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RESEARCH ARTICLE

RANK and RANK Ligand Expression in Parotid Gland Carcinomas

Alessandro Franchi, MD, Cecilia Taverna,* Antonella Simoni,* Monica Pepi,* Giuditta Mannelli,† Martina Fasolati,† and Oreste Gallo†*

Abstract: Recently, it has been reported that deregulation of the receptor activator of NFkB ligand (RANKL)/RANK signaling axis results in salivary gland tumor development in a mouse transgenic model. The aim of this study was to ascertain RANKL and RANK protein expression in a series of primary parotid gland carcinomas and to correlate it with clinicopathologic parameters. Formalin-fixed paraffin-embedded tumor samples from 46 consecutive cases of parotid gland carcinoma were selected for this study. For comparison, we examined a group of 40 randomly chosen parotid gland adenomas, including 20 pleomorphic adenomas, 10 myoepitheliomas, and 10 Warthin tumors. Immunohistochemical analysis for RANK and RANKL was conducted on tissue microarrays. Overall, 33 carcinomas (71.7%) were scored as positive for RANK and 25 (54.3%) for RANKL. The expression of both RANK and RANKL was significantly higher in carcinomas than in adenomas as only 6 (15%) adenomas were positive for RANK, and RANKL was negative in all benign tumors ($P < 0.001$ for both, Fisher exact test). Some histologic types, including salivary duct carcinoma, mucoepidermoid carcinoma, and carcinoma ex pleomorphic adenoma presented a high frequency of RANK and RANKL expression. No significant correlation was observed between RANK/RANKL expression and clinical parameters. Our study indicates that the expression of RANK and RANKL in parotid gland neoplasms is associated with the acquisition of a malignant phenotype and this pathway may represent an attractive therapeutic target in patients with parotid gland carcinomas.

Key Words: parotid gland, carcinoma, RANK, RANKL, immunohistochemistry

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Salivary gland carcinomas are rare tumors representing < 5% of all head and neck malignancies, with an estimated age-adjusted incidence rate of 11.95/1,000,000 person-years in the United States.[1] The majority of salivary gland carcinomas occur in the parotid gland, and currently over 20 different histologic subtypes are recognized in the WHO Classification, which show a highly variable clinical behavior. Surgery remains the mainstay for the treatment of parotid gland carcinomas, and radiation therapy is administered on the basis of evaluation of clinicopathologic risk factors. Chemotherapy may be utilized as palliative treatment, although its efficacy has not been proven in clinical trials.[2,3] However, in the past years, no significant improvement has been obtained in the overall survival of patients affected by parotid gland carcinomas. Therefore, there is urgent need of new treatment strategies possibly based on the identification of specific targets.

Recently, it has been reported that deregulation of the receptor activator of NFkB ligand (RANKL)/RANK signaling axis results in salivary gland tumor development in a mouse transgenic model.[4] Moreover, the RANKL/RANK signaling axis could elicit an aggressive salivary gland tumor phenotype at both the histologic and molecular level, whereas early blockade of RANKL/RANK signaling markedly attenuated the development of malignant salivary gland neoplasms in this model.[4] On the basis of these results, the RANK/RANKL pathway may represent a novel therapeutic target in salivary gland carcinomas. As there are no data currently available on the status of this signaling pathway in human salivary tumors, we aimed to ascertain RANKL and RANK protein expression in a series of primary parotid gland carcinomas in this study. In addition, we examined the impact of RANK and RANKL expression on patients' outcome.

