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Expression Pattern of Receptor Activator of NFκB (RANK) in a Series of Primary Solid Tumors and Related Bone Metastases

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Receptor activator of NFκB ligand (RANKL), RANK, and osteoprotegerin (OPG) represent the key regulators of bone metabolism both in normal and pathological conditions, including bone metastases. To our knowledge, no previous studies investigated and compared RANK expression in primary tumors and in bone metastases from the same patient. We retrospectively examined RANK expression by immunohistochemistry in 74 bone metastases tissues from solid tumors, mostly breast, colorectal, renal, lung, and prostate cancer. For 40 cases, tissue from the corresponding primary tumor was also analyzed. Sixty-six (89%) of the 74 bone metastases were RANK-positive and, among these, 40 (59.5%) showed more than 50% of positive tumor cells. The median percentage of RANK-positive cells was 60% in primary tumors and metastases, without any statistically significant difference between the two groups ($P = 0.194$). The same percentage was obtained by considering only cases with availability of samples both from primary and metastasis. Our study shows that RANK is expressed by solid tumors, with high concordance between bone metastasis and corresponding primary tumor. These data highlight the central role of RANK/RANKL/OPG pathway as potential therapeutic target not only in bone metastasis management, but also in the adjuvant setting. J. Cell. Physiol.

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Receptor activator of NFκB ligand (RANKL)/RANK/osteoprotegerin (OPG) pathway represents a key regulator of bone metabolism both in normal and pathological conditions, including bone metastases. RANK is expressed at the surface of osteoclasts and it is an essential signaling receptor in osteoclast differentiation. RANKL is preferentially expressed on committed pre-osteoblastic cells and, upon binding to RANK, has been shown to both activate mature osteoclasts and mediate osteoclastogenesis in presence of macrophage-colony stimulating factor (M-CSF). OPG, produced by osteoblast lineage cells, acts as a decoy receptor and inhibits osteoclast formation, function, and survival by preventing RANKL binding to RANK (Boyce and Xing, 2008). RANK expression has been found in osteosarcoma cell lines from human origin together with some human osteosarcoma specimens, both at transcriptional and protein level (Wittrant et al., 2006; Mori et al., 2007a).

However, RANK expression is not restricted to bone cells and has been also observed in other tissues including epithelial

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cells of mammary gland, lung, brain, and kidney (Nakagawa et al., 1998; Hsu et al., 1999; Srivastava et al., 2003). Moreover, RANK is also expressed in several cancer cell lines where seems to play a central role in tumor cell migration and invasion (Mori et al., 2007b). As recently demonstrated, this pathway has been found to be disregulated in several solid tumors, such as breast cancer, malignant bone tumors, multiple myeloma, giant cell tumors of bone, chondroblastoma, neuroblastoma, squamous cell carcinoma, and Hodgkin disease (Mori et al., 2007b).

Additionally, Jones et al. (2006) have reported the expression of functional RANK in human breast and prostate cancer and mouse melanoma cell lines. Moreover, Chen et al. studied RANKL, RANK, and OPG expression in primary and metastatic prostate cancer samples. They found that the expression of these molecules was significantly higher in metastatic prostate cancer than in primary carcinoma (Chen et al., 2006). Similarly, Brown et al. (2004) analyzed primary and metastatic prostate cancer samples and showed that the proportion of tumor cells expressing RANKL was significantly increased in bone metastases compared with metastases in other sites or with the primary tumor. Furthermore, Mori et al. evaluated cases of primary HCC and observed that RANKL expression in HCC cells is correlated with the development of bone metastases after hepatic resection (Sasaki et al., 2007). Moreover, it has been demonstrated that RANKL triggers cytoskeletal changes and migration of several human epithelial tumor cells (including primary breast cancer cells) expressing RANK (Jones et al., 2006).

Taken together, these findings suggest that, upon RANKL activation, RANK may stimulate primary tumor cells to migrate into the bone and may induce the bone metastases process, with additional autocrine and paracrine mechanisms.

