High expression levels of Wnt5a and Ror2 in laryngeal squamous cell carcinoma are associated with poor prognosis

WEI ZHANG1*, YONGBING YAN1*, MIAO GU1, XUDONG WANG2, HUIJUN ZHU3, SHU ZHANG3 and WEI WANG3

Departments of 1Otorhinolaryngology, 2Surgical Comprehensive Laboratory and 3Clinical Pathology, Affiliated Hospital of Nantong University, Nantong, Jiangsu 226001, P.R. China

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Abstract. The present study investigated the prognostic significance of Wnt family member 5a (Wnt5a) and receptor tyrosine kinase-like orphan receptor 2 (Ror2) expression in laryngeal squamous cell carcinoma (LSCC). The protein expression levels of Wnt5a and Ror2 were analyzed in specimens from 137 patients with LSCC, using immunohistochemical staining of tissue microarrays and pairs of LSCC and adjacent tissue samples, and examined the associations between the two markers and various clinicopathological parameters. The Wnt5a and Ror2 expression levels were significantly higher in LSCC tissues than in normal tissue samples (Wnt5a, P=0.015; Ror2, P=0.039), and were significantly associated with high tumor stage (P<0.001), lymph node metastasis (Wnt5a, P=0.029; Ror2, P=0.018), and with each other (P=0.002). Patients with LSCC with high Wnt5a or Ror2 expression had poorer prognosis compared with those with low Wnt5a (P=0.022) or Ror2 (P=0.038) expression. Thus, Wnt5a and Ror2 may affect LSCC development, and are potential biomarkers in LSCC.

Introduction

Laryngeal squamous cell carcinoma (LSCC) is a common type of head and neck malignancy, and is also the second most common malignancy of the respiratory tract (1,2). The prognosis of LSCC is associated with the primary site, clinical stage, pathological differentiation and lymph node metastasis (3). Despite advancements in the available treatments for LSCC, its survival rate has not improved significantly. Therefore, the identification of cancer-associated biomarkers is warranted to diagnose LSCC and improve its prognosis.

Wnt proteins, a large family of cysteine-rich, secreted molecules, function by activating various intracellular signaling pathways (4). Wnt interactions are classified as belonging to the canonical or non-canonical pathways (4,5). The canonical pathway is β-catenin-dependent (4), whereas the non-canonical pathway is β-catenin-independent, and includes planar cell polarity (PCP) and Wnt/Ca2+ signaling pathways (5). Mutations of the canonical Wnt signaling pathway have been demonstrated to be associated with head and neck carcinogenesis, particularly in oral cancer (7). However, the role of the Wnt signaling pathway specifically in LSCC remains unclear.

Wnt family member 5a (Wnt5a) is a Wnt ligand that affects almost all aspects of the non-canonical pathway (8-10). Wnt5a has an important role in the progression of malignancies; for example, in head and neck squamous cell carcinoma (HNSCC), it has been reported that Wnt5a is highly expressed and may act as a tumor promoter (11,12). However, its role varies in different types of cancer, and its function is significantly altered by its receptors (13-15). Receptor tyrosine kinase-like orphan receptor 2 (Ror2) mediates the Wnt5a-dependent non-canonical pathway, and is required for Wnt5a-mediated inhibition of the canonical pathway (16,17).

Ror2, an orphan tyrosine kinase, is characterized by intracellular tyrosine kinase domains (related to those of the Trk-family receptor tyrosine kinases), extracellular Frizzled (Fz)-like cysteine-rich domains (CRD), and membrane-proximal Kringle domains (18,19). Ror2 affects diverse cellular processes, including the cell cycle, cell proliferation, differentiation and migration (18). As the receptor for Wnt5a, Ror2 mediates the PCP pathway and inhibits the β-catenin/T-cell factor (TCF) pathway (16). Ror2 is reportedly overexpressed in renal cell carcinoma, squamous cell carcinoma and metastatic melanoma (20-22), but downregulated in hepatocellular carcinoma, medulloblastoma, and colon cancer (23-25). Accordingly, as with Wnt5a, the functions of Ror2 vary depending on the cancer type and signaling pathway.

