Hyaluronan in the cerebrospinal fluid of patients with spinal tumor

Endogenous Secretory Receptor for Advanced Glycation End-products as a Novel Prognostic Marker in Chondrosarcoma

Running title: esRAGE as a novel diagnostic and prognostic marker in chondrosarcoma

Akihiko Takeuchi MD [1, 2, 5], Yasuhiko Yamamoto MD, PhD [2], Koichi Tsuneyama MD, PhD [3], Chunmei Cheng MD, PhD [3], Hideto Yonekura PhD [2, 4], Takuo Watanabe MD, PhD [2], Katsuji Shimizu MD, DMSc [5], Katsuro Tomita MD, PhD [1], Hiroshi Yamamoto MD, PhD [2], and Hiroyuki Tsuchiya MD, PhD [1 *]

1. Department of Orthopaedic Surgery, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan

2. Department of Biochemistry and Molecular Vascular Biology, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan

3. Department of Pathology, School of Medicine, University of Toyama, Toyama, Japan

4. Department of Biochemistry, Kanazawa Medical University, Uchinada, Japan

5. Department of Orthopaedic Surgery, Gifu University Graduate School of Medicine, Gifu, Japan.

* Correspondence to Hiroyuki Tsuchiya MD, PhD, Department of Orthopaedic Surgery, Kanazawa University Graduate School of Medical Science, 13-1 Takara-machi, Kanazawa 920-8641, Japan;

e-mail: tsuchi@med.kanazawa-u.ac.jp

telephone: +81-76-265-2374

fax: +8176-234-4261

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ABSTRACT

BACKGROUND.

Chondrosarcoma, the second most frequent primary malignant bone tumor, is classified into three grades according to histologic criteria of malignancy. However, a low-grade lesion can be difficult to distinguish from a benign enchondroma, while some histologically low-grade lesions may carry a poor prognosis. We quantified the receptor for advanced glycation end-products (RAGE) and its ligand, high-mobility group box (HMGB) 1 in enchondromas and chondrosarcomas, to determine whether these markers were associated with histological malignancy and prognosis.

METHODS.

Enchondromas (n=20) and typical chondrosarcomas (n=39) were evaluated for RAGE, endogenous secretory RAGE (esRAGE, a splice variant form), and HMGB1 protein expression by immunohistochemistry including laser confocal microscopy. Content of esRAGE in resected specimens was measured with an enzyme-linked immunosorvent assay. Associations of these molecules with histology and clinical behavior of tumors were analyzed.

RESULTS.

Expression of esRAGE and HMGB1 was observed in all specimens. Numbers of cells positive for esRAGE and HMGB1 expression were positively associated with histologic grade. Expression of esRAGE was significantly higher in chondrosarcomas than in enchondromas ($P < .001$). Tissue esRAGE content also was significantly higher in grade 1 and 2 chondrosarcomas than enchondromas ($P = .0255$ and $P = .008$, respectively). High expression of esRAGE in grade 1 chondrosarcoma was associated with subsequent recurrence ($P = .0013$), lung metastasis ($P = .0071$), and poor survival ($P < .001$).

CONCLUSIONS.

Assessment of esRAGE expression should aid in diagnostic and prognostic determinations in

chondrosarcoma.

Key words: receptor for advanced glycation endproducts (RAGE), endogenous secretory RAGE (esRAGE), high-mobility group box 1 (HMGB1), enchondroma, chondrosarcoma, diagnosis, prognostic marker

INTRODUCTION

Chondrosarcomas are the second most frequent primary tumor of bone.[1-4] On the basis of histologic features, typical chondrosarcomas are divided into grade 1 to 3,[3, 5, 6] a distinction long considered to correlate with clinical behavior.[3, 7] However, distinguishing a low-grade chondrosarcoma from a benign enchondroma solely from microscopic cytologic features often is impossible.[8, 9] Grade 1 chondrosarcomas tend toward slow growth, infrequent metastasis, and an overall favorable prognosis. However, since an occasional histologically low-grade tumor shows metastatic potential associated with poor prognosis, new diagnostic and prognostic markers for chondrosarcomas are needed.

The receptor for advanced glycation end-products (RAGE) is a multiligand receptor which has an extracellular region, consisting of one V-type and two C-type immunoglobulin-like domains, a transmembrane region, and a short C-terminal intracellular portion.[10, 11] The V-domain of RAGE is critical for binding of various ligands including advanced glycation end-products (AGE),[10-14] S100,[15, 16] β–amyloid,[17, 18] Mac-1,[19] and high-mobility group box-1 (HMGB1),[19-23] which have been implicated in diabetic vascular complications, neurodegenerative disorders, proinflammatory reactions, and cancer.[18] Among these ligands, HMGB1 has been linked with tumor growth, invasion, and metastasis through interactions with RAGE[20, 24] ; RAGE expression has been examined in gastric,[25] colorectal,[26] prostatic,[27, 28] lung,[26] and breast[26] cancers.

Recently, Yonekura *et al.*[29] I dentified a novel splice variant of RAGE - endogeneous secretory RAGE (esRAGE), which lacked a transmembrane region and represented a soluble form. This variant is considered to act as a modulating factor opposing RAGE-associated conditions mentioned above and various diseases including cancers. Immunohistochemistry using domain-specific antibodies for RAGE or esRAGE showed esRAGE to be distributed in a wide variety of normal human organs and tissues.[30] However, examination comparing RAGE to esRAGE expression have

not yet been reported in any type of cancer, including bone tumors.

In this study we investigated expression of RAGE and esRAGE as well as HMGB1 in surgical specimens of enchondroma and chondrosarcomas. We sought associations between expression of these molecules and the clinical behavior of these tumors.

PATIENTS AND METHODS

Patients

Resection sprecimens containing enchondromas of long bones (n=20) and specimens including typical chondrosarcomas (n=39, grade 1, 24cases; grade 2, 13; and grade 3, 2). Of 39 specimens in chondrosaroma, 30 primary tumors and individual 9 recurrent lesions were obtained from 30 patients. The data from the recurrent tumors were not used for further clinical outcome analyses. Specimens studied were obtained from 50 patients (27 male and 23 female), all treated at the Kanazawa University Hospital from 1988 to 2004. Locations of enchondromas and chondrosarcomas are shown in Table 1.

After histologically establishing the diagnosis, all enchondromas were treated by curettage and bone grafting. Chondrosarcomas were treated by en-bloc excision. The study was approved by the Ethics Committee for Medical Studies at the Kanazawa University Graduate School of Medical Science. Written informed consent was obtained from each subject, or their guardian.