PATIENTS AND METHODS

Patients

A total of 46 consecutive patients treated at our hospital for parotid gland carcinoma between 2001 and 2011 were selected for this study. In each case, all available histologic slides were reviewed and tumors were classified according to the 2005 WHO Classification scheme[5] and assigned to low-risk and high-risk categories as previously described.[6] The study group included 9 mucoepidermoid carcinomas, 9 carcinomas ex pleomorphic adenoma, 7 adenoid cystic carcinomas, 5 acinic cell carcinomas, 4 salivary duct carcinomas, 3 squamous cell carcinomas, 3 myoepithelial carcinomas, 2 basal cell adenocarcinomas, 2

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TABLE 1. Summary of the Main Clinical Features of 46 Parotid Carcinomas

Age (Mean)	61.3 y (Range, 26-87 y)
Sex (male:female)	31:15
T stage	
I-II	27
III-IV	19
Lymph node metastasis	2
Distant metastasis	11
Local recurrence	5
Proportion disease free at 5 y (%)	72.1
Proportion surviving at 5 y (%)	73.9

adenocarcinomas NOS, 1 undifferentiated carcinoma, and 1 epithelial-myoepithelial carcinoma. The salient clinicopathologic characteristics of this series are summarized in Table 1. For comparison, we examined a group of 40 randomly chosen parotid gland adenomas, including 20 pleomorphic adenomas, 10 myoepitheliomas, and 10 Warthin tumors.

Immunohistochemistry

For tissue microarray construction, areas of interest rich in non-necrotic tumor were identified on corresponding hematoxylin and eosin-stained sections and marked on the source paraffin block. The source block was cored and a 1-mm core was transferred to the recipient master block using the Beecher Tissue Microarrayer (Beecher Instruments, Silver Spring, MD). Three cores from different areas of the same tissue block were arrayed for each case. Sections (5-μm thick) were obtained from the block, which were stained with hematoxylin and eosin, or utilized for the immunohistochemical analysis.

For immunohistochemical staining, tissue sections (5 μm) were deparaffinized, hydrated, and after endogenous peroxidase inactivation immunostained with BenchMark Ultra stainer (Ventana, Tucson, AZ), and revealed with iVIEW DAB detection kit, providing a brown reaction product. Table 2 shows the antibody source, dilution, and antigen retrieval protocol. After completing the staining process, the slides were removed from the autostainer, counterstained with hematoxylin, dehydrated, and mounted with a permanent medium. As a negative control, we substituted the primary antibody with a Ventana dispenser filled with nonimmune serum at the same concentration for each immunohistochemical reaction. As a positive control, we used reactive bone and bone fracture specimens.

The semiquantitative evaluation of the results of the immunohistochemical studies was conducted considering both the staining intensity and the percentage of positive cells, as previously described.[6] The staining intensity was evaluated on a 4-point scale (0 to 3), and the proportion of positive cells was evaluated according to the scale 0, 1 (1%), 2 (2% to 10%), 3 (11% to 30%), 4 (31% to 60%), and 5 (100%).[7] The 2 values were summed to obtain a total score ranging between 0 and 8. For statistical analysis, cases with score ≥ 4 were considered positive.

Statistical Analysis

All statistical tests were performed using SPSS software (release 12.0). Associations between categorical variables were assessed by means of the χ^2 test. For analysis of survival, the endpoints considered in this study were rates of developing first local recurrence and rate of any disease-related mortality. Local recurrence-free survival and disease-specific survival were modeled using the Kaplan-Meier method and analyzed by the log-rank test. χ^2 $P < 0.05$ were considered significant.

RESULTS

The intensity score for RANKL was weaker than that of RANK, whereas the proportion of positive cells was similar for the 2 factors. Overall, 33 carcinomas (71.7%) were scored as positive for RANK and 25 (54.3%) for RANKL. The expression of both factors was significantly higher in carcinomas than in adenomas as only 6 (15%) adenomas were positive for RANK, whereas RANKL was negative in all cases ($P < 0.001$ for both, Fisher exact test).

The distribution of RANK and RANKL positivity in carcinomas according to histologic type is shown in Table 3. Although no significant difference was observed, some histologic types, including salivary duct carcinoma, mucoepidermoid carcinoma, and carcinoma expleomorphic adenoma, presented a high frequency of RANK and RANKL expression. Figure 1 illustrates the results of the immunohistochemical studies in the main histologic types. Moreover, high-grade tumors tended to express RANKL more frequently than low-grade ones (66.6% vs. 36.8%; $P = 0.07$, Table 4).

