into 96-well plates. Cells were allowed to rest for 24 h and subsequently exposed to 100 μl of LRRK-2-in-1 containing medium for 72 h. Concentrations of LRRK-2-in-1 ranged from 1.25 μM to 20 μM. DMSO-treated cells at corresponding concentrations served as a control group. For combination experiments, cells were also exposed to different doses of irradiation, ranging from 2 to 8 Gy. Subsequently, growth inhibition was evaluated using a Cell Counting Kit -8 (Dojindo Laboratories, Kumamoto, Japan). Combination Index (CI) blots according to the Chou-Talalay method were