Immunohistochemistry

Specimens used had been fixed in 20% formalin and embedded in paraffin. These were retrieved from the surgical pathology files of the Pathology Section of Kanazawa University Hospital, School of Medicine, Kanazawa University, Kanazawa, Japan. For each case one representative block of formalin-fixed, paraffin-embedded tumor tissue was selected. All specimens were decalcified. We found that the decalcification step did not influence the IHC for each of the stains. All sections were cut

at a 4-μm thickness for immunohistochemistry. As a control we used a uninvolved epiphyseal cartilage resected for osteosarcoma from a distal femur in an 8-year-old girl, because the epiphyseal chondrocytes has the active growth and differentiation potential, and some primary bone tumors such as osteosarcoma and chondrosarcoma often occur around the growth plate. Immunostaining procedures included a new microwave technique for antigen retrieval as described previously.[31] Primary antibodies used in this study included a goat polyclonal antibody against the V domain of RAGE proteins (Chemicon, Temecula, CA; dilution, 1:800) that recognized both full-length and esRAGE; a rabbit polyclonal antibody against the C-terminal 16 amino acids of esRAGE (dilution, 1:1000), recognizing only esRAGE; a rabbit polyclonal antibody against 20 C-terminal amino acids of RAGE representing the intracellular domain (Santa Cruz Biotech, Santa Cruz, CA; dilution, 1:100), which recognizing the full-length RAGE[29]; and goat polyclonal antibody against HMGB1 (Santa Cruz Biotech.; dilution, 1:50). Peroxidase-labeled polymers of antibodies raised against rabbit polyclonal antibody (EnVision, DAKO, Carpinteria, CA) or goat polyclonal antibody (Histone, Simple Stain., Nichirei, Tokyo, Japan) were used as a secondary antibody. After visualization of reaction product,, sections were counterstained with Meyer's hematoxylin and then coverslipped for microscopic observation. Areas with apparent brown staining were evaluated for positive immunostaining based on the manufacturer's instructions (Envision System, DAKO). In each case, all of positive and negative cells were counted in 5 non-overlapping visual fields at ×200 magnification. Labeling index (LI) for esRAGE and HMGB1 were calculated as a percentage of positive cells among the total number of cells counted at least 250 tumor cells.[32]

ELISA for esRAGE

We examined frozen tissue samples from 10 cases of enchondroma and 11 cases of chondrosarcoma (5 grade 1 cases, 6 grade 2 cases) from among the cases used for immunohistochemistry. Tissue samples were dissected and homogenized in a lysis buffer (1%

Nonidet P-40, 0.5% deoxycholate, 10 mM EDTA, 0.1% Sodium Dodecyl Sulfate, and 1.0 mM Phenylmethylsulfonyl Fluoride) also containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MI). The homogenate then was cleared of debris by centrifugation at 12,000 rpm for 30 min at 4°C. The supernatant was collected, and its total protein concentration was determined (BCA assay kit, Model 550, BioRad Laboratories, Hercules, CA). Consentrations pf esRAGE protein were determined using a human esRAGE enzyme-linked immunosorbent assay (ELISA) system (B-Bridge international, Sunnyvale, CA).[33] Twenty micrograms of protein (100 μl of homogenate) were used for this assay.. Content of esRAGE protein in tumor tissue is reported as picograms per gram of protein.

Confocal Microscopy

The procedure for tissue section treatment was the same as for immunohistochemistry. Primary antibodies used included goat polyclonal antibody against the V domain of RAGE (dilution, 1:800); rabbit polyclonal antibody specific for esRAGE (dilution, 1:1000); and goat polyclonal antibody against HMGB1 (dilution, 1:50). Secondary antibodies were chicken anti-rabbit IgG labeled with Alexa Fluor 488 (A-21441, Molecular Probes, Eugene, OR; dilution, 1:200) for esRAGE and donkey anti-goat IgG labeled with Alexa Fluor 556 (A-11056, Molecular Probes; dilution, 1:200) for the V domain of RAGE and HMGB1. A zeiss confocal microscope (objective lens power, x 63) and Bio-Rad 1024 software (Hercules, CA) were used to produce images.

Statistical Analysis

Analyses were performed using Stat View (Version 5.0; SAS Institute, Cary, NC). LI of esRAGE and HMGB1 obtained by immunostaining and concentrations of esRAGE protein according to ELISA were compared with histologic tumor grades of enchondroma and chondrosarcomas by Fisher's exact test. Correlations between the two variables (esRAGE LI and HMGB1 LI) were analyzed using the Pearson correlation coefficient. Associations esRAGE and

HMGB1 LI with subsequent tumor recurrence and distant metastasis were analyzed by Fisher's exact test. Univariate analysis of time to death as a result of chondrosarcomas was carried out using a product-limit procedure (Kaplan-Meier method) and a log-rank test, based on esRAGE and HMGB1 LI.

RESULTS

Immunohistochemistry for RAGE, esRAGE, and HMGB1

We detected expression of esRAGE (Fig. 1a to e) and HMGB1 (Fig. 1f to j) in all specimens examined in this study, including normal epiphyseal cartilage, enchondroma, and chondrosarcoma. In an epiphyseal cartilage, the expressions of esRAGE and HMGB1 were observed in the chondrocytes from the proliferating through the hypertrophic zones to the calcifying zone, but very weak signals in the resting zone. Contrary to our expectation, reaction product staining intensity was very weak in enchondroma and chondrosarcoma when specimens were stained with antibody against the intracellular domain of RAGE (data not shown). Confocal microscopic distribution of the goat polyclonal antibody against the V domain of RAGE proteins, which recognized both RAGE and esRAGE proteins, was compared with distribution of the esRAGE-specific antibody, indicating that total RAGE signals overlapped with esRAGE signals (Fig. 2a-c). A diffuse cytoplasmic esRAGE staining pattern was observed in all tumor cells, but strong staining occupying the entire cytoplasm was present only in chondrosarcomas. The esRAGE LI owas 15.7% in normal epiphyseal cartilage (control), 33.5 ± 11.4% in enchondroma, 67.7 ± 12.1% in grade 1 chondrosarcoma, 79.0 ± 7.7% in grade 2, and 93.6 ± 2.2% in grade 3 (mean ± SD; Fig. 1k). The esRAGE LI was found to increase as tumor grade advanced. Significant differences in esRAGE LI were seen not only between grade 1 chondrosarcomas and enchondromas ($P < .0001$) but also between in grade 1 and grade 2 chondrosarcomas ($P = .0039$; Fig. 1k). Faint cytoplasmic staining for esRAGE was encountered in

scattered chondrocytes of the normal control cartilage.

HMGB1 LI was 6.0% in normal epiphyseal cartilage, 42.2 ± 19.0% in enchondroma, 61.8 ± 19.5% in grade 1 chondrosarcoma, 75.9 ± 11.1% in grade 2 and 77.9 ± 7.7% in grade 3 (mean ± SD; Fig. 1l). HMGB1 LI also was found to increase as tumor grade advanced;. HMGB1 LI was significantly higher in grade 1 chondrosarcoma than in enchondroma (P = .0006), and a significant difference in HMGB1 LI was noted between grade 1 and grade 2 chondrosarcomas (P = .024; Fig. 1l). A significant positive correlation was found between HMGB1 and esRAGE expression in enchondromas and chondrosarcomas ($y = 0.532x + 27.283$, $r = 0.547$, $P < .0001$; Figure 1m).