To our knowledge, there are no previous studies investigating RANK expression in primary tumors and corresponding bone metastases. Thus, we studied RANK expression by immunohistochemistry (IHC) in a large heterogeneous cohort of human primary solid tumors and related bone metastases, in order to compare RANK expression between the two sites of tumoral disease. These results drive us to investigate if RANK expression depends more on bone microenvironment or on primary tumor histology and whether these molecules could represent ubiquitariously expressed targets for anticancer therapies.

Materials and Methods

Patients

Surgical biopsy samples from 74 patients with bone metastases were examined in this study. The period of accrual was March 2006–January 2009. All patients had histological diagnosis of solid tumor (regardless of treatment performed) and signed the informed consent. For 40 patients, the paraffined samples of both primary and metastatic tumors were available. For 34 patients we only examined the samples from bone metastasis. Exclusion criteria were any previous hematological disease (myelodisplastic, lymphoproliferative, and myeloproliferative syndromes) and assumption of drugs with high osteotrophism, such as bisphosphonates, calcitonin, and Vitamin D. Table 1 shows the distribution of patients included in the study, by site of primary tumor.

Immunohistochemistry

IHC experiments were performed on surgical pathology specimens used for diagnosis. Samples were fixed in 4% neutral buffered formaldehyde and, in case of bone biopsies, were demineralized by EDTA solution (1,000 ml water, 37,22 g ethylenediaminetetraacetic acid disodium salt dehydrate, 70 ml HCL 37%) for less than 3 h. Bones specimens were washed in water before paraffin embedding.

Representative tumor blocks were sectioned at 3 μm thickness. IHC was performed by the streptavidin–biotin method. Endogenous peroxidase in the section was blocked by incubation with 3% hydrogen peroxide. A mouse monoclonal antibody against RANK protein (clone 80707, R&D Systems, Inc., Minneapolis, MN) was used as primary antibody at a concentration of 25 μg/ml. Sections were incubated with LSAB2 (Dakocytomation, Carpinteria, CA). 3-3′-Diaminobenzidine (DAB) was used for color development and hematoxylin was used for counterstaining. Negative controls were obtained by omitting primary antibodies.

Scoring for RANK was based on the relative staining intensity of tumor cells compared to the RANK staining of osteoclasts (bone metastatic lesions) and tissue associated macrophages (primary tumors). These internal references were then used as internal positive controls between slides and samples as well as for the staining procedure. Each pathologic tissue was evaluated by comparison with the internal controls. Staining intensity was graded as absent (0), positive but less intense than internal control tissue (1+), positive like internal control tissue (2+), positive but more intense than internal control tissue (3+). Samples with

TABLE 1. Number of samples for each type of primary tumor and metastases

	Primary		Metastatic	
	No. of lesions	No. (%) of lesions RANK positive in >50% of tumor tissue	No. of lesions	No. (%) of lesions RANK positive in >50% of tumor tissue
Breast	14	8 (57.1%)	19	13 (68.4%)
Colorectal	6	4 (66.6%)	8	5 (62.5%)
Renal	5	2 (40%)	6	2 (33.3%)
Lung	1	1 (100%)	8	7 (87.5%)
Prostate	4	2 (50%)	7	2 (28.5%)
Thymic	1	0 (0%)	1	0 (0%)
Esophageal	1	1 (100%)	1	1 (100%)
Bladder	1	0 (0%)	2	1 (50%)
Thyroid	2	0 (0%)	5	0 (0%)
Hepatic	1	1 (100%)	2	1 (50%)
Cervical	2	2 (100%)	2	2 (100%)
Endometrial	2	1 (50%)	2	2 (100%)
Biliary tract			1	0 (0%)
Salivary glands			2	1 (50%)
Squamous			1	1 (100%)
Malignant melanoma			1	1 (100%)
Unknown			6	1 (16.6%)

regions of heterogeneous staining intensities of RANK were scored and the percentage of each area was recorded. Tumor cells with an immunostaining intensity of 2+ and 3+ were considered as positive. Immunostaining was assessed by two independent pathologists blinded to clinical characteristics and outcomes.

