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RON (recepteur d'origine nantais) expression and its association with tumor progression in laryngeal squamous cell carcinoma

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ABSTRACT

Objectives: Several human tumor tissues show an aberrant expression and activation of recepteur d'origine nantais (RON). In this paper, we investigate the expression of RON in human laryngeal squamous cell carcinoma (SCC) and evaluate whether RON affects the tumor cell progression in human laryngeal SCC cell line.
Methods: Immunohistochemistry was used to assess RON expression in human laryngeal SCC. To evaluate the impact of RON knockdown, the cell invasion assay and cell migration assay using small-interfering RNA were performed.
Results: The expression of RON protein was dominantly observed in laryngeal SCC tissues relative to adjacent normal mucosa in all cases. Knockdown of RON resulted in significantly reduced cell invasion in human laryngeal SCC cells. Cell migration showed a marked decrease in RON knockdown laryngeal SCC cells compared to the negative control laryngeal SCC cells. Laryngeal SCC cell migration was enhanced by incubation with macrophage stimulating protein (MSP).
Conclusion: RON is highly expressed in human laryngeal SCC. We suggest that RON plays an important role in invasion, and metastasis of laryngeal SCC.

1. Introduction

Laryngeal squamous cell carcinomas (SCCs) represent the vast majority (approximately 96%) of laryngeal malignancies [1]. In the United States, laryngeal SCC is estimated to account for almost 1% of all new cases of malignancy, with an incidence of about 10,000 cases per year, and causes 0.7% of cancer deaths in 2009 [2]. In accordance with the American Cancer Society data, a 5-year disease-specific survival (DSS) for laryngeal cancer is about 65% [2]. Despite the evident technical, technological, and methodological advances of head and neck oncology over the past 30 years, the prognosis of laryngeal SCC have not shown a satisfactory improvement [3]. In addition, advanced stage is associated with a higher rate of relapse and disease-related death, and the number of advanced (especially stage IV) cases at diagnosis seem to be increasing [1]. For this reason, the improvement of survival in advanced cases (stage III and IV) would have the most decisive impact on the overall prognosis of laryngeal SCC. Thus, in order to improve survival rate in patients with laryngeal SCC, particularly patients with advanced stage, a new treatment modalities as the molecular target therapy are clearly needed.

Receptor tyrosine kinases (RTKs) mediate numerous cellular processes known to be critical to cancer cell proliferation, invasiveness, apoptotic resistance, and metastasis [4–6]. Recently, members of the MET proto-oncogene family, a subfamily of RTKs, have drawn special attention due to the association between invasion and metastasis [7]. The MET family, including MET and the recepteur d'origine nantais (RON), can function as oncogenes, like most tyrosine kinases [8]. Macrophage stimulating protein (MSP) is the only ligand identified for RON [6]. Upon ligand binding, RON is activated and mediates multiple signaling cascades involved in cell motility, adhesion, proliferation, and survival, including the Ras/mitogen-activated protein kinase (MAPK),

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phosphatidylinostiol-3 kinase (PI3K)/Akt, β-catenin, and nuclear factor-kappaB (NF-κB) [9–12]. Several human tumor tissues show the aberrant expression and activation of RON, including tumors of the breast, colon, prostate, ovary, pancreas, and thyroid [13–18]. Recently, we revealed that RON expression play an important role in more aggressive tumor behavior such as tumor invasion, metastasis, and tumorigenicity in hypopharyngeal SCCs [19]. However, there are no reports on the role of RON in laryngeal SCC.

Determining the presence of RON and its function in laryngeal SCCs may identify an important mediator of the aggressive behavior of laryngeal SCCs and a potential target for molecular therapy. This current study is the first report to demonstrate the RON expression and its association with tumor progression in laryngeal SCC.

2. Materials and methods

2.1. Patients and tumor specimens

Clinical data and paraffin tissue sections were collected from 28 patients who underwent diagnostic biopsy for advanced laryngeal SCC (stage III and IV). In our Institution, Chonnam National University Hwasun Hospital (Jeonnam, Korea), most of patients with advanced laryngeal SCC except patients with major laryngeal cartilage invasion or compromised airway had been treated with neoadjuvant chemotherapy followed by concurrent chemoradiation. In order to decrease the influence according to variable treatment strategies, patients who were treated with the same chemoradiation regimen were included. Between July 2004 and February 2009, 37 consecutive patients were newly diagnosed with advanced laryngeal SCC (stage III or IV). Among these patients, 28 were included in this study. Nine patients excluded with radiation alone therapy, follow-up loss, or loss of paraffin tissue section. All 28 patients were treated with 3 cycles of docetaxel, cisplatin, and 5-fluorouracil-based neoadjuvant chemotherapy (NAC). It was followed by cisplatin-based concurrent chemoradiation therapy (CCRT) in 25 patients with complete response (CR) or partial response (PR) after NAC and one patient who refused salvage total laryngectomy despite of stable disease (SD) after NAC. Other two patients with SD after NAC underwent salvage total laryngectomy.