HMGB1-positive cells also were stained by esRAGE-specific antibody. In contrast, signals for esRAGE and HMGB1 were not colocalized in chondrosarcoma cells examined by confocal microscopy (Fig. 2d-f). A cultured chondrosarcoma cell line H-EMC-SS showed diffuse cytoplasmic staining by esRAGE- and HMGB1- specific antibody, but these signals did not necessarily merge (data not shown).

Quantitative Evaluation of esRAGE Protein by ELISA

To quantitatively evaluate the esRAGE expression in surgical specimens, an esRAGE ELISA was performed in extracts from enchondroma (n=10), and from grade 1 (n=5) and grade 2 (n=6) chondrosarcomas. Concentrations of esRAGE protein in enchondroma were significantly lower than in grade 1 or grade 2 chondrosarcomas (P = .0255 and P = .008, respectively) (Fig. 3). These findings support observations made by esRAGE immunostaining.

Correlation between esRAGE Expression and Clinical Outcome of Chondrosarcomas

A significant association was present between esRAGE LI and subsequent tumor recurrence in grade 1 chondrosarcoma (P = .0013; Fig. 4a). Mean esRAGE LI in chondrosarcomas with metastasis was significantly higher than in those without metastasis (P = .0071; Fig. 4d); this difference was most evident for grade 1 chondrosarcomas (P = .0033; Fig. 4c). A significant difference in survival

time was evident between high (≥ 70%) and low (< 70%) esRAGE LI in patients with chondrosarcomas (P = .0017; Fig. 5a). HMGB1 LI was not significantly associated with clinical features or prognosis in patients with chondrosarcoma (Figs. 4a to d and 5b).

DISCUSSION

RAGE has been associated with prognosis in several types of cancers such as those of lung,[26] breast,[26] and prostate[27, 28] as well as melanoma.[26] However, RAGE expression and its clinical importance in bone tumors had not yet been analyzed. In this study, we selectively detected protein expression of esRAGE and RAGE as well the expression of their ligand, HMGB1, in human enchondroma and chondrosarcoma specimens. Recently esRAGE has been identified as a novel splice variant of RAGE mRNA coding for a soluble form of RAGE.[29] Considered a modulating factor in cancer phenotypes, esRAGE can trap ligands outside of cells without initiating signal transduction within cells. When Cheng, *et al*.[30] performed a systemic analysis of esRAGE expression in various human tissues and organs, esRAGE expressions showed patterns distinctive for individual organs including brain, thyroid, salivary gland, lung, pancreas, and others. We hypothesized that a balance between RAGE and esRAGE expression might be a factor modifying RAGE-associated malignant phenotypes.

Relative expression of esRAGE in chondrosarcoma was associated with histologic grade, recurrence, lung metastasis, and clinical outcome; yet RAGE expression was very weak in both enchondromas and chondrosarcomas. Confocal microscopic analysis comparing reactive to a pan-RAGE antibody and to an esRAGE-specific antibody indicated that the form of RAGE predominantly expressed in bone tumors was esRAGE as opposed to membrane-bound RAGE in bone tumors. Relative expression of HMGB1 in chondrosarcomas was associated with histologic grade but not with lung metastasis or clinical outcome. Colocalization of esRAGE and HMGB1 was

not seen, suggesting that their actions were distinct and that esRAGE could not take part in ligand-initiated signal transduction. Mechanisms causing esRAGE and HMGB1 up-regulation in chondrosarcoma remain to be investigated.

We next focused on esRAGE expression as a diagnostic marker and as a predictor of clinical features including prognosis. Histopathologically, chondrosarcomas are divided into three grades based on cellularity, atypia, and pleomorphism.[3-5] The multiple subtypes of chondrosarcoma based on histologic grades have been considered predictive of clinical outcome.[3, 7, 34, 35] However, difficulty often is encountered even in distinguishing, a low-grade chondrosarcoma from an enchondroma.[8, 9] on the other hand, even though, grade 1 chondrosarcomas have been considered to have a favorable prognosis, occasional grade 1 lesions develop metastases resulting in a poor survival outcome. Accordingly, many markers and tests have been examined for ability to distinguish low-grade chondrosarcoma from enchondroma and to predict clinical outcome in chondrosarcoma. Methods have involved DNA content,[36, 37] molecular markers (MIB-1,[38, 39] p53,[40] or hTERT[41]), cytogenetics,[42] and morphometry.[43] Previously, Tsuchiya *et al.*[44] reported histological and clinical findings in six cases of borderline chondrosarcoma in which they examined expression of type I, II, III, V, and VI collagen, immunohistochemically. Absence of tumor lobule rims containing collagen types I and V was useful in distinguishing borderline chondrosarcoma from enchondroma. In our present study, two grade 1 chondrosarcomas (in the radius of a 58-year-old man, case 1; in the femur of a 25-year-old woman, case2) appeared to be borderline chondrosarcomas. LIs for esRAGE and HMGB1 representively were 58.2% and 61.5% in case 1 and 64.1% and 69.8% in case 2. The patients were treated by curettage and bone grafting based on a frozen-section diagnosis of enchondroma. Fortunately no recurrence or metastasis occurred subsequently in either case. Further investigation is required concerning expression of esRAGE in very low-grade chondrosarcomas such as borderline[44] or grade 1/2 lesions.[45] Distinguishing an enchondroma from low-grade

chondrosarcoma is among the most important aspects of diagnosis of chondrosarcoma, both for planning the surgical procedure and for predicting outcome.

In this study, esRAGE protein was detected in enchondroma and chondrosarcoma by immunohistochemistry and ELISA. The esRAGE LI rose as the histologic grade of the chondrosarcoma advanced. A few esRAGE-immunopositive cells were observed in normal epiphyseal cartilage used as a control. Staining patterns of esRAGE included a punctuate paranuclear pattern and diffuse cytoplasmic staining. Various intensities of diffuse cytoplasmic staining were observed in enchondroma and chondrosarcoma cells. Strong staining occupying the entire cytoplasm area was detected only in chondrosarcomas. Accordingly, esRAGE LI should be a useful marker for distinguishing low-grade from high-grade tumors. Further, esRAGE LI correlated with tumor recurrence and lung metastasis in grade 1 chondrosarcomas (P=.0013 and .0033). Generally, grade 1 chondrosarcoma is considered to grow slowly, metastasize infrequently, and carry a favorable prognosis.[8] However, in our series 4 of the 17 patients with grade 1 chondrosarcoma developed lung metastasis (25%), underscoring that even when the histological diagnosis is grade 1, a few cases might have potential for progression. All 4 of these cases showed an esRAGE LI of at least 70%. Analysis of cumulative survival rates based on esRAGE LI demonstrated that patients with a high esRAGE of at least 70% may have a poor prognosis. Although further studies are required to understand the functional roles of esRAGE and mechanisms of its induction in chondrosarcoma, esRAGE expression appears to be a promising marker in predicting clinical outcome.