In 5 of 9 cases of carcinoma expleomorphic adenoma, we also examined RANK and RANKL expression in whole histologic sections including both the benign and the malignant components of the tumor. In 2 cases, both the adenoma and the carcinoma were scored as negative for RANK and RANKL. In 3 cases, the malignant component of the tumor showed positive staining for RANK whereas the adenoma was negative, and an increase in RANKL expression from adenoma to carcinoma was observed in 2 of these cases (Fig. 2).

As summarized in Table 4, no significant correlation was observed between RANK/RANKL expression and

TABLE 2. Features of the Antibodies Used in This Study

Antibody	Clone and Provider	Species and Dilution	Antigen Retrieval
RANKL	Polyclonal, Abcam, Cambridge, UK	Rabbit, 1:2000	Citrate buffer, pH 6
RANK	64C1385, Abcam, Cambridge, UK	Mouse, 1:200	Citrate buffer, pH 6

RANKL indicates receptor activator of NFkB ligand.

TABLE 3. Correlation Between Histologic Type and RANK and RANKL Expression in 46 Salivary Gland Carcinomas

Histologic Type	RANK+ (%)	RANKL+ (%)
Mucoepidermoid carcinoma (N = 9)	7 (77.8)	5 (55.6)
Carcinoma expleomorphic adenoma (N = 9)	8 (88.9)	7 (77.8)
Adenoid cystic carcinoma (N = 7)	2 (28.6)	1 (14.3)
Acinic cell carcinoma (N = 5)	4 (80)	1 (20)
Salivary duct carcinoma (N = 4)	4 (100)	3 (75)
Squamous cell carcinoma (N = 3)	2 (66.7)	2 (66.7)
Myoepithelial carcinoma (N = 3)	2 (66.7)	2 (66.7)
Basal cell adenocarcinoma (N = 2)	0	0
Adenocarcinoma NOS (N = 2)	2 (100)	2 (100)
Undifferentiated carcinoma (N = 1)	1 (100)	1 (100)
Epithelial-myoepithelial carcinoma (N = 1)	1 (100)	1 (100)

RANKL indicates receptor activator of NFkB ligand.

clinical parameters, although the majority of patients with facial nerve involvement (85.7%) had tumors positive for both factors. Similarly, no correlation was found between the expression of the 2 factors and disease-free interval ($P = 0.13$ for RANK and $P = 0.76$ for RANKL) as well as with overall survival ($P = 0.18$ for RANK and $P = 0.81$ for RANKL).

DISCUSSION

To the best of our knowledge, this is the first analysis of the immunohistochemical expression of RANK and RANKL in parotid gland tumors. Our study indicates that the expression of RANK and RANKL is associated with the acquisition of a malignant phenotype, as we observed that parotid carcinomas present a significantly higher expression than adenomas. Accordingly, we also observed an increased expression of both factors in the