To the best of our knowledge, the roles of Wnt5a/Ror2 in the non-canonical pathway in LSCC have not been previously investigated. Thus in the present study, the expression and

Correspondence to: Professor Miao Gu, Department of Otorhinolaryngology, Affiliated Hospital of Nantong University, 20 Xisi Road, Nantong, Jiangsu 226001, P.R. China
E-mail: 13862932120@163.com

*Contributed equally

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clinical significance of Wnt5a and Ror2 were investigated in LSCC.

Materials and methods

Patient selection and data collection. A total of 137 LSCC tissue samples and 28 adjacent non-tumor tissues were collected from a cohort of laryngeal carcinoma patients following surgical excision at the Department of Otolaryngology, Head and Neck Surgery, Affiliated Hospital of Nantong University (Nantong, China) between January 2005 and December 2013. The patients included 130 males and 7 females aged 46-80 years old (mean age, 66.8 years). All patients were diagnosed with LSCC according to the World Health Organization criteria (26) and Tumor Node Metastasis (TNM) classification (Union for International Cancer Control, 2009). Patients underwent laryngectomy and neck dissection (unilateral or bilateral, radical or functional, based on clinical and surgical findings) at the Affiliated Hospital of Nantong University (Nantong, China). No patients had received radiation therapy, chemotherapy or immunotherapy prior to surgery. The present study was approved by the Ethics Committee of the Affiliated Hospital of Nantong University and written informed consent was obtained from all patients.

Tissue microarray (TMA). In the current study, 137 formalin-fixed paraffin-embedded LSCC and 28 tumor-adjacent normal tissue samples were prepared as TMAs, which were generated using a manual Tissue Micro-arrayer Quick-Ray system (UT06; Unitma Co., Ltd., Seoul, Korea). A total of three TMAs were produced, whereby 4-μm sections were cut and placed on Superfrost charged glass microscope slides.

Immunohistochemistry (IHC). The TMA slides for IHC were deparaffinized and rehydrated through an alcohol gradient. Endogenous peroxidase activity was blocked through incubation of slides in 3% H2O2 at room temperature for 15 min. Antigen retrieval was performed by incubation with citrate buffer (pH 6.0) at 95°C for 15 min. Wnt5a expression was detected using a mouse monoclonal anti-human Wnt5a primary antibody (dilution, 1:200; cat no. ab110073; Abcam, Cambridge, UK), and Ror2 was detected using a rabbit polyclonal anti-human Ror2 primary antibody (dilution, 1:100; cat no. LS-C99125; Lifespan BioSciences, Inc., Seattle, WA, USA), which were incubated at 4°C overnight. Then reactions were detected with EnVision™ + Peroxidase kit (no. K165212F; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) at room temperature for 30 min. Sections were stained with 3,3’-diaminobenzidine (Dako; Agilent Technologies, Inc.), counterstained with hematoxylin, dehydrated through an alcohol gradient, cleared with xylene and cover-slipped with permanent mounting media using a previously described protocol (27,28).

Results were determined using a double-blind method by two investigators. The results were reevaluated by a third investigator when disagreement between the primary two investigators occurred, until a consensus was established. Expression levels of Wnt5a and Ror2 were assessed by observing the incidence and staining intensity of IHC-stained cells. The intensity of staining was scored as follows: 0, no staining; 1, weak positive; 2, moderate positive; and 3, strong positive. The final staining score was estimated by multiplying the staining intensity score by the staining area percentage, producing a score in the range of 0 (no staining) to 300 (100% of cells with strong staining), following a previously described method (29).

Statistical analysis. For statistical analysis, continuous Wnt5a and Ror2 expression data from LSCC were first converted into dichotomized data (low vs. high) using specific cutoff values, which were assessed with regard to their ability to predict overall survival (OS) using the X-tile software program (Rimm Lab, Yale University, New Haven, CT, USA; http://www.tissuarray.org/rimmlab) (29-31). Using the X-tile software program for TMA data analysis (http://www.tissuarray.org/rimmlab), we first identified significant cutoff point in terms of OS in LSCC. For Wnt5a, the cutoff 180 was selected: Score 0-180 was considered low expression, while 180-300 was considered high expression. For Ror2, the cutoff point 192 was selected: Score 0-192 was considered low expression, while 192-300 was considered high expression. For all subsequent analyses, Wnt5a and Ror2 protein expression levels were considered either as ‘low’ or ‘high’ using these cutoff values.