In conclusion, we first found that esRAGE indeed was expressed in tumors of cartilage, and that relative expression of this protein was associated with histologic tumor grade and cumulative survival rate. The esRAGE labeling index could be a useful complement to routine histologic grading as a predictor of lung metastasis and survival outcome. Our data therefore supported esRAGE as a novel diagnostic and prognostic marker in chondrosarcoma. Further large-scale prospective studies

are warranted to further validate this marker as a routine diagnostic and prognostic tool in assessment of cartilage neoplasms.

REFERENCES

1. Bauer HC, Brosjo O, Kreicbergs A, Lindholm J. Low risk of recurrence of enchondroma and low-grade chondrosarcoma in extremities. 80 patients followed for 2-25 years. *Acta Orthop Scand.* 1995;66:283-288.

2. Campanacci M. *Bone and Soft Tissue Tumors*. 1st ed. New York: Springer-Verlag, Inc.,1990:265-338.

3. Evans HL, Ayala AG, Romsdahl MM. Prognostic factors in chondrosarcoma of bone: a clinicopathologic analysis with emphasis on histologic grading. *Cancer.* 1977;40:818-831.

4. Healey JH, Lane JM. Chondrosarcoma. *Clin Orthop Relat Res*. 1986;204:119-129.

5. Enneking WF. A system of staging musculoskeletal neoplasms. *Clin Orthop Relat Res*. 1986;204:9-24.

6. Mankin HJ, Cantley KP, Schiller AL, Lippiello L. The biology of human chondrosarcoma. II. Variation in chemical composition among types and subtypes of benign and malignant cartilage tumors. *J Bone Joint Surg Am*. 1980;622:176-188.

7. Dahlin DC, Henderson ED. Chondrosarcoma, a surgical and pathological problem; review of 212 cases. *J Bone Joint Surg Am*. 1956;38-A:1025-1038.

8. Dorfman HD, Czerniak B: *Bone Tumors*. St. Louis, Mosby, Inc., 1998: 353-440.

9. Lane JM: Malignant bone tumors. In Alfonson AE, and Gardner, B, editors. *The Practice of Cancer Surgery*. New York, Appleton-Century-Cofts, Inc., 1982: 307-324.

10. Schmidt AM, Yan SD, Yan SF, Stern DM. The multiligand receptor RAGE as a progression factor amplifying immune and inflammatory responses. *J Clin Invest.* 2001;1087:949-955.

11. Neeper M, Schmidt AM, Brett J, et al. Cloning and expression of a cell surface receptor for advanced glycosylation end products of proteins. *J Biol Chem*. 1992;267:14998-15004.

12. Schmidt AM, Mora R, Cao R, et al. The endothelial cell binding site for advanced glycation end

products consists of a complex: an integral membrane protein and a lactoferrin-like polypeptide. *J Biol Chem*. 1994;269:9882-9888.

13. Brownlee M, Cerami A, Vlassara H. Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. *N Engl J Med*. 1988;318:1315-1321.

14. Thornalley PJ. Cell activation by glycated proteins. AGE receptors, receptor recognition factors and functional classification of AGEs. *Cell Mol Biol (Noisy-le-grand)*. 1998;44:1013-1023.

15. Hofmann MA, Drury S, Fu C, et al. RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. *Cell*. 1999;97:889-901.

16. Moroz OV, Antson AA, Dodson EJ, et al. The structure of S100A12 in a hexameric form and its proposed role in receptor signalling. *Acta Crystallogr D Biol Crystallogr*. 2002;58:407-413.

17. Du Yan S, Zhu H, Fu J, et al. Amyloid-beta peptide-receptor for advanced glycation endproduct interaction elicits neuronal expression of macrophage-colony stimulating factor: a proinflammatory pathway in Alzheimer disease. *Proc Natl Acad Sci U S A*. 1997;94:5296-5301.

18. Yan SD, Chen X, Fu J, et al. RAGE and amyloid-beta peptide neurotoxicity in Alzheimer's disease. *Nature*. 1996;382:685-691.

19. Chavakis T, Bierhaus A, Al-Fakhri N, et al. The pattern recognition receptor (RAGE) is a counterreceptor for leukocyte integrins: a novel pathway for inflammatory cell recruitment. *J Exp Med*. 2003;198:1507-1515.

20. Taguchi A, Blood DC, del Toro G, et al. Blockade of RAGE-amphoterin signalling suppresses tumour growth and metastases. *Nature*. 2000;405:354-360.

21. Hori O, Brett J, Slattery T, et al. The receptor for advanced glycation end products (RAGE) is a cellular binding site for amphoterin. Mediation of neurite outgrowth and co-expression of rage and amphoterin in the developing nervous system. *J Biol Chem*. 1995;270:25752-25761.

22. Wang H, Bloom O, Zhang M, et al. HMG-1 as a late mediator of endotoxin lethality in mice.

Science. 1999;285:248-251.

23. Treutiger CJ, Mullins GE, Johansson AS, et al. High mobility group 1 B-box mediates activation of human endothelium. *J Intern Med*. 2003;254:375-385.

24. Huttunen HJ, Fages C, Kuja-Panula J, Ridley AJ, Rauvala H. Receptor for advanced glycation end products-binding COOH-terminal motif of amphoterin inhibits invasive migration and metastasis. *Cancer Res*. 2002;62:4805-4811.

25. Kuniyasu H, Oue N, Wakikawa A, et al. Expression of receptors for advanced glycation end-products (RAGE) is closely associated with the invasive and metastatic activity of gastric cancer. *J Pathol*. 2002;196:163-170.

26. Hsieh HL, Schafer BW, Sasaki N, Heizmann CW. Expression analysis of S100 proteins and RAGE in human tumors using tissue microarrays. *Biochem Biophys Res Commun*. 2003;307:375-381.

27. Kuniyasu H, Chihara Y, Kondo H, Ohmori H, Ukai R. Amphoterin induction in prostatic stromal cells by androgen deprivation is associated with metastatic prostate cancer. *Oncol Rep*. 2003;10:1863-1868.

28. Hermani A, Hess J, De Servi B, et al. Calcium-binding proteins S100A8 and S100A9 as novel diagnostic markers in human prostate cancer. *Clin Cancer Res*. 2005;11:5146-5152.

29. Yonekura H, Yamamoto Y, Sakurai S, et al. Novel splice variants of the receptor for advanced glycation end-products expressed in human vascular endothelial cells and pericytes, and their putative roles in diabetes-induced vascular injury. *Biochem J*. 2003;370:1097-1109.