Statistical analysis

Wilcoxon signed-rank test was used to assess differences in RANK expression between primary and related metastatic lesions. $P = 0.05$ was considered as statistically significant. SPSS software (version 14.00; SPSS, Inc., Chicago, IL) was used for statistical analysis.

Results

RANK protein expression

RANK immunostaining, if present, was observed both at the plasma membrane and the cytoplasm of tumor cells (Fig. 1). Consistently with findings from other groups, RANK staining was also constantly found in osteoclast cells of bone tissue around metastatic lesions (Nakagawa et al., 1998) and in tissue associate macrophages within and around primary cancers (van Ravenswaay Claasen et al., 1992; Lau et al., 2007). Thus, both cell types were used as internal positive controls.

RANK expression in bone metastases

Sixty-six (89%) of the 74 bone metastasis samples were RANK-positive and, among these, 40 (59.5%) showed more than 50% of positive tumor cells.

The median percentage of RANK-positive cells seemed to be dependent on the tumor histotype, being 70% for breast, 60% for colorectal, 30% for renal, 100% for lung, and 40% for prostate cancer. We did not perform analysis of differences because of the heterogeneous distribution of the cases.

To note, the only case of bone metastasis from thymoma was RANK-negative, whereas the median percentage of RANK-positive cells was between 30% for tumor of unknown origin and 100% for metastases from esophageal cancer. Specifically, the median RANK expression was 40% in bladder cancer, thyroid cancer and cholangiocarcinoma, 55% in salivary glands tumors, 60% in hepatocarcinoma, 70% in cervical cancer, 75% in endometrial cancer, 80% in squamous cancer, and 90% in melanoma (Fig. 2).

RANK expression in primary tumors

Considering only the 40 primary tumors samples, 27 (67.5%) were RANK-positive and 22 (55%) showed more than 50% of positive tumor cells.

Also at primary sites, the median percentage of RANK-positive cells seemed to be dependent on the tumor histotype, being 65% for breast, 75% for colorectal, 25% for renal, 90% for lung, and 55% for prostate cancer. The number of primary lesions did not allow a reliable difference analysis evaluation. Considering all the histotypes, we observed that RANK expression was considerably variable among tumors. For example, thymoma and bladder cancers were completely negative, whereas the median percentage of RANK-positive cells was between 15% and 85% in the other tumors (thyroid cancer and cervical cancer, respectively). Specifically, RANK expression was 30% in endometrial cancer, 60% in hepatocarcinoma and 80% in esophageal cancer (Fig. 3).

Comparison of RANK expression between primary tumors and bone metastases

To compare the expression of RANK in primary tumor and metastasis, we calculated the median percentage of RANK-positive cells in all the samples from the primaries versus metastases. The median value of RANK-positive cells was 60% in both groups. In terms of percentage of RANK-positive cells, we did not find any statistically significant difference between primary and metastases group ($P = 0.194$) (Fig. 4). Moreover, we repeated the analysis including only patients with availability of matched primary and metastasis tissue samples and we