Statistical analysis was performed using SPSS software (version 20.0; IBM Corp., Armonk, NY, USA). Pearson’s χ² test was used to analyze differences in Wnt5a and Ror2 expression in LSCC and adjacent normal tissue samples, and the correlations between the two markers and clinicopathological parameters. Survival curves were calculated using the Kaplan-Meier estimator method and the log-rank test was used for statistical analysis. Univariate and multivariate analyses were performed using Cox’s regressions models. P<0.05 was considered to indicate a statistically significant difference.

Results

Wnt5a and Ror2 expression in LSCC tissue. Wnt5a and Ror2 protein were localized in the cytoplasm (Fig. 1). The frequencies of high Wnt5a expression (P=0.015) and high Ror2 expression (P=0.039) were significantly higher in LSCC tissues compared with those in tumor-adjacent normal tissue samples (Table I). Notably, high Wnt5a and Ror2 coexpression (Wnt5a*/Ror2*) was only detected in LSCC tissue, although not all LSCC samples exhibited coexpression of Wnt5a and Ror2.

Association between Wnt5a and Ror2 expression and clinicopathological characteristics in LSCC. In the present study, the associations between Wnt5a or Ror2 protein expression and clinical parameters among patients with LSCC were examined. High Wnt5a and high Ror2 expression levels were significantly associated with TNM stage (III/IV vs. I/II; both P<0.001) and lymph node metastasis (Wnt5a, P=0.029; Ror2, P=0.018; Table II). High Wnt5a*/Ror2* coexpression was significantly associated with high TNM stage (P<0.001). In addition, a significant and positive correlation between Wnt5a and Ror2 expression was detected (P=0.002; Table II).
Prognostic value of Wnt5a and Ror2 protein expression in LSCC.

Prognostic factors in LSCC were determined using univariate and multivariate analyses. High Wnt5a expression (P=0.022), high Ror2 expression (P=0.002) and high Wnt5a + Ror2 coexpression (P<0.001) were significantly associated with poor OS according to univariate analysis. In addition, sex (P=0.003), TNM stage (P=0.028), lymph node metastasis (P=0.013) and high histopathological grade (P=0.011) were significantly associated with poor OS in the univariate analysis. Following multivariate analysis, high Wnt5a [hazard ratio (HR), 2.540; 95% confidence interval (CI), 1.146-5.630; P=0.022] and high Ror2 (HR, 2.290; 95% CI, 1.049-5.000; P=0.038) expression remained significantly associated with poor OS (Table III). The Kaplan-Meier analyses revealed that patients in the high Wnt5a expression group (Wnt5a+) had significantly poorer prognosis compared with those in the low-expression group (Wnt5a−; P=0.020; Fig. 2A), and patients in the high Ror2 expression group (Ror2+) also had significantly poorer prognosis compared with those in the low-expression group (Ror2−; P=0.001; Fig. 2B). More importantly, patients in the high Wnt5a and high Ror2 expression level group (Wnt5a+/Ror2+) demonstrated significantly poorer prognosis compared with those in the high Wnt5a or high Ror2 expression level group (Wnt5a+/Ror2− or Wnt5a−/Ror2+) and those in the low-expression group (Wnt5a−/Ror2−; P=0.001; Fig. 2C).