30. Cheng C, Tsuneyama K, Kominami R, et al. Expression profiling of endogenous secretory receptor for advanced glycation end products in human organs. *Mod Pathol*. 2005;18:1385-1396.

31. Kumada T, Tsuneyama K, Hatta H, Ishizawa S, Takano Y. Improved 1-h rapid immunostaining method using intermittent microwave irradiation: practicability based on 5 years application in

Toyama Medical and Pharmaceutical University Hospital. *Mod Pathol*. 2004;17:1141-1149.

32. Scotlandi K, Serra M, Manara MC, et al. Clinical relevance of Ki-67 expression in bone tumors. *Cancer*.1995;75:806-814.

33. Sakurai S, Yamamoto Y, Tamei H, et al. Development of an ELISA for esRAGE and its application to type 1 diabetic patients. *Diabetes Res Clin Pract*. 2006;73:158-165.

34. Henderson ED, Dahlin DC. Chondrosarcoma of Bone--a Study of Two Hundred and Eighty-Eight Cases. *J Bone Joint Surg Am*. 1963;45:1450-1458.

35. Kreicbergs A, Boquist L, Borssen B, Larsson SE. Prognostic factors in chondrosarcoma: a comparative study of cellular DNA content and clinicopathologic features. *Cancer*. 1982;50:577-583.

36. Herget GW, Neuburger M, Adler CP. Prognostic significance of nuclear DNA content in chondrosarcoma. *Ann Diagn Pathol*. 2000;4:11-16.

37. Lee FY, Mankin HJ, Fondren G, et al. Chondrosarcoma of bone: an assessment of outcome. *J Bone Joint Surg Am*. 1999;81:326-338.

38. Nawa G, Ueda T, Mori S, et al. Prognostic significance of Ki67 (MIB1) proliferation index and p53 over-expression in chondrosarcomas. *Int J Cancer*. 1996;69:86-91.

39. Rizzo M, Ghert MA, Harrelson JM, Scully SP. Chondrosarcoma of bone: analysis of 108 cases and evaluation for predictors of outcome. *Clin Orthop Relat Res*. 2001;391:224-233.

40. Oshiro Y, Chaturvedi V, Hayden D, et al. Altered p53 is associated with aggressive behavior of chondrosarcoma: a long term follow-up study. *Cancer*. 1998;83:2324-2334.

41. Martin JA, DeYoung BR, Gitelis S, et al. Telomerase reverse transcriptase subunit expression is associated with chondrosarcoma malignancy. *Clin Orthop Relat Res*. 2004;426:117-124.

42. Dijkhuizen T, van den Berg E, Molenaar WM, et al. Cytogenetics as a tool in the histologic subclassification of chondrosarcomas. *Cancer Genet Cytogenet*. 1994;76:100-105.

43. Gruber HE, Marshall GJ, Kirchen ME, Menendez LR, Schwinn CP. Bone remodelling in the presence of chondrosarcoma: histomorphometry. *Acta Anat (Basel)*. 1993;148:1-7.

44. Tsuchiya H, Ueda Y, Morishita H, et al. Borderline chondrosarcoma of long and flat bones. *J Cancer Res Clin Oncol*. 1993;119:363-368.

45. Springfield DS, Gebhardt MC, McGuire MH. Chondrosarcoma: a review. *Instr Course Lect*. 1996;45:417-424.

FIGURE LEGENDS

Fig. 1. Immunohistochemistry for esRAGE (a to e) and HMGB1 (f to j). (a) and (f), normal epiphyseal cartilage; (b) and (g), enchondroma; (c) and (h), grade 1 chondrosarcoma; (d) and (i), grade 2 chondrosarcoma; (e) and (j), grade 3 chondrosarcoma. (k), esRAGE labeling index and histologic tumor grade. (l), HMGB1 labeling index and histologic tumor grade. (EN, enchondroma). (m), correlation between HMGB1 and esRAGE in enchondroma and chondrosarcoma ($y=0.532x+27.283$; $r=0.547$; $P<.0001$).

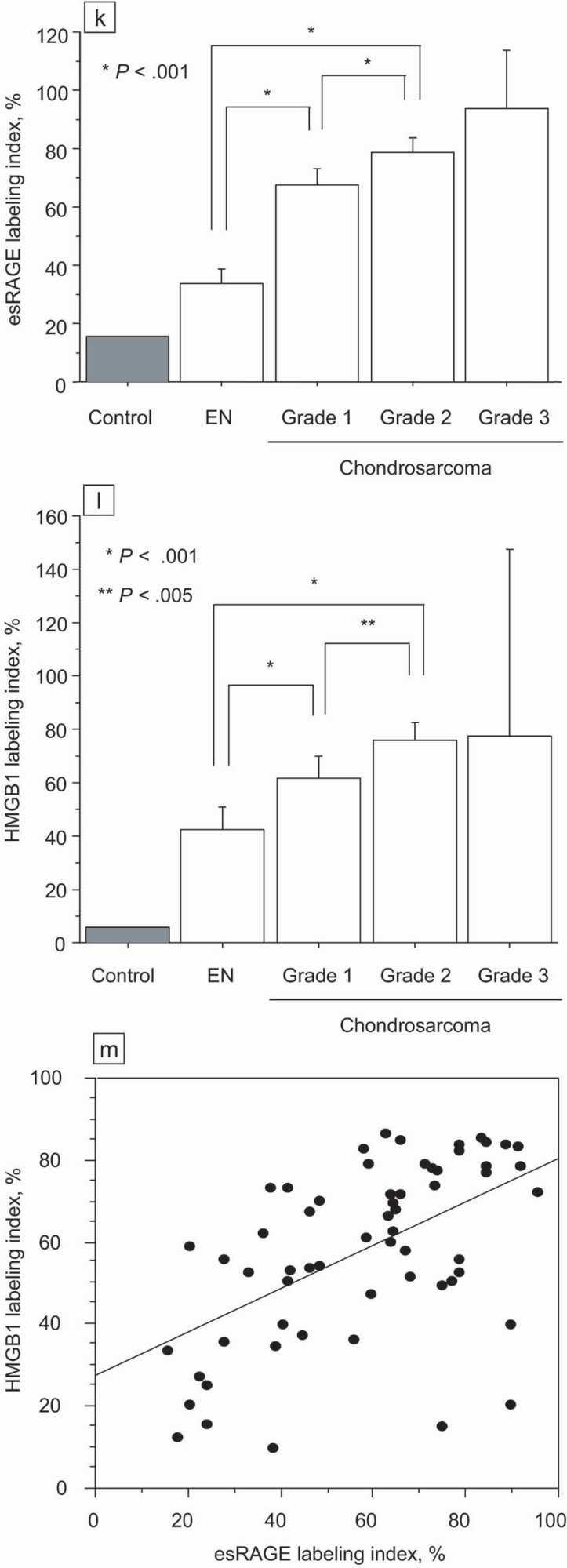
Fig. 2. Confocal microscopy. Total RAGE signal [red, (a)] and esRAGE signal [green, (b)] were colocalized in a chondrosarcoma specimen [(c), merged image]. Signals for HMGB1 [red, (d)] and esRAGE [green, (e)] were not colocalized in chondrosarcoma cells [(f), merged image].

