Receptor activator of nuclear factor κB expression is a prognostic factor in human osteosarcoma

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Abstract. Receptor activator of nuclear factor κB (RANK), a member of the tumour necrosis factor family, is activated by its ligand and regulates the differentiation of osteoclasts and dendritic cells. Local growth of osteosarcoma involves destruction of the host bone by osteoclasts and proteolytic mechanisms. Although the prognosis of patients with osteosarcoma has been improved by advances in chemotherapy over the last four decades, the issues of non-responders, and the lack of effective prognostic markers have remained. The present study aimed to investigate the prognostic and predictive value of RANK expression in human osteosarcoma. The expression of RANK was immunohistochemically evaluated in biopsies of 43 patients (mean age 25.4 years) with high-grade osteosarcoma, and was found to be correlated with histological response to chemotherapy, disease-free status and overall survival. RANK expression was detected in eight of the 43 osteosarcoma specimens (18%), whereas the remaining specimens were negative for RANK. A statistically significant correlation was detected between RANK expression and the overall survival of patients. A total of 7/8 patients with RANK-expressing tumours succumbed to the disease (88% mortality rate amongst patients with RANK-positive tumours vs. 37% with RANK-negative tumours; $P<0.05$). No significant difference was found when comparing RANK expression status with response to chemotherapy; 50% of RANK-positive patients exhibited a poor response to chemotherapy, compared with 66% in the RANK negative group. In addition, the appearance of metastases was not correlated with RANK expression status (38% metastases in RANK-positive tumours vs. 34% in RANK-negative tumours). In conclusion, the results of the present study suggested that RANK expression is likely to be of prognostic, but not of predictive, value.

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Introduction

Osteosarcoma is the most common malignant bone tumour, and is responsible for 20% of all primary bone sarcomas (1). Although treatment strategies have been improved over the past four decades, osteosarcoma remains associated with a high mortality rate, particularly amongst children and young adults. The peak incidence rate of osteosarcoma is detected in the second decade of life. The majority of osteosarcomas are characterized by highly malignant tumours, which arise within the bone. The general treatment strategy for osteosarcoma is comprised of preoperative chemotherapy, followed by surgical resection and a postoperative chemotherapy regimen determined in accordance with the extent of tumour necrosis (2). Risk adaptation of chemotherapy is not currently possible in individual cases, while postoperative clinical stratification is calculated according to patient response to chemotherapy and tumour size. Identification of patients exhibiting good or poor responses to treatment may aid the modification of these treatment regimens (1-3).

Receptor activator of nuclear factor κB (RANK) is a key protein involved in bone development and osteoclastogenesis (4). Activation of RANK, which is expressed on the surface of osteoclast precursors, by the binding of its ligand results in activation of the nuclear factor κB (NF-κB) and c-Jun N-terminal kinase signalling pathways, leading to differentiation of osteoclast precursor cells into pro-osteoclasts, which mature into active osteoclasts (5). RANK is activated by its ligand RANKL, a member of the TNF ligand superfamily (also known as OPGL or TRANCE), which is negatively controlled by osteoprotegerin (OPG). OPG is an inhibitor of osteoclastogenesis, functioning as a soluble decoy receptor for RANKL. When OPG is bound to RANKL, it circumvents the binding of RANKL to RANK and therefore inhibits the biological activation of RANKL, preventing osteoclastogenesis (6,7). Previous studies have identified a significant role for RANK in the formation of bone. In osteosarcoma, the characteristic bone destruction is mediated by the expression of RANKL, which induces osteoclastic activity (8-10).

A recent study revealed poor patient prognosis in RANK-positive osteosarcomas, and suggested further investigations which were undertaken in the current study (11).

Patients and methods

Patients and tissue specimens. Tissue specimens from 43 patients with high-grade osteosarcoma (mean age 25.4 years; range 7-68 years; 27 male, 16 female) were immunohistochemically investigated. Of the 43 patients, a total of 32 patients were recruited at the Medical University of Vienna (Vienna, Austria) and 11 patients were recruited at Klinikum Frankfurt (Oder) (Frankfurt, Germany) between June and November 2007. Patient consent was obtained from all patients prior to the study and the study was approved by the ethics committee of Klinikum Frankfurt (Oder). All patients received neoadjuvant, multiagent chemotherapy immediately following biopsy and diagnosis. The anatomical locations of the tumours, included the femur (24 cases), the tibia and fibula (12 cases), the humerus (3 cases) and the pelvis (4 cases).

Limb salvage was able to be performed in 42 cases, while one patient received an amputation. The latter was an 18-year-old mentally disabled patient, and the tumour was located in the distal tibia. Surgical margins were defined histologically according to criteria outlined by Enneking *et al* (12), and were wide in all cases; no marginal resection was required.

The resected tumour specimens were histologically analysed to determine the response to preoperative chemotherapy, according to the following criteria outlined by Salzer-Kuntschik *et al* (13): Grade I, no viable cells; grade II, single tumour cells or one tumour isle <0.5 cm in size; grade III, <10% viable tumour cells; grade IV, 10-50% viable tumour cells; grade V, >50% viable tumour cells; grade VI, no effect of chemotherapy. Grades I-III were considered to indicate a good response to chemotherapy, whereas grades IV-VI, with >10% viable tumour cells, were considered to indicate a poor response to chemotherapy (13).