Discussion

To the best of our knowledge, the present study is the first to investigate Wnt5a and Ror2 protein expression and their prognostic value in LSCC. The results demonstrated that Wnt5a and Ror2 protein expression levels are significantly higher in LSCC compared with in adjacent normal tissue samples. High
| Clinicopathological characteristic | Total no. | High expression, n (%) | χ² | P-value | High expression, n (%) | χ² | P-value | High expression, n (%) | χ² | P-value |
|-----------------------------------|-----------|------------------------|-----|---------|------------------------|-----|---------|------------------------|-----|---------|
| Total                             | 137       | 58 (42.3)              | -  | -       | 34 (24.8)              | -  | -       | 22 (16.1)              | 5.552 | 0.018 |
| Age, years                        |           |                        |    |         |                        |    |         |                        |      |         |
| ≥60                               | 49        | 19 (38.8)              | 0.447 | 0.504 | 10 (20.4)              | 1.006 | 0.316 | 8 (10.1)              | 0.000 |         |
| >60                               | 85        | 38 (44.7)              |      |         | 24 (28.2)              |      |         | 14 (25.5)              |      |         |
| Unknown                           | 3         | 1 (33.3)               |      |         | 0 (0.0)                |      |         | 0 (0.0)                |      |         |
| Tobacco consumption               |           |                        | 0.025 | 0.874 |                        | 1.145 | 0.285 | 0.135 | 0.714 |
| Yes                               | 73        | 33 (45.2)              |      |         | 26 (35.6)              |      |         | 16 (21.9)              |      |         |
| No                                | 32        | 15 (46.9)              |      |         | 8 (25.0)               |      |         | 6 (18.8)               |      |         |
| Unknown                           | 32        | 10 (31.3)              |      |         | 0 (0.0)                |      |         | 0 (0.0)                |      |         |
| Alcohol consumption               |           | 0.0152 | 0.902 |         | 0.384 | 0.535 | 0.654 | 0.419 |
| Yes                               | 51        | 23 (45.1)              |      |         | 18 (35.3)              |      |         | 9 (17.7)               |      |         |
| No                                | 54        | 25 (46.3)              |      |         | 16 (29.6)              |      |         | 13 (24.1)              |      |         |
| Unknown                           | 32        | 10 (31.3)              |      |         | 0 (0.0)                |      |         | 0 (0.0)                |      |         |
| TNM stage                         |           | 13.352 | <0.001 |         | 31.404 | <0.001 | 29.441 | <0.001 |
| Stage I/II                        | 70        | 23 (32.9)              |      |         | 10 (14.3)              |      |         | 4 (5.71)               |      |         |
| Stage III/IV                      | 35        | 25 (71.4)              |      |         | 24 (68.6)              |      |         | 18 (51.4)              |      |         |
| Unknown                           | 32        | 10 (31.3)              |      |         | 0 (0.0)                |      |         | 0 (0.0)                |      |         |
| Lymph node metastasis            |           | 4.794 | 0.029 |         | 5.606 | 0.018 | 0.764 | 0.382 |
| Yes                               | 22        | 14 (63.6)              |      |         | 10 (45.5)              |      |         | 5 (22.7)               |      |         |
| No                                | 112       | 43 (38.4)              |      |         | 24 (21.4)              |      |         | 17 (15.2)              |      |         |
| Unknown                           | 3         | 1 (33.3)               |      |         | 0 (0.0)                |      |         | 0 (0.0)                |      |         |
| Histopathological grade          |           | 0.211 | 0.646 |         | 2.506 | 0.113 | 1.192 | 0.275 |
| High                              | 62        | 25 (40.3)              |      |         | 12 (19.4)              |      |         | 8 (12.9)               |      |         |
| Moderate/low                      | 70        | 31 (44.3)              |      |         | 22 (31.4)              |      |         | 14 (20.0)              |      |         |
| Unknown                           | 5         | 2 (40.0)               |      |         | 0 (0.0)                |      |         | 0 (0.0)                |      |         |
| Ror2                              |           | 9.270 | 0.002 |         | - | - | - | - |
| Low                               | 103       | 36 (35.0)              |      |         | - | - | - | - |
| High                              | 34        | 22 (64.7)              |      |         | - | - | - | - |
| Wnt5a                             |           | - | - |         | 9.270 | 0.002 | - | - |
| Low                               | 79        | - | - |         | 12 (15.2) | - | - | - |
| High                              | 58        | - | - |         | 22 (37.9) | - | - | - |

*Statistically significant (P<0.05). χ² based on Pearson's χ² test. Wnt5a+/Ror2+ indicates high expression of both markers. Wnt5a, Wnt family member 5a; Ror2, receptor tyrosine kinase-like orphan receptor 2; LSCC, laryngeal squamous cell carcinoma; TNM, tumor, node, metastasis.
Wnt5a and Ror2 protein expression levels were associated with high TNM stage and lymph node metastasis. Furthermore, a positive correlation was identified between Wnt5a and Ror2 expression. Univariate and multivariate analyses revealed that high Wnt5a and Ror2 expression were significantly associated with poor OS.