Fig. 3. Quantitative evaluation of esRAGE protein in surgical specimens using an ELISA. Concentrations of esRAGE protein in enchondroma were significantly lower than in grade 1 or grade 2 chondrosarcomas ($P = .0255$ and $P = .008$, respectively).

Fig. 4. LI for esRAGE and HMGB1 in patients with chondrosarcoma showing tumor recurrence [(a), all grades; (b), grade 1] and lung metastasis [(c), all grades; (d), grade 1]. A significant association was present between esRAGE LI and subsequent tumor recurrence in grade 1 chondrosarcoma [$P = .0013$; (a)]. Mean esRAGE LI in chondrosarcomas with metastasis was significantly higher than in those without metastasis [$P = .0071$; (d)]; this difference was most evident for grade 1 chondrosarcomas [$P = .0033$; (c)].

Fig.5. Overall survival in patients with all grades of chondrosarcoma according to (a) esRAGE and

(b) HMGB1. A significant difference in survival time was evident between high (≥ 70%, a dotted line and filled square) and low (< 70%, a solid line and filled circles) esRAGE LI in patients with chondrosarcomas [*P* = .0017; (a)]. HMGB1 LI was not significantly associated with prognosis (b).


k
* P < .001
esRAGE labeling index, %
120
100
80
60
40
20
0
Control
EN
Grade 1
Grade 2
Grade 3
Chondrosarcoma
l
* P < .001
** P < .005
HMGB1 labeling index, %
160
140
120
100
80
60
40
20
0
Control
EN
Grade 1
Grade 2
Grade 3
Chondrosarcoma
m
HMGB1 labeling index, %
100
80
60
40
20
0
0
20
40
60
80
100
esRAGE labeling index, %

