Int. J. Oral Maxillofac. Surg. 2017; 46: 1205–1219
http://dx.doi.org/10.1016/j.ijom.2017.03.035, available online at http://www.sciencedirect.com

International Journal of Oral & Maxillofacial Surgery

Clinical Paper
Head and Neck Oncology

Cytoplasmic neuropilin 2 is associated with metastasis and a poor prognosis in early tongue cancer patients

H. S. Ong[1], S. Gokavarapu[2], Q. Xu[3], Z. Tian[4], J. Li[4], T. Ji[1], C. P. Zhang[1]
[1]Department of Oral and Maxillofacial–Head and Neck Oncology, Shanghai Ninth People's Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai, China; [2]Head and Neck Oncology Reconstructive Surgery, Department of Surgical Oncology, Krishna Institute of Medical Science, Hyderabad, Telangana, India; [3]Shanghai Key Laboratory of Oral and Maxillofacial–Head and Neck Oncology and Shanghai Research Institute of Stomatology, Shanghai Ninth People's Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai, China; [4]Department of Oral Pathology, Shanghai Ninth People's Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai, China

H. S. Ong, S. Gokavarapu, Q. Xu, Z. Tian, J. Li, T. Ji, C. P. Zhang: Cytoplasmic neuropilin 2 is associated with metastasis and a poor prognosis in early tongue cancer patients. Int. J. Oral Maxillofac. Surg. 2017; 46: 1205–1219.

Abstract. Neuropilin 2 (Nrp2) plays an important role in regulating lymphangiogenesis. Nrp2 expression in early tongue cancer was investigated to predict lymph node metastasis and the long-term prognosis. The relationships between clinicopathological variables of cT1–T2N0 tongue squamous cell carcinoma (SCC) and overexpression of Nrp2, vascular endothelial growth factor C (VEGFC), vascular endothelial growth factor receptor 3 (VEGFR3), and semaphorin 3F (Sema3F) were analyzed. Expression levels were compared using oral SCC cell lines. The *Nrp2* gene was silenced to determine the impact of Nrp2. Cytoplasmic Nrp2 overexpression predicted regional metastasis with sensitivity and specificity of 90.3% and 42.1%, respectively. Cytoplasmic Nrp2 overexpression ($P < 0.001$) and VEGFC overexpression ($P = 0.006$) were significantly related to regional metastasis (Student *t*-test). However, only cytoplasmic Nrp2 overexpression was an independent prognostic factor for both disease-free survival (DFS; $P = 0.008$) and overall survival (OS; $P = 0.016$) (Cox regression); the risk of recurrence was 12-times higher ($P = 0.015$) and risk of mortality was 8-times higher ($P = 0.016$). Co-localization of Nrp2 and VEGFC was greater within the cytoplasm of aggressive cell lines (HN12 and RCa-T). Nrp2 plays a role in tumourigenesis; VEGFC supplementation cannot rescue the biological function of Nrp2 in Nrp2-depleted cell lines. Cytoplasmic Nrp2 overexpression is associated with decreased OS and DFS. Cytoplasmic Nrp2 overexpression may be a reliable diagnostic and prognostic marker for early tongue SCC.

Key words: neuropilin; VEGFC; head neck neoplasm; metastasis; neck dissection; tumourigenesis.

Accepted for publication 28 March 2017
Available online 9 June 2017

Tongue and floor of the mouth cancers together comprise about 50% of oral squamous cell carcinomas (OSCC)[1]. Relative to the other sites of the oral cavity, tongue cancers are associated with a high rate of occult regional lymph node metastasis. This rate has been reported to be as high as 34% on final histopathology, which is almost double that of other oral primary sites[2]. Additionally, false-positive results by radiological evaluation are frequent[3], even with more advanced imaging methods such

as positron emission tomography–computed tomography (PET-CT), and the identification of occult lymph node metastasis preoperatively is difficult[4].

The presence of regional lymph node metastasis is a reliable indicator of the prognosis in oral cancer patients[5–7], and the appropriate staging of early cancers is very important in determining whether neck dissection should be performed in such patients.

Research on the metastatic potential of tumours has demonstrated that tumour proliferation, migration, and invasion promotes the dissemination of the tumour cells[8]. Vascular endothelial growth factor receptor 3 (VEGFR3) is the receptor for vascular endothelial growth factor C (VEGFC). Molecular studies have emphasized that VEGFC–VEGFR3 binding promotes the lymphatic dissemination of tumour cells[9]. VEGFC is known to bind to at least two receptor families: VEGFR2/VEGFR3 and neuropilin 2 (Nrp2)[10]. The role of Nrp2 expression in the lymphatic system has been shown in embryological studies, which have revealed a striking deficiency of lymphatic capillaries and retardation in lymphatic development in homozygous Nrp2 mutants[11]. This work helps to explain the interaction between the VEGF family and Nrp2 in the promotion of lymphangiogenesis. Interestingly, neuropilins (Nrps) are often the only receptor expressed by tumour cells when VEGFR is missing[11–14].