Table III. Univariate and multivariate analyses of prognostic factors for overall survival in LSCC.

| Factor                              | Univariate analysis | Multivariate analysis |
|-------------------------------------|---------------------|-----------------------|
|                                     | P-value  | HR    | 95% CI     | P-value | HR    | 95% CI     |
| Wnt5a expression: High vs. low      | 0.022a   | 2.092 | 0.254-9.091 | 0.022a   | 2.540 | 1.146-5.630 |
| Ror2 expression: High vs. low       | 0.002a   | 2.746 | 1.462-5.158 | 0.038a   | 2.290 | 1.049-5.000 |
| Wnt5a+/Ror2+ vs. non-Wnt5a+/Ror2+  | <0.001a  | 3.285 | 1.683-6.411 | -        | -     | -          |
| Gender: Male vs. female             | 0.003a   | 0.106 | 0.025-0.454 | 0.001a   | 0.047 | 0.008-0.264 |
| Age, years: ≤60 vs. >60             | 0.274    | 1.463 | 0.740-2.891 | 0.209    | 1.762 | 0.728-4.264 |
| Tobacco consumption: Yes vs. no     | 0.303    | 0.687 | 0.336-1.404 | 0.187    | 0.518 | 0.195-1.375 |
| Alcohol consumption: Yes vs. no     | 0.476    | 0.787 | 0.408-1.520 | 0.826    | 0.905 | 0.372-2.200 |
| TNM stage: III/IV vs. I/II          | 0.028a   | 2.084 | 1.083-4.010 | 0.649    | 0.823 | 0.355-1.906 |
| Lymph node metastasis: Yes vs. no   | 0.013a   | 2.428 | 1.202-4.904 | 0.002a   | 3.899 | 1.647-9.229 |
| Histopathological grade: High vs. moderate/low | 0.011a | 2.423 | 1.226-4.788 | 0.164    | 1.706 | 0.804-3.619 |

*Statistically significant (P<0.05). Wnt5a+/Ror2+ indicates high expression of both markers. Wnt5a, Wnt family member 5a; Ror2, receptor tyrosine kinase-like orphan receptor 2; LSCC, laryngeal squamous cell carcinoma; TNM, tumor-node-metastasis; HR, hazard ratio; CI, confidence interval.

Figure 2. Survival curves of laryngeal squamous cell carcinoma determined using the Kaplan-Meier estimator method and the log-rank test. Overall survival curves are shown for (A) Wnt5a+ (green line, 1) and Wnt5a- (blue line, 0), patients in the high-expression group (Wnt5a+) had significantly poorer prognosis compared with those in the low-expression group (Wnt5a-; P=0.020, log-rank test); (B) Ror2+ (green line, 1) and Ror2- (blue line, 0), patients in the high-expression group (Ror2+) had significantly poorer prognosis compared with those in the low-expression group (Ror2-; P=0.001, log-rank test); and (C) Wnt5a+/Ror2+ (red line, 2), Wnt5a+/Ror2- or Wnt5a-/Ror2+ (green line, 1) and Wnt5a-/Ror2- (blue line, 0), patients in the high Wnt5a and high Ror2 expression group (Wnt5a+/Ror2+) had significantly poorer prognosis compared with both those in the high Wnt5a or high Ror2 expression group (Wnt5a+/Ror2- or Wnt5a-/Ror2+ or Wnt5a-/Ror2-) and those in the low-expression group (Wnt5a-/Ror2-; P=0.001, log-rank test). Wnt5a, Wnt family member 5a; Ror2, receptor tyrosine kinase-like orphan receptor 2.

Wnt5a and Ror2 protein expression levels were associated with high TNM stage and lymph node metastasis. Furthermore, a positive correlation was identified between Wnt5a and Ror2 expression. Univariate and multivariate analyses revealed that high Wnt5a and Ror2 expression were significantly associated with poor OS.