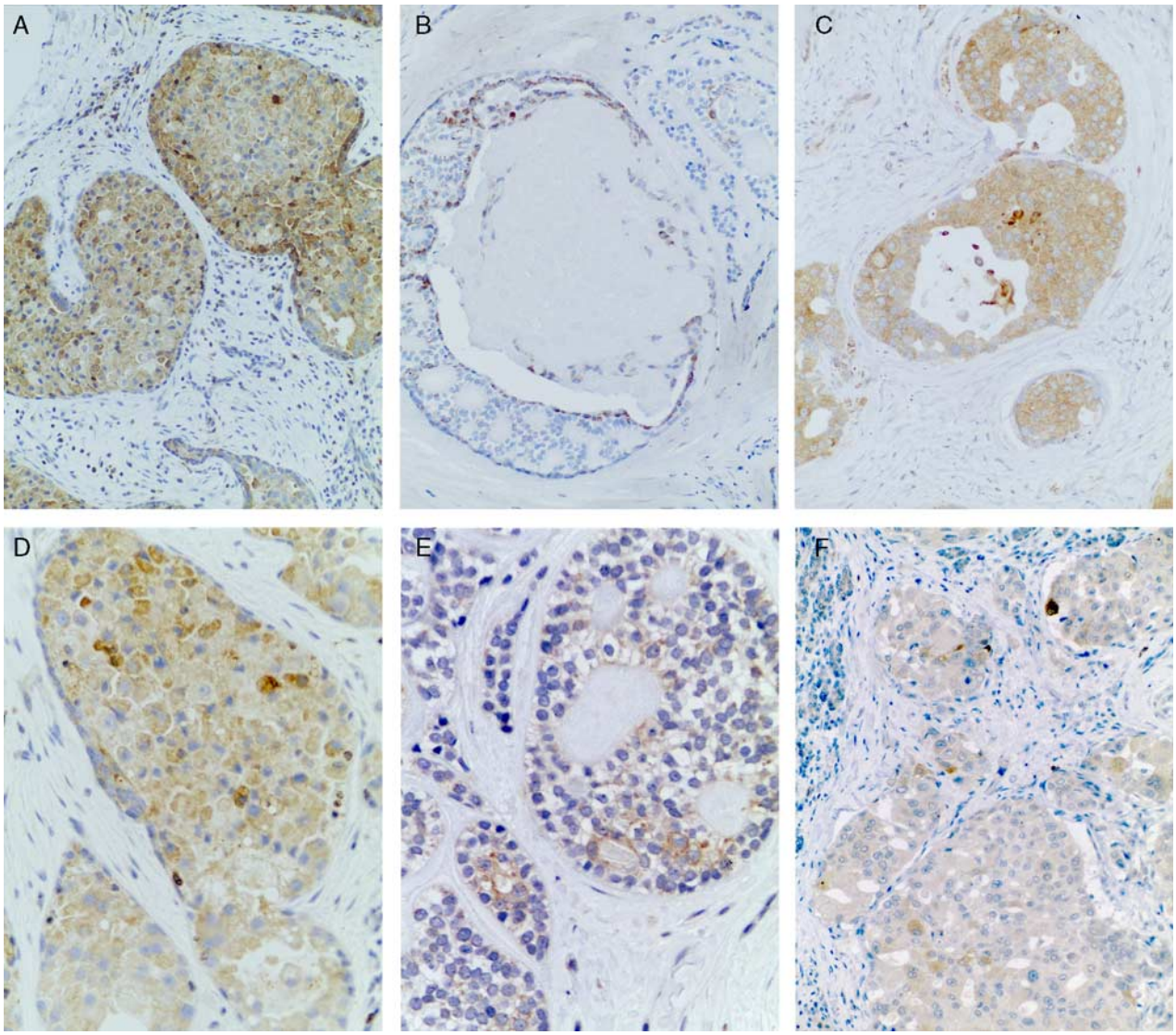

FIGURE 1. Immunohistochemical expression of RANK (A–C) and RANKL (D–F). Representative images from mucoepidermoid carcinoma (A, D), adenoid cystic carcinoma (B, E), and salivary duct carcinoma (C, F).

TABLE 4. Correlation Between Clinicopathologic Features and Expression of RANK and RANKL in 46 Parotid Gland Carcinomas

	RANK + N (%)	*P*	RANKL + N (%)	*P*
Histologic grade				
Low (19)	12 (63.1)		7 (36.8)	
High (27)	21 (77.7)	0.3	18 (66.6)	0.07
T stage				
I-II (27)	19 (70.3)		14 (51.8)	
III-IV (19)	14 (73.6)	1.0	11 (57.8)	0.7
Local recurrence				
No (41)	31 (75.6)		23 (56.1)	
Yes (5)	2 (40.0)	0.15	2 (40.0)	0.65
Lymph node metastases				
No (44)	31 (70.4)		24 (54.5)	
Yes (2)	2 (100)	1.0	1 (100)	0.48
Distant metastases				
No (35)	28 (80.0)		21 (60.0)	
Yes (11)	5 (45.5)	0.67	4 (36.4)	0.30
Facial nerve involvement				
No (39)	27 (69.2)		19 (48.7)	
Yes (7)	6 (85.7)	0.65	6 (85.7)	0.11