Nrp2 is a non-tyrosine kinase glycoprotein shown to act as a receptor for members of the VEGF family and semaphorin 3F (Sema3F)[10,12,15]. When Nrp2 binds to VEGFC/VEGFR3, VEGFR is phosphorylated, which subsequently promotes tumour lymphangiogenesis via the extracellular signal-regulated kinase (ErK) or phosphatidylinositol 3-kinase (PI3K) pathway[15]. On the other hand, Sema3F has an inhibitory effect on VEGF. These studies suggest that Nrp2, VEGFR3, VEGFC, and Sema3F may have prognostic value.

Such a prognostic factor may be particularly useful in OSCC. Many of these cancers present as N0 cancers without overt metastatic disease, and a molecular marker to detect the probability of regional lymph node metastasis would be useful to decide on the need for elective neck dissection. Tongue cancers are notorious for occult lymph node metastasis in clinically/radiologically negative patients. In fact, regional relapse has been reported in 30–40% of cN0 tongue cancers when prophylactic neck dissection was deferred[4].

The possibility of distant metastasis and death due to regional failure has been debated by proponents of elective neck dissection, and it has been reported that the 5-year survival rate is decreased to <20% in such patients[16]. However, Fakih et al. have pointed out that an elective neck dissection in early oral cancer will increase the rate of metastasis to the contralateral neck[17]. This theory is based on the idea that disruption of the normal lymphatic pathways will result in a diversion of lymphatics towards the alternate lymph node basins and cause metastasis to the contralateral neck.

Other methods (e.g., sentinel lymph node biopsy) have produced inconsistent results in this setting[18]. Nevertheless, a predictor of metastasis would allow improved prognostication of tumours and the delivery of a customized treatment plan for patients with no clinical or radiological evidence of metastases. In the current study, the roles of Nrp2, VEGFR3, VEGFC, and Sema3F were evaluated in patients with early tongue cancer (cT1–T2N0). Additionally, the significance of Nrp2, VEGFR3, VEGFC, and Sema3F expression in OSCC cell lines with different biological behaviours was investigated. Finally, the effect of knocking down Nrp2 on the cellular behaviour of OSCC cell lines was also assessed.

Methods

Patient selection, clinical review, and follow-up

The institutional review board of the study institution approved this study. Informed consent was obtained from all patients and the study was performed in accordance with the Declaration of Helsinki.

A prospective study on patients with clinical early stage tongue cancer (cT1–T2N0) was conducted at the institution in 2009. The end-point for inclusion was December 2012, to ensure that all subjects could be followed up for more than 2 years. This study had a single inclusion criterion: the cohort was limited to patients with primary cT1–T2N0 disease to ensure the clinical applicability of this research. Therefore, all patients underwent a thorough clinical evaluation, ultrasound (US) examination, and radiological imaging (CT) to ensure that only patients with a preoperative cN0 status were included.

Patients with enlarged neck nodes on clinical examination and radiological evidence of frank metastatic disease on CT or ultrasonography were excluded. Additionally, only patients with primary tongue squamous cell carcinoma (SCC) were included in the study.

Patients with radiological imaging revealing tongue cancer extending into the tongue base, floor of the mouth, or other sub-sites were excluded. Furthermore, patients with a history of previous head and neck cancer in the past 5 years and patients who had received radiation or chemotherapy were excluded. Patient consent was obtained before they were considered eligible to be enrolled in this study. All patient data were managed with confidentiality.

A total of 100 consecutive cN0 tongue cancer patients were randomized in a double-blinded manner into a prophylactic neck dissection arm and a watchful waiting arm (50 patients in each arm). For those in the prophylactic neck dissection arm, subjects underwent primary radical tongue cancer resection with simultaneous selective neck dissection of levels I to III. For those in the watchful waiting arm, patients underwent transoral resection and primary closure without interruption of the neck.

All patients were followed up closely to confirm disease status (progression) or death. All patients were followed up every 2 months in the first 2 years, every 3 months in the third year, and every 6 months in the subsequent years. A clinical examination and neck US was performed routinely at every follow-up visit; CT was performed routinely every 6 months, or whenever the clinician suspected a relapse. A biopsy and additional magnetic resonance imaging (MRI) scan was ordered on clinical suspicion of recurrence and patients were treated accordingly without delay.

The primary pathology slides were reviewed by a panel of pathologists to determine the histological grading, perineural invasion status, lymph node metastasis status, and immunohistochemical score. Additionally, histological slides of the primary lesion and metastatic lymph node with the appropriate tumour were selected for immunochemistry staining.

For the purpose of this study, regional metastasis was defined as either occult lymph node metastasis (i.e., a positive node at the time of pathological examination) after prophylactic neck dissection, or the occurrence of regional lymph node metastasis without local relapse during the first 12 months of follow-up. Patients fulfilling the above regional metastasis criteria were considered as pN+.

Immunochemistry

Histological sections (4 μm thick) were obtained and stained with anti-Nrp2 (1:300; Abcam, Cambridge, MA, USA),