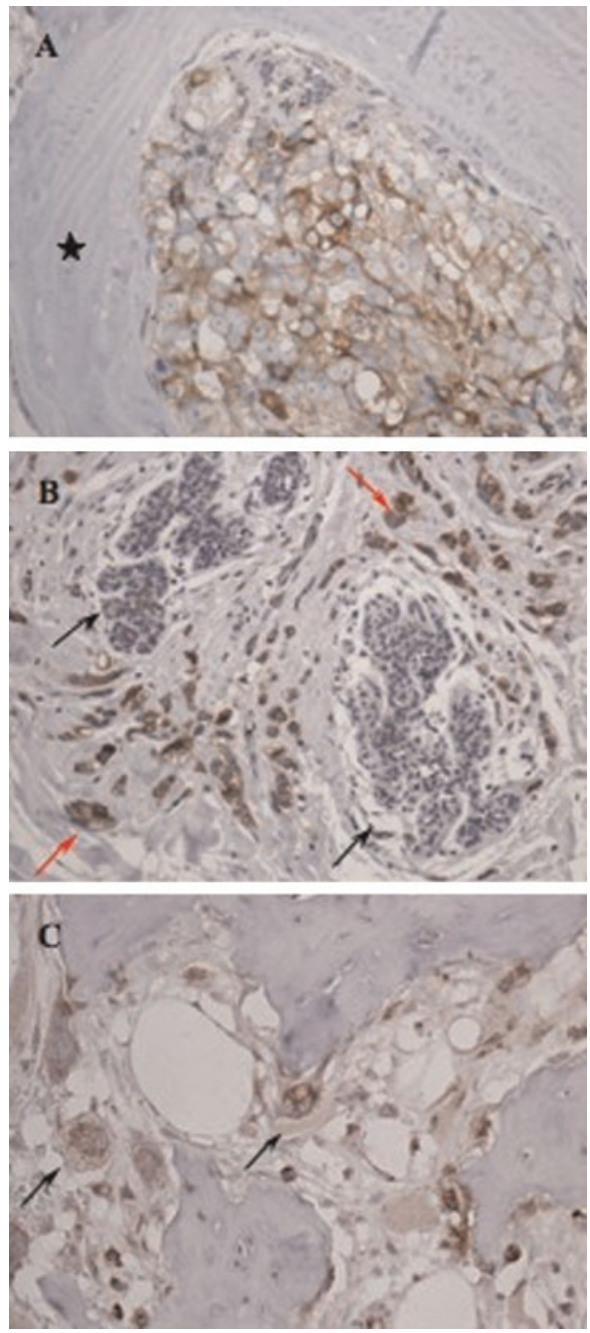

Fig. 1. Immunohistochemical results. Image A shows a prostatic bone metastasis in which the majority of tumor cells are positive for RANK immunostaining. The black star is placed on the peripheral bone tissue. In image B, one of the breast cancer studied. The tumor cells are strongly positive for RANK immunostaining (red arrows). The normal glandular epithelium is negative or weakly positive (black arrows) confirming the RANK protein overexpression of the tumor tissue. Image C shows osteoclast cells positive for RANK staining (black arrows). This picture is taken from a perimetastatic area. Original magnification A,C 400×, B 100×. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