Immunohistochemistry. Tumour sections (2-3 μm thickness) were subjected to antigen retrieval in a microwave (600 W; Siemens AG, Munich, Germany), with 1 mM EDTA pH 8.0 buffer (Gibco Life Technologies, Paisley, UK) four times for 5 min. Blocking of nonspecific binding was conducted with 5% Tris-buffered saline/bovine serum albumin (TBS/BSA; Merck Millipore) for 1 h at room temperature. Mouse RANK affinity purified polyclonal antibody, goat IgG (1:500; cat. no. AF692) and isotype control polyclonal goat IgG_1 (1:250; cat. no. AF108) (R&D Systems, Inc., Minneapolis, MN, USA) were applied and incubated at 4˚C overnight. Subsequently, biotinylated anti-goat secondary antibody (1:100; cat. no. BA5000; Vector Laboratories, Inc., Burlingame, CA, USA) in 1% TBS/BSA/10% human serum (Dako, Glostrup, Denmark) was applied for 1 h at room temperature, followed by alkaline phosphatase-conjugated Streptavidin-AP/10% human serum (1:250; Dako) for 1 h at room temperature. Visualisation was performed using fast red (Sigma-Aldrich, St. Louis, MO, USA) and counterstained by haemalaun (Merck Millipore). The immunohistochemical expression of RANK was reviewed independently by two observers under a microscope (Axio Examiner; Zeiss GmbH, Jena, Germany), who had no prior knowledge of the clinicopathological data.

Table I. Clinical results in correlation with the expression of RANK.

Clinical factor	RANK (+), n/total (%)	RANK (-), n/total (%)
Survival		
Dead	7/8 (88)	13/35 (37)
Alive	1/8 (13)[a]	22/35 (63)
Response to chemotherapy		
Good	3/6 (50)	12/35 (34)
Poor	3/6 (50)	23/35 (66)
Metastases		
Yes	3/8 (38)	12/35 (34)
No	5/8 (63)	23/35 (66)

[a]P<0.05 vs. dead. RANK, receptor activator of nuclear factor κB.

Statistical analysis. The osteosarcoma specimens were divided into two groups: RANK-expressing and non RANK-expressing specimens. The disease-free status and overall survival rates were estimated using the Kaplan-Meier method and analysed by the log-rank test (2). The statistical significance of the differences between the expression of RANK and response to chemotherapy was determined with the χ^2 test. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of RANK on osteosarcoma cells. Of the 43 osteosarcomas studied, eight cases (18%) were classified as RANK positive, identified by an intense immunostaining of RANK on the cell surface. The remaining 35 cases (82%) were classified as RANK negative.

RANK expression is not correlated with response to preoperative chemotherapy. The osteosarcoma specimens were divided into RANK-positive and RANK-negative groups and their response to preoperative chemotherapy was compared. The results suggested that there was no significant difference in response to chemotherapy between the two groups: 50% of RANK-positive tumours exhibited a poor response to chemotherapy, while 34% of RANK-negative tumours exhibited a good response and 66% exhibited a bad response. This difference was not statistically significant (Table I).

Tumour RANK status is associated with overall survival in osteosarcoma. Comparison of the survival of the 43 patients with osteosarcoma revealed that the disease-free and overall survival rate of the eight patients with RANK positive tumours was significantly lower than that of the patients with

tumours negative for RANK. The overall survival rate for patients with RANK-positive tumours was 13%, compared with 63% amongst RANK-negative patients (P<0.05; Table I).

RANK expression is not correlated with metastatic status. A total of 15 patients were identified with metastases. Three of these were detected in the RANK-positive group (38%) and 12 in the RANK-negative group (34%), indicating no significant difference in the incidence rate between the two groups (Table I).

Discussion

Osteosarcoma is the most frequent primary malignant bone tumour, is highly aggressive in its nature and typically occurs in adolescents and young adults. Following the introduction of neoadjuvant chemotherapy, the prognosis for patients with osteosarcoma has significantly increased over the last four decades (1,2). The most significant prognostic factor is the response to chemotherapy, with subsequent tumour cell necrosis (13). In osteosarcoma, a pre-chemotherapy stratification of patients based on prognostic factors may aid the identification of non-responders at the initial stage and therefore increase therapeutic success. Multiple markers have been evaluated with respect to their prognostic value (3,11,14-18).

One of these markers is the RANK/RANKL cascade, which has a crucial role in mediating bone resorption. RANK/RANKL belongs to the tumour necrosis factor superfamily, which includes RANK, its ligand RANKL and OPG. RANK is expressed on the surface of osteoclast precursors, and the binding of RANKL induces their differentiation into mature osteoclasts. OPG is a decoy receptor for RANKL and prevents the binding of RANKL to osteoclast precursors, thereby inhibiting osteoclast differentiation. Dysregulation of this system has been observed in multiple tumours of various origins, including malignant bone tumours, multiple myeloma, breast cancer and prostate cancer. The RANK/RANKL system induces osteolytic bone lesions, and blocking of this system prevents bone destruction (4-10). The results of the present study confirm previous data indicating that the chemotherapy responsiveness of patients with tumours that are RANK positive is equal to that of patients with RANK-negative tumours, whereas RANK-positive tumours are associated with a significantly higher mortality rate (11). A significantly higher mortality rate was detected amongst patients with RANK-positive osteosarcoma, but no differences were detected in the response to chemotherapy or rate of metastases. These results confirm those of a recent study of RANK expression regarding overall survival and response to chemotherapy (11). In addition, RANKL expression was lower and did not demonstrate any statistically significant correlation with disease outcome. Taken together, these results indicated that RANK expression has a prognostic value but is not able to function as a predictive factor. This has been confirmed by the results of the present study and the study by Bago-Horvath *et al* (11), and should be further analysed in order to identify additional predictive factors in human osteosarcoma.

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