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DICKKOPF-RELATED PROTEIN 1 EXPRESSION IN BONE MARROW OF MULTIPLE MYELOMA PATIENTS: CORRELATION WITH BONE DISEASE AND PLASMA CELL MALIGNANCY TYPE

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***Background*: Previous studies have pointed out the role of dickkopf-related protein 1 (DKK 1) — Wnt inhibitor, which is essential for osteoblast functioning, in the development of osteolytic lesions in multiple myeloma (MM). *Aim*: To assess the DKK 1 expression displayed by myeloma cells in bone marrow trephine biopsies of patients with and without osteolytic lesions, and in different malignancy grades of the disease. *Methods*: The expression level of DKK 1 was assessed immunohistochemically in bone marrow of 49 MM patients presented with and without osteolytic lesions (the 1st and the 2nd group, respectively). *Results*: Levels of weak, moderate, and strong DKK 1 expression were distributed — as 43.33, 27.78 and 25.56%, and 63.91, 18.80, and 1.50%, respectively when evaluating the samples obtained from the 1st group and 2nd group. Statistically significant differences were found when the levels of DKK 1 expression in the 1st and the 2nd group were compared ($\chi^2 = 51$; df = 3; $p < 0.001$). *Conclusions*: DKK 1 contributes to the development of osteolytic lesions in MM. The present study provides morphological evidence that inhibition in Wnt signaling may lead to bone damage observed in the advanced stage of the disease.**

***Key Words*: multiple myeloma, osteolytic lesions, DKK 1, immunohistochemistry.**

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A growing body of evidence suggests an increase in the incidence of multiple myeloma (MM). It accounts for approximately 10% of all hematological neoplasms and is the second most frequent hematological malignancy [1–3]. According to WHO 2018 data, 159,985 MM cases were diagnosed in 2018 causing 106,105 deaths worldwide.

One of the most common MM complications is a bone disease frequently presented with osteolytic lesions [4, 5]. The etiology of osteolytic bone damage in MM is not fully understood but seems to involve interaction between myeloma cells and bone marrow microenvironment [6, 7]. It has been recognized that receptor activator of nuclear factor kappa β ligand and osteoprotegerin produced by the bone marrow microenvironment and osteoblasts are locally acting factors of osteoclast regulation [8, 9].

Recently, the role of dickkopf-related protein 1 (DKK 1) has come into focus as a target for the immunotherapy of MM [10]. The Wnt signaling pathway is an important regulator of the growth and differentiation of osteoblasts, and DKK 1 is a Wnt signaling pathway inhibitor [11]. Previous studies have suggested that a shift in the receptor activator of nuclear factor kappa β ligand/osteoprotegerin levels found in MM subjects is mediated by disruption of Wnt signaling in osteoblasts and their precursors, thus providing a rationale for the development of therapeutic strategies that target DKK 1 in the disease.

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Abbreviations used: DKK 1 – dickkopf-related protein 1; MM – multiple myeloma.

Furthermore, other studies have demonstrated DKK 1 overexpression in MM cells, and especially in patients with the severe bone destruction [12]. Osteoblast inhibition and osteoclast activation by DKK 1 have been shown in several experimental studies and preclinical trials suggesting that this Wnt inhibitor is the driving force in osteolytic bone damage.

The purpose of this study was to assess the DKK 1 expression displayed by myeloma cells in bone marrow trephine biopsies of patients with and without osteolytic lesions, and in different malignancy grades of the disease suggesting the contribution of DKK 1 to bone damage demonstrated in MM.

MATERIALS AND METHODS

***Patients*.** Forty-nine patients recruited from Riga East University Hospital, Oncology Center of Latvia, with primary diagnosed MM were enrolled in the study between June 2014 and June 2016. The study was approved by the Ethical Committee of Riga Stradins University, and it was conducted according to the Declaration of Helsinki. Of 49 MM patients, 51% were women, and 49% men. The median age was 67.35 years, with the youngest patient — 33 years and the oldest — 86 years (SD 11.9). All MM subjects fulfilled relevant International Myeloma Working Group criteria for the disease. Salmon — Durie staging system and the International Staging System for clinical classification of MM were used on standardized clinical data that were collected from medical records. All patients were subdivided into two groups — subjects with (the 1st group) and without (the 2nd group) osteolytic lesions, accordingly. The average age of MM subjects of the 1st group was 64.14 years (SD 13.5) whereas the 2nd group — 72.78 years (SD 5.5).

Conventional radiography, computed tomography, and magnetic resonance imaging were used for the confirmation of multiple destructive lytic bone lesions, as well as severe compression fractures of the vertebrae or ribs. According to the International Myeloma Working Group criteria, > 1 focal lesions on magnetic resonance imaging about 5 mm or greater in size was considered a true abnormal.