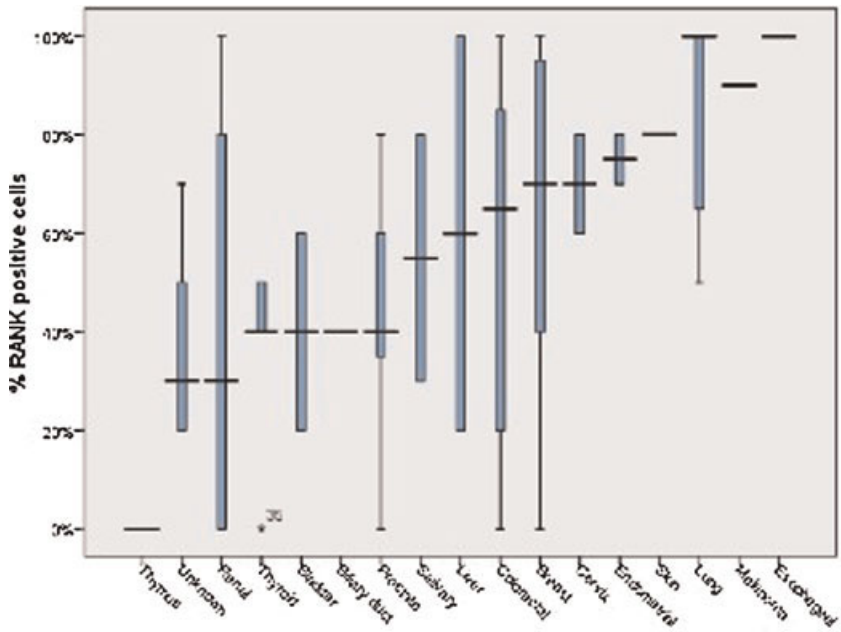

Fig. 2. shows the distribution of RANK-positive cells among the bone metastases.

observed a median percentage of RANK-positive cells of 60% for the "primary" and 55% for the "metastasis" group ($P = 0.528$) (Fig. 5). Moreover, considering only patients with breast cancer (14 primaries and 19 bone metastases), we did not observe any statistically significant difference ($P = 0.854$) between number of RANK-positive cells in primary (65%) versus bone metastasis (70%). No statistical differences were found between primary and metastatic prostate and renal cancers ($P = 0.775$ and 0.925, respectively).

Conclusions

RANK/RANKL/OPG pathway represents a new therapeutic target in the treatment of osteoporosis and in the risk reduction of skeletal related events in bone metastatic cancer patients (Cummings et al., 2009; Fizazi et al., 2009; Smith et al., 2009). Our study shows that RANK is expressed in a wide percentage of bone metastases deriving from several different primary histotypes and in a large part of corresponding primary solid

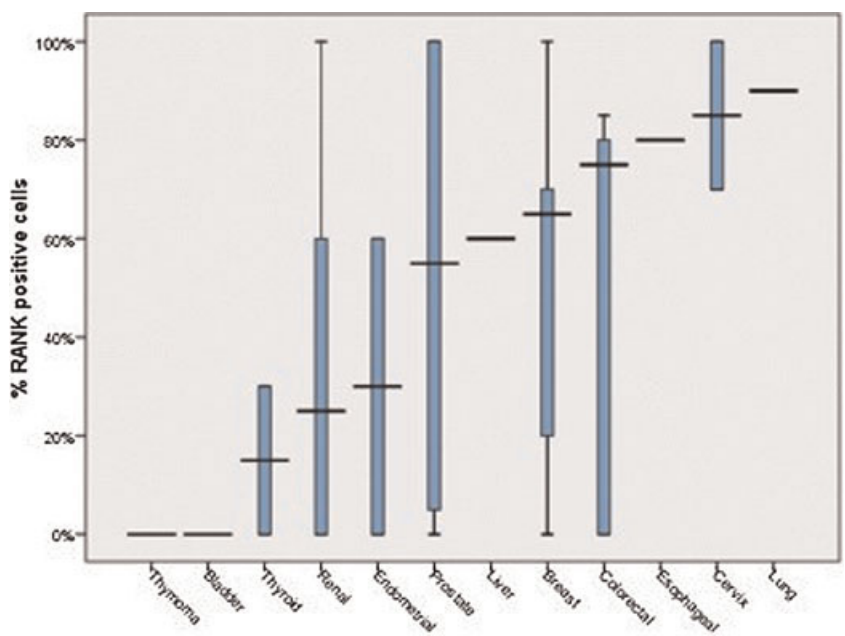

Fig. 3. Distribution of RANK-positive cells among the primitive tumors.