RANKL indicates receptor activator of NFkB ligand.

malignant component of 2 cases of carcinoma expleomorphic adenoma, and of RANK only in a further example. Moreover, some histologic types presented a high prevalence of positive cases, including salivary duct carcinoma, mucoepidermoid carcinoma, and carcinoma expleomorphic adenoma, although the differences were not statistically significant, probably because of the high number of histologic variants and the low number of cases in each category.

These results are in keeping with recent experimental studies showing that activation of the RANKL/RANK signaling axis in a transgenic mouse model results in rapid tumor development in major salivary glands.[4] These tumors were histologically similar to poorly differentiated mucoepidermoid carcinomas, and expressed several molecular markers associated with advanced-stage malignancies and poor prognosis.[4] Moreover, RANKL elicited tumor cell proliferation with overexpression of markers of cell division, including cyclin D1 and proliferation cell nuclear antigen.[4]

RANK and RANKL are members of the TNF superfamily and are key regulators of immunity and bone

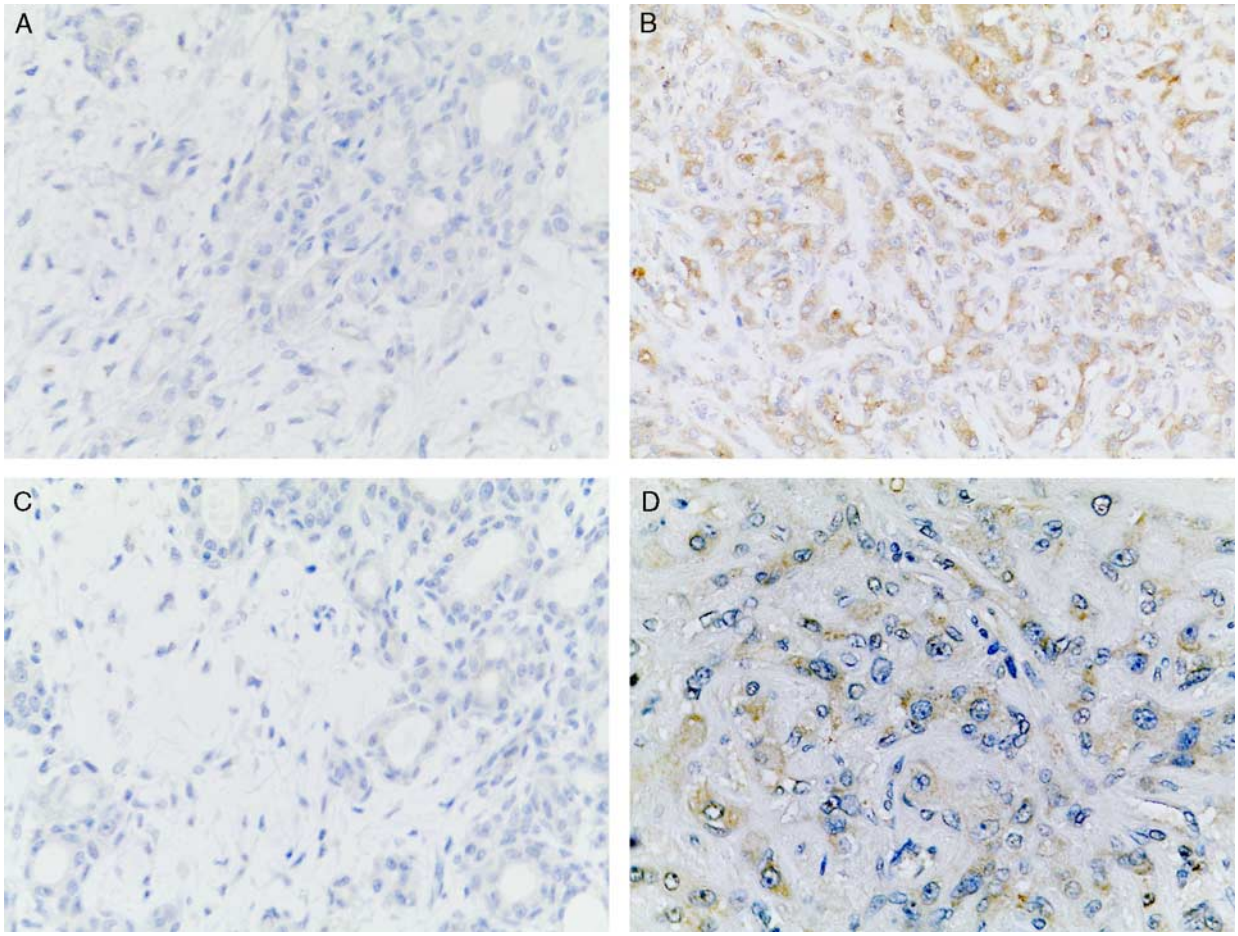

FIGURE 2. Comparison of RANK (A, B) and RANKL (C, D) expression in carcinoma expleomorphic adenoma. A–C, Absent expression in the adenoma. B–D, High expression in the malignant component. full color online

remodeling.[8–10] This signaling system is involved in the control of osteoclast activity and in the development of skeletal disorders such as osteoporosis and bone metastasis. The RANK/RANKL pathway is also involved in the growth of giant cell tumor of bone.[11] However, there is increasing evidence that the RANK/RANKL system is involved in the regulation of several key aspects of the development of epithelial tissues such as the mammary gland, and it is implicated in the acquisition of aggressive malignant features in other epithelial tumors, including breast and prostate carcinomas,[12,13] renal cell carcinoma,[14] and lung carcinoma.