Patient's clinico-pathologic characteristics, including age, sex, tumor location, stage, tumor invasion, lymph node metastasis, distant metastasis, tumor response after treatment, survival, and follow-up information were all obtained from hospital records. Patients' characteristics are summarized in Table 1. The group of patients selected for this study includes 28 men. The average age was 66.3 ± 7.2 years (mean ±standard deviation), ranging from 49 to 77 years. The mean follow-up period was 42.7 months, ranging from 8.1 to 92.4 months. All patients were staged again according to the seventh edition of the American Joint Committee on Cancer (AJCC) staging system [20]. Response to therapy was assessed by physical examination, laryngoscopy, and computed tomography. A CR was defined as no visible or palpable disease. A PR was defined as a >50% decrease in tumor size, compared with the initial measurement. SD was defined as stationary or progressive disease. Overall survival was calculated from the start date of chemotherapy to the date of death or date last seen. This study was approved by the Institutional Review Board of Chonnam National University Hwasun Hospital.

Table 1
Clinicopathological variables of 28 patients with laryngeal SCC.

Variables	Value
Age (yr): mean ±SD (range)	66.3 ± 7.2 (49–77)
Sex (male:female)	28:0
Location (TVC:FVC:Arytenoid, AEF:Epiglottis)	2:6:5:15
T stage (T1:T2:T3:T4)	1:8:16:3
N stage (N0:N1:N2:N3)	12:5:11:0
Distant metastasis (M0:M1)	23:5
Tumor response after NAC (CR,PR:SD)	25:3
Overall tumor response after CRT (CR:PR, SD)	20:6
Recurrence (negative: positive)	14:6
5** year overall survival (%)	40
RON expression	
Low (%)	13 (46.4%)
High (%)	15 (53.6%)

TVC = true vocal cord; FVC = false vocal cord; AEF = aryepiglottic fold; NAC = neoadjuvant chemotherapy; CR = complete response; PR = partial response; SD = stable disease; CRT = chemoradiation.

2.2. Immunohistochemistry

An horseradish peroxidase (HRP)/3,3′-diaminobenzidine (DAB) immunohistochemical staining method was used on formalin-fixed paraffin-embedded tissues. Tissue sections of 5 μm were cut from each paraffin block, and then mounted and dried on glass slides. Tissues were deparaffinized using xylene, then rehydrated in graded alcohol solutions and retrieved with retrieval buffer. Endogenous peroxidase activity was blocked with peroxidase-blocking solution (Dako, Carpinteria, CA, USA), followed by incubation with polyclonal rabbit anti-mouse RON (Invitrogen, Carlsbad, CA, USA) overnight at 4 °C. After washing in TBS-Tween 20 buffer (TBST), tissues were stained using the DakoReal™ Envision HRP/DAB detection system (Dako). Tissues were counter-stained with hematoxylin and mounted with coverslips. Stained tissues were viewed and photographed using a light microscope.

Assessment of staining was given to two observers, who do not have knowledge of clinical information, for interpretation. Staining intensity was scored from 0 (no staining) to 3+ (strong staining). The percentage of immunostaining area was graded on a scale with fifth grades: no staining, 0; <25%, 1; 25–49%, 2; 50–74%, 3; ≥75%, 4. A staining index was calculated as the multiplication of staining intensity and staining area. Tumors were categorized as high RON expression (staining index ≥6) or low expression (staining index <6).

2.3. Cell culture and Transfection

Human laryngeal SCC cell line, PCI 1 were kindly provided by Dr. Sung MW (Seoul National University, Seoul, South Korea). Cell lines were cultured in RPMI1640 (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 50 units/ml penicillin, and 50 μg/ml streptomycin (Gibco, Grand Island, NY, USA) in a humidified atmosphere of 5% CO_2 at 37 °C. For transfection, cells were prepared and maintained in culture dishes with media and cells were seeded on 6-well plates at 2×10^5 cells per well at the time of transfection. RON-specific small interfering RNA (siRNA) (Santa Cruz biotechnology, Santa Cruz, CA, USA) and negative control siRNA (Quiagen, MD, USA) were transfected with Lipofectamine™ 2000 (Invitrogen) into the cells. RON knockdown was identified by RT-PCR and Western blotting.

2.4. Cell invasion assay

Cell invasion was measured using a transwell invasion apparatus (Costar Inc., Cambridge, United Kingdom). Transwell filters (8.0 μm pores) were coated with 1% gelatin solution on both the top and bottom surfaces and dried at room temperature. Cells transfected with each siRNA (RON siRNA, negative control siRNA) were seeded at 2×10^5 cells in 120 μl 0.2% bovine serum albumin (BSA) medium in the upper chamber. Subsequently, 400 μl 0.2% BSA medium containing 100 ng/ml MSP (R&D system, Minneapolis, MN, USA, Calbiochem, LaJolla, CA, USA), as the chemoattractant,