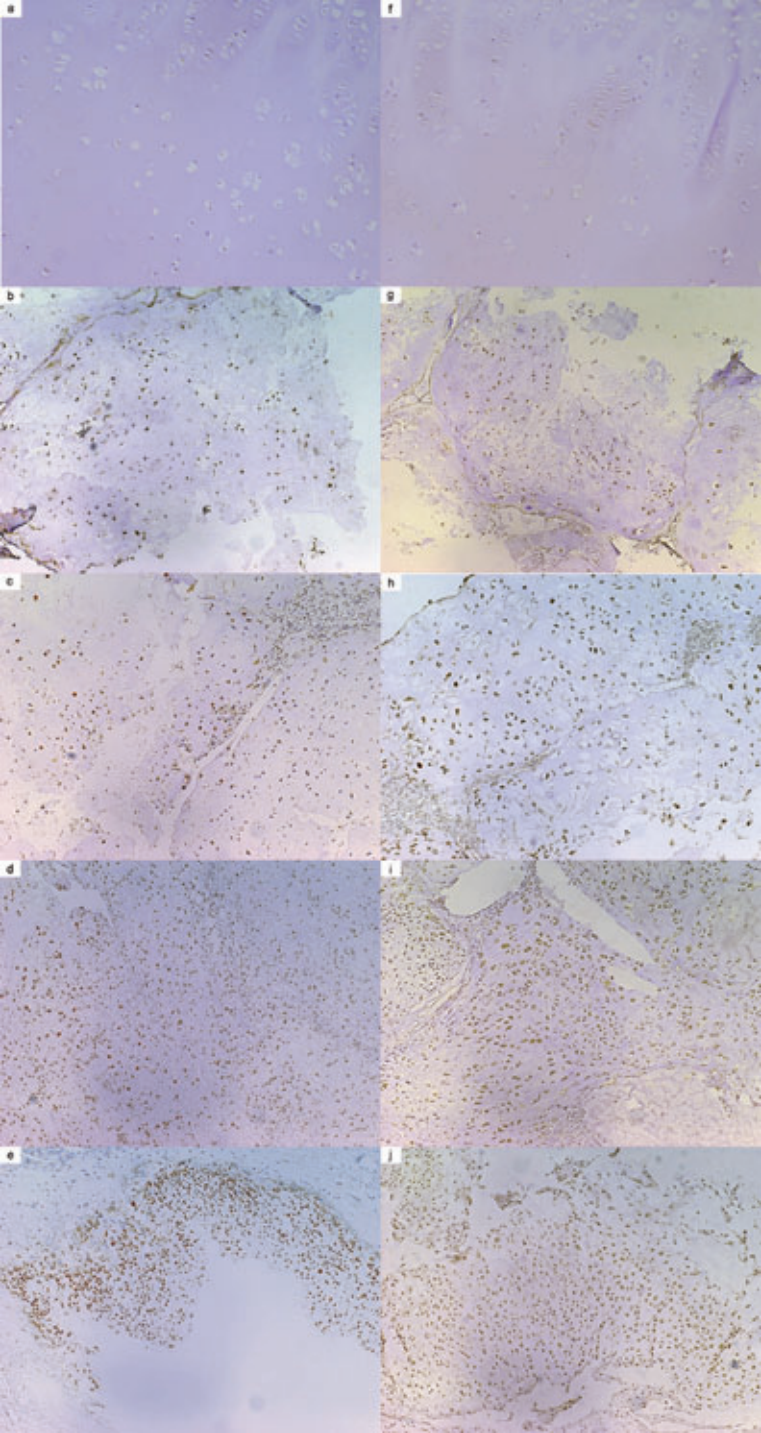
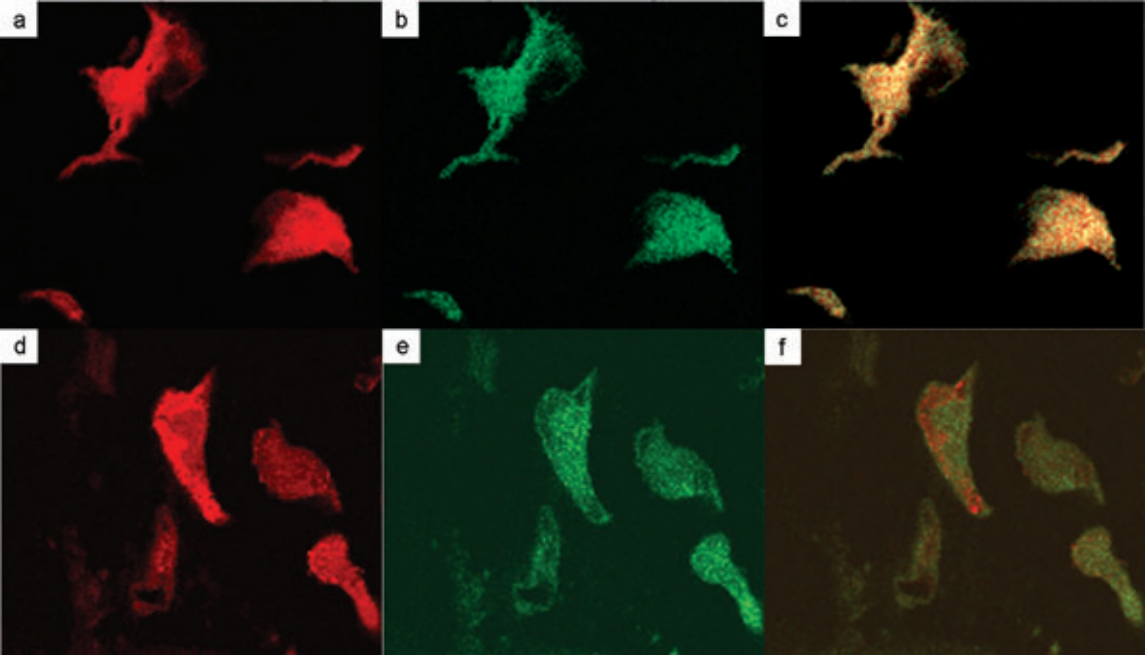
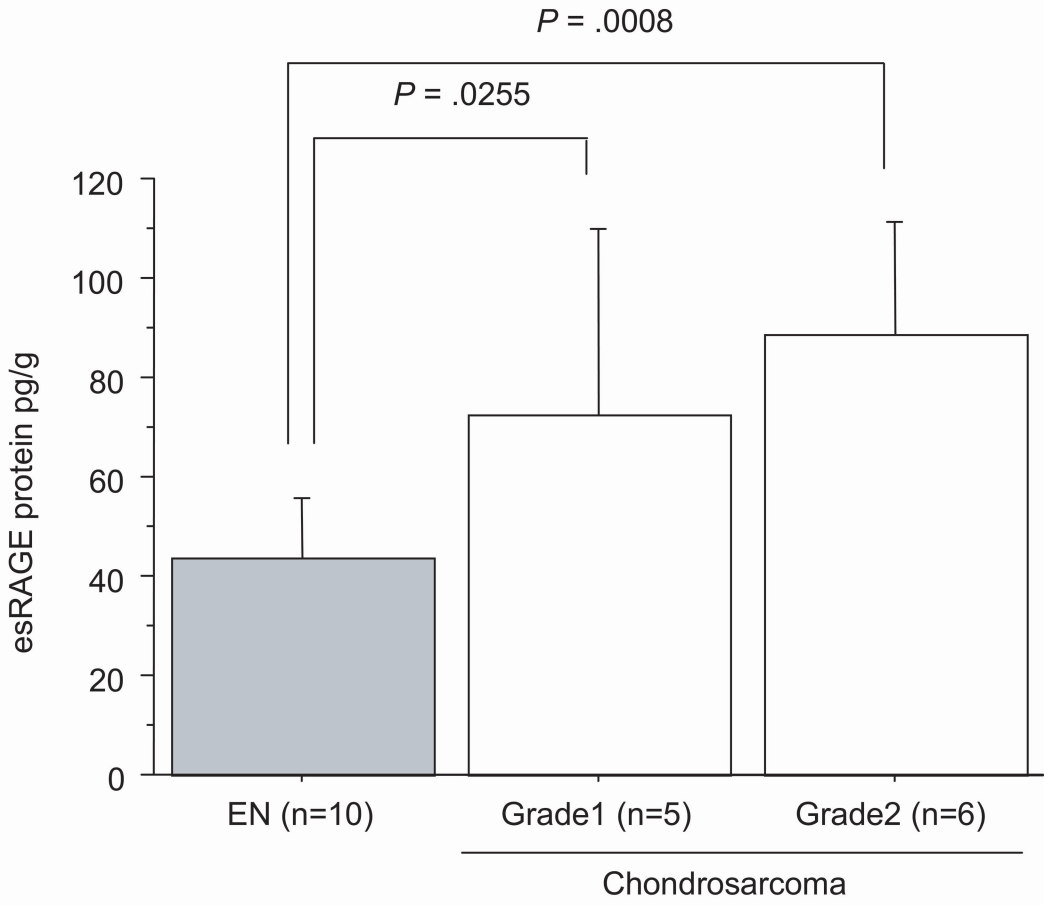
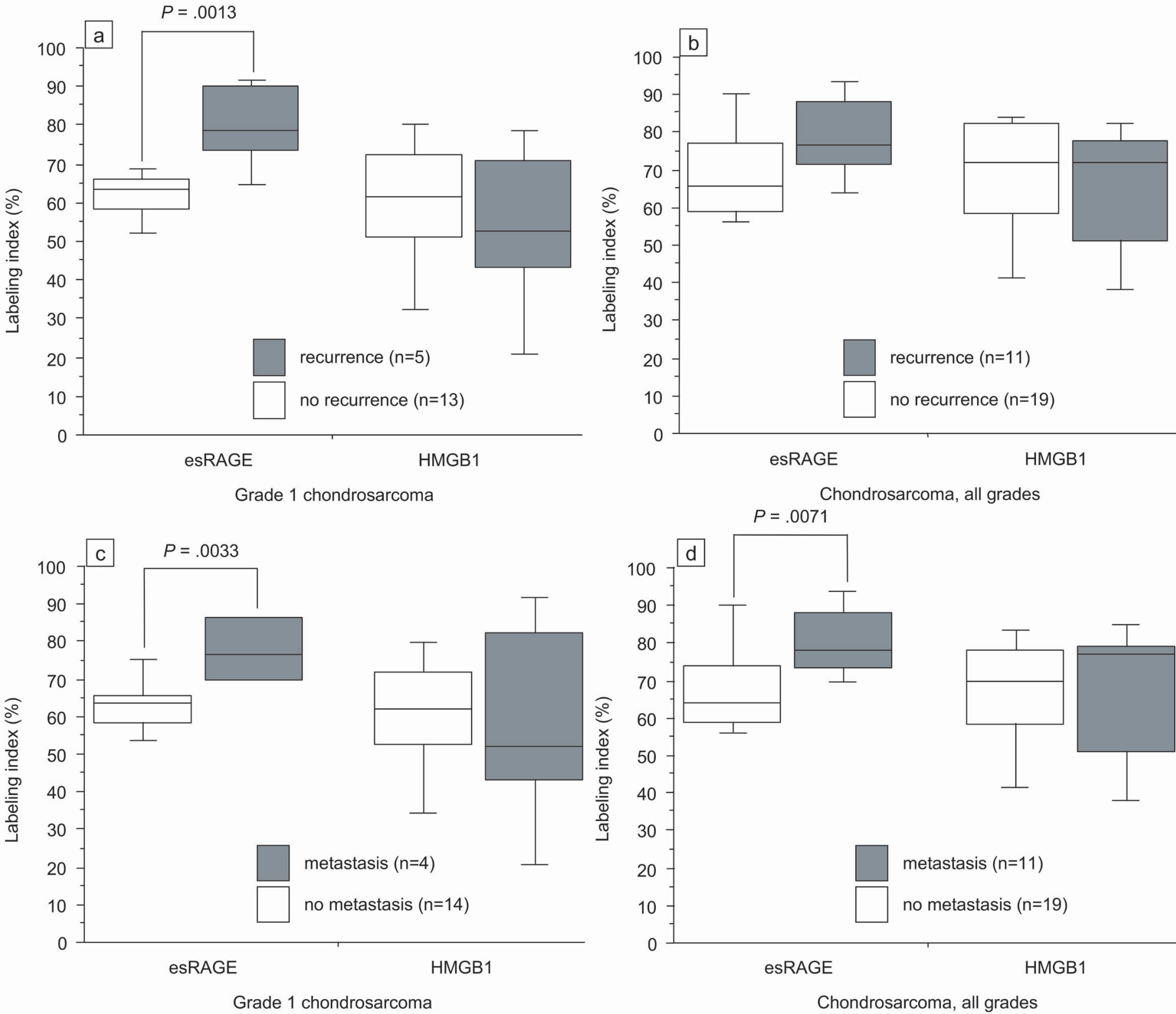