Wnt5a, a member of Wnt family, is located at the chromosomal site 3p14.2-p21.1, which was revealed by Clark et al (32). Wnt5a interacts with G-proteins to activate the non-canonical Wnt/Ca²⁺ pathway. This signaling pathway can activate or inhibit the canonical Wnt/β-catenin pathway, depending on the interaction of Wnt5a with various receptors. For example, when Wnt5a interacts with Ror2, the Wnt/β-catenin pathway is inhibited. Conversely, when Wnt5a combines with Fz or low-density lipoprotein receptor-related protein, the Wnt/β-catenin pathway is activated (33). Wnt5a affects cell proliferation, migration, invasion and angiogenesis, and is also involved in the genesis and development of various types of carcinoma through its combination with different receptors (34,35). In the present study, Wnt5a protein expression was identified to be significantly higher in LSCC compared with in adjacent normal tissue samples. Thus, Wnt5a may act as a tumor promoter for LSCC. Similar results have been observed in non-small cell lung carcinoma, metastatic melanoma and gastric cancer (15,36,37). Furthermore, the current study investigated the associations of Wnt5a and Ror2 protein expression with OS in 137 patients with LSCC. High Wnt5a expression was significantly associated with poor OS on univariate and multivariate analyses, indicating that Wnt5a is an independent prognostic factor, and may be a tumor
promoter in LSCC. However, previous studies have indicated that Wnt5a is a tumor suppressor in certain types of cancer, including hepatocellular carcinoma, thyroid carcinoma and colon carcinoma (23,38,39). These findings suggest that the Wnt5a-regulated pathways and the functional role of Wnt5a depend on the cancer cell type.

Ror2 is an orphan tyrosine kinase, belonging to the Ror family and acts as a receptor for Wnt5a (40). The biological function of Ror requires its presence in the cell membrane; however, in the present study, Wnt5a and Ror2 protein were localized in the cytoplasm. Since Ror2 possesses an extracellular CRD that resembles the Wnt-binding sites of the Fz proteins (41,42), it may be assumed that Wnt5a binds to the CRD of Ror2. This hypothesis was confirmed in a study performed by Oishi et al (40), which also demonstrated that Ror2 associated with receptor Fz2, a putative receptor for Wnt5a, via its CRD (43). Ror2 is expressed in the face, limbs, heart and lungs during mouse embryogenesis (44). Mice that lack Ror2 expression exhibit facial abnormalities, dwarfism, short limbs and tails, dysplasia of lungs and genitals, abnormalities in axial skeletons, and ventricular septal defects, resulting in neonatal mortality (45,46). Mutations of Ror2 that result in misfolded protein or premature truncation have been associated with human diseases, including dominant Brachydactyly type B, a dominant skeletal disorder characterized by hypoplasia or aplasia of distal phalanges (47,48); and Robinow syndrome, a recessive condition characterized by short stature, segmental defects of the spine, limb bone shortening, and a dysmorphic facial appearance (49). Thus, Ror2 is essential in embryogenesis. Together, Wnt5a/Ror2 have been revealed to mediate the non-canonical Wnt signal pathway and simultaneously suppress Wnt/β-catenin activity. In addition, it has been demonstrated that the Ror2 receptor requires tyrosine kinase activity to mediate the Wnt5a signal pathway (16,50). In the current study, Ror2 protein was highly expressed in LSCC tissue samples. Furthermore, following univariate and multivariate analyses, high Ror2 expression was significantly associated with poor OS, which demonstrated that Ror2 was also an independent prognostic factor, and a possible tumor promoter in LSCC. These results were consistent with previous studies (20-22); however, other studies have demonstrated that Ror2 expression is reduced in hepatocellular carcinoma, medulloblastoma and colon cancer (23-25). Thus, Ror2 may serve different roles in different cancer types, as with Wnt5a.

Wnt5a and Ror2 are important in developmental morphogenesis, particularly in skeletal development (51,52). Notably, Ror2-/- and Wnt5a-/- mice exhibit similar defect phenotypes (40). Previous studies have demonstrated that Brachydactyly type B and Robinow syndrome are associated with the non-canonical Wnt5a/Ror2 signaling pathway (53). The current study identified a positive correlation between Wnt5a and Ror2 expression and, these results suggested that Wnt5a and Ror2 may be associated with the development of LSCC.

In conclusion, the overexpression of Wnt5a and Ror2 was detected in LSCC tissue samples, and was significantly associated with tumor stage, lymph node metastasis and poor prognosis of LSCC. These findings provide a novel insight into the mechanisms underlying tumorigenesis and have identified two potential therapeutic targets for LSCC.

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