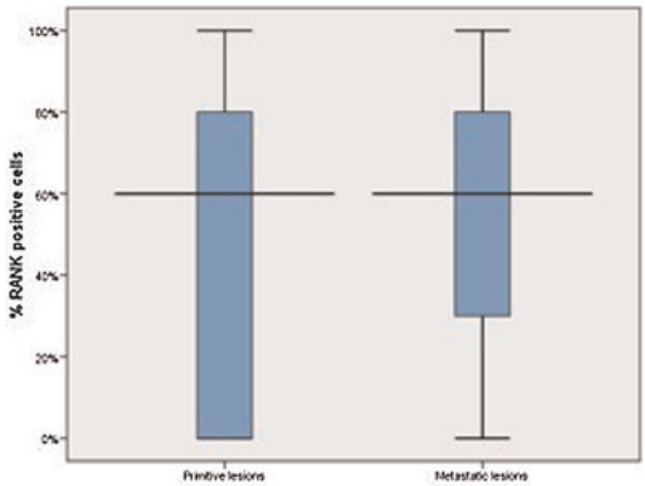

Fig. 4. Comparison of the RANK expression between "primary" and "metastasis" considering all the samples. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

tumors. Further studies are obviously necessary to confirm these data and to verify the functional importance of RANK expression in each histotype. However, these results open a new scenario in which RANK/RANKL/OPG pathway could become an optimal therapeutic target for anticancer therapy. Moreover, we observed that RANK expression in primary tumors and in the related bone metastases differs according to the histotype and not according to the metastatization process or to the bone biological microenvironment. There are tumors expressing high levels of RANK both in primary and bone metastasis (e.g., breast and colorectal cancers) compared to others expressing low levels in both sites (e.g., prostate cancer). Unfortunately, the heterogeneous distribution of primaries did not allow a statistical analysis to compare the different levels of RANK expression according to the histological origin of tumors.

However, this study clearly shows for the first time in literature that, considering all primary sites, RANK expression

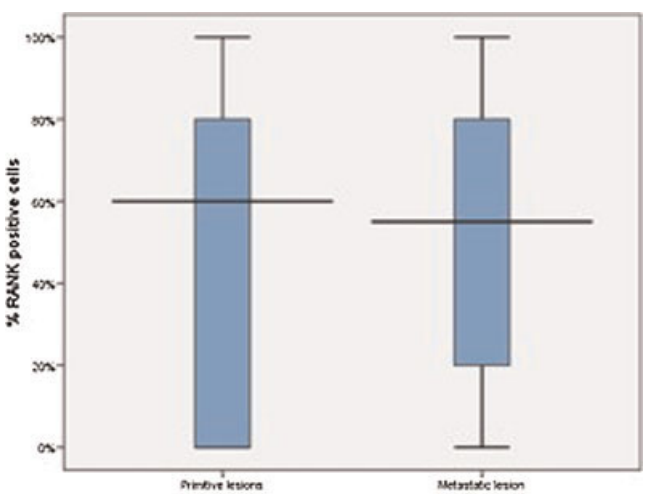

Fig. 5. Comparison of the RANK expression between "primary" and "metastasis" considering only the samples with both the "primary" and the "metastasis" available. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

level in bone metastases is, on average, not significantly different from the level detected in the related primary tumors. For this reason, the amount of RANK expression in bone metastases seems to be histotype-dependent. In fact, we found the same concordance in RANK expression when we compared only primary and related bone metastasis pairs, excluding the bone metastases whose the corresponding primary tumors samples were not available.

There are no previous studies comparing RANK expression in bone metastatic tissue versus primary tumor tissue from the same patient. If we consider previous series (comparing bone metastasis with primary tumor from different patients), as for prostate cancer, our results differ from those obtained by Chen et al. (2006). In particular, their study showed a greater RANK expression in bone metastases compared with primary cancer, while our work demonstrated a very similar level of expression in primary cancer and in bone metastatic lesions. On the contrary, regarding breast cancer, our data are consistent with the observations from Bhatia et al. (2005), showing an almost complete concordance of RANK expression between primary tumors and related bone metastases.

In conclusion, on the basis of our data, RANK is expressed in a large range of primary tumors and related bone metastases. Whether this expression could represent a valid therapeutic target, not only in bone metastasis management, but also as treatment in advanced and adjuvant setting, is still an unanswered question that needs to be investigated by prospective randomized clinical trials.

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