a
f
b
g
c
h
d
i
e
j

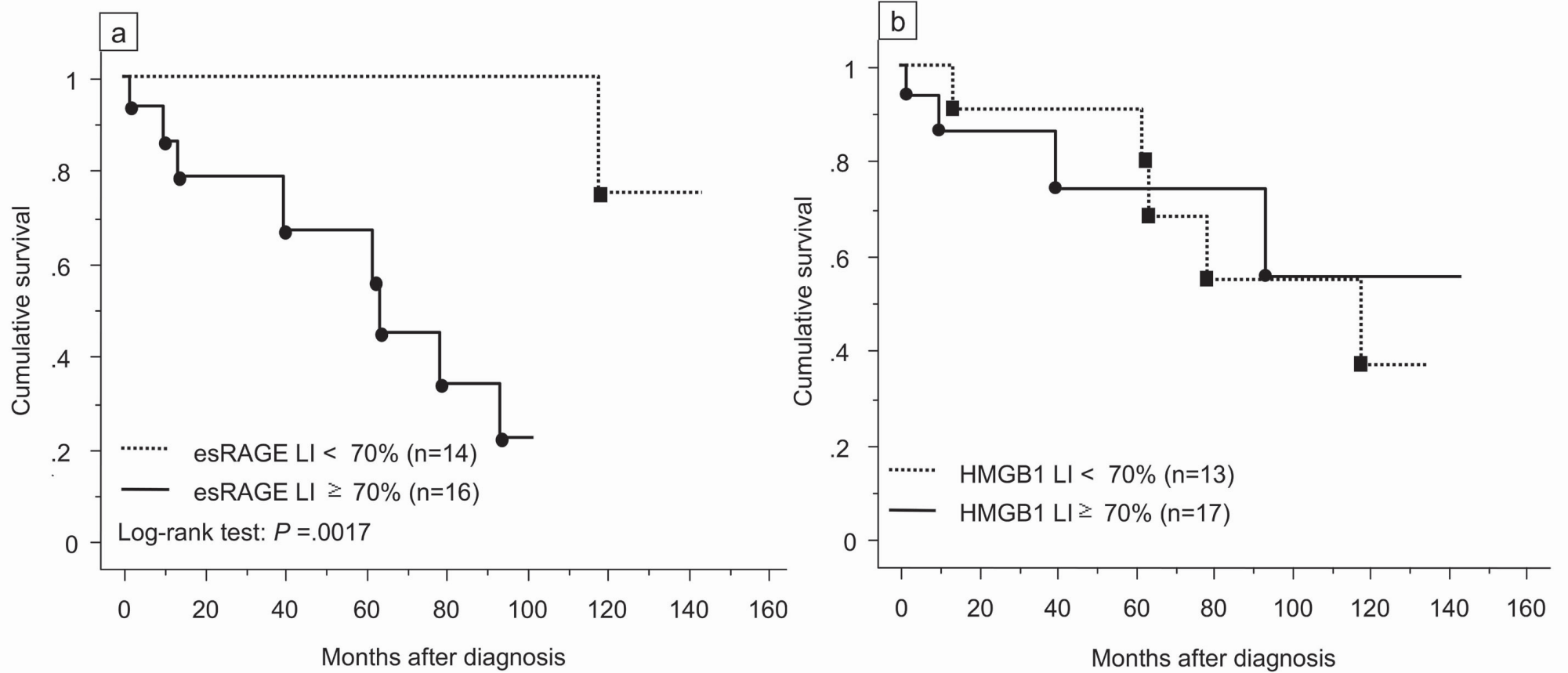
Table 1. Patient characteristics

	enchondroma	chondrosarcoma
Median age, years (range)	44.5 (6-63)	49.6 (7-81)
Gender		
male	10	17
female	10	13
Tumor Status		
primary	20	30
recurrence	0	9
Histologic grade		
1		24
2		13
3		2
Location		
humerus	7	4
forearm	1	1
femur	9	6
tibia	3	1
spine	0	4
scapula	0	2
rib	0	1
hand	0	2
pelvis	0	8
sternum	0	1


a
b
c
d
e
f


P = .0008
P = .0255
120
100
80
60
40
20
0
esRAGE protein pg/g
EN (n=10)
Grade1 (n=5)
Grade2 (n=6)
Chondrosarcoma


a
P = .0013
Labeling index (%)
recurrence (n=5)
no recurrence (n=13)
esRAGE
HMGB1
Grade 1 chondrosarcoma
b
Labeling index (%)
recurrence (n=11)
no recurrence (n=19)
esRAGE
HMGB1
Chondrosarcoma, all grades
c
P = .0033
Labeling index (%)
metastasis (n=4)
no metastasis (n=14)
esRAGE
HMGB1
Grade 1 chondrosarcoma
d
P = .0071
Labeling index (%)
metastasis (n=11)
no metastasis (n=19)
esRAGE
HMGB1
Chondrosarcoma, all grades


a
Cumulative survival
1
.8
.6
.4
.2
0
esRAGE LI < 70% (n=14)
esRAGE LI ≥ 70% (n=16)
Log-rank test: P =.0017
0
20
40
60
80
100
120
140
160
Months after diagnosis
b
Cumulative survival
1
.8
.6
.4
.2
0
HMGB1 LI < 70% (n=13)
HMGB1 LI ≥ 70% (n=17)
0
20
40
60
80
100
120
140
160
Months after diagnosis