[15] In particular, considering the similarities between mammary gland tumors and salivary gland tumors, with significant overlaps in their histopathologic features and regulatory mechanisms, it is of interest that expression studies in human breast carcinoma have shown that RANK protein expression is associated with hormone receptor–negative status, high-pathologic grade, and poor survival.[16] In the present study, no correlation could be found between RANK/RANKL expression and clinical parameters of tumor aggressiveness, including stage, facial nerve involvement, nodal and distal metastases, as well as survival. This may be due to the intrinsic variability of clinical behavior among the several different histologic subtypes of parotid gland carcinomas and the relatively small number of cases analyzed.

In the recently reported preclinical mouse model, early therapeutic targeting of the RANK/RANKL signaling axis significantly attenuated salivary gland tumor progression,[4] suggesting that this pathway may represent a novel area of therapeutic intervention in salivary gland carcinomas. Denosumab, a monoclonal antibody directed against RANKL, is currently used in the management of osteoporosis, bone metastases, and giant cell tumor of bone, mainly for its role of inhibitor of bone resorption through its effects on osteoclastogenesis.[17] However, there is increasing evidence that inhibition of the RANK/RANKL axis may offer new therapeutic chances in cancer patients. Indeed, in a phase III clinical trial of patients affected by lung cancer, Denosumab determined an increase not only in bone metastasis–free survival but also in overall survival,[15] indicating a possible direct antitumor effect of this drug. In addition, 1 patient with AQ4 metastatic adenocarcinoma of the lung with ALK gene rearrangement responded to treatment with Denosumab.[18] In this context, our preliminary results support the hypothesis that targeting the RANK/RANKL signaling pathway may represent a valid option to be tested either alone or in combination with other therapies for the treatment of parotid gland carcinomas. Further studies on larger series of patients are needed to explore this attractive hypothesis.

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