Light microscopy and immunohistochemistry. Bone marrow trephine biopsy specimens (n = 49) were obtained routinely from the posterior iliac crests of patients with primary diagnosed MM. Two series of histological sections of 4–5 μm were cut from 10% formalin-fixed, paraffin-embedded biopsy samples, and mounted on SuperFrost Plus slides (Germany Menzel GmbH, Germany) slides for routine histopathological and immunohistochemical evaluation. Before immunostaining, deparaffinization and hydration were done in xylene and graded alcohol to distilled water. During hydration, a 5-min blocking for endogeneous peroxidase is done with 0.3% (v/v) H_2O_2 in 95% ethanol. Heat-induced epitope retrieval was accomplished with the sections immersed in 10 mM sodium citrate buffer, pH 6.0, at 100 °C for 5 min in a vapor lock.

Immunohistochemistry was performed conventionally using polyclonal rabbit anti-human DKK 1 antibody (Sigma Life Science, Inc., Sigma-Aldrich, USA, HPA018995, 1:20) raised against DKK 1 precursor recombinant protein epitope signature tag. The amplification of the primary antibody and visualization of reaction products was performed applying the HiDef Detection HRP Polymer system and diaminobenzidine tetrahydrochloride substrate kit (Cell Marque, USA). The sections were counterstained with Mayer's hematoxylin, washed, mounted, and covered with coverslips. Immunohistochemical controls included the omission of the primary antibody. Sections were photographed by a Leitz DMRB brightfield microscope using a DFC 450C digital camera and a Glissando Slide Scanner (Objective Imaging Ltd., UK) 0.5 μm/pixel resolution with 20× objective, 0.275 μm/pixel resolution with 40× objective. The assessment of conventional histopathology and immunostaining was performed by two independent observers blinded to clinicopathological data.

Assessment of histopathology and immunohistochemistry findings. Routinely processed specimens were evaluated applying hematoxylin and eosin staining and classified according to Bartl grading system: Marschalko type, small cell, cleaved, polymorphous, asynchronous, and plasmablastic. These six histopathological types were stratified into three malignancy grades: low (Marschalko and small cell types), intermediate (cleaved, polymorphous, and asynchronous types), and high (plasmablastic type) grade MM [13].

Cells that were labeled with DKK 1 antibody and displayed brown reaction products were considered as immunopositive. The levels of immunopositivity for DKK 1 were defined semiquantitatively and graded into four groups: no cells stained (0%); weak staining: few cells stained (1–10%); moderate: a moderate number of cells stained (11–50%), and strong: the majority of cells stained (51–100%).

Statistical data analysis. Statistical analysis was performed using the SPSS system (version 23.0). Descriptive statistics were used to analyze the clinical and laboratory parameters of the study groups. The quantitative data were expressed as means ± standard deviation, whereas categorical parameters were expressed as frequencies and percentages. Correlation between DKK 1 antigen expression and clinical data was studied by χ^2 statistics. *P* values of < 0.05 were considered significant.

RESULTS

Patients' characteristics. We found that among patients with osteolytic lesions, MM stage IIA (90.3%) was the most common whereas in patients without osteolytic lesions — IA (44.4%). Radiologically, 17 patients (34.7%) were presented without any detectable bone lesion, whereas 28 (57.1%) demonstrated severe MM-associated bone lesions. One-fifth (18.4%) of osteolytic patients revealed hypercalcemia, and it was demonstrated significantly more often ($p < 0.05$) when compared to the 2nd group.

MM malignancy grades. By summing up, low-grade malignancy MM was confirmed in 28 (57.14%) of cases — 18 (36.73%) and 10 (20.40%), in the 1st and 2nd group, respectively when the intermediate grade MM — in 14 (28.57%) of cases — 7 (14.29%) and 7 (14.29%), in the 1st and 2nd group, respectively. Finally, the high-grade malignancy MM was diagnosed in 7 (14.29%) of cases presented exclusively in subjects with osteolytic damage (Table 1).

Table 1. Plasma cell malignancy grade in MM patients groups

MM group	Plasma cell malignancy grade		
	Low	Intermediate	High
1st group	18 (36.73%)	7 (14.29%)	7 (14.29%)
2nd group	10 (20.40%)	7 (14.29%)	-

Immunohistochemistry. When studying the histopathological appearance of specimens in MM subjects, and the contribution of DKK 1 by immunohistochemistry, we found that levels of the antigen expression greatly varied from almost nil to weak, moderate and strong (Table 2). We found statistically significant differences when the levels of DKK 1 expression in the 1st and the 2nd group were compared ($\chi^2 = 51$; df = 3; $p < 0.001$). In a group of patients with osteolytic lesions, less than one-half of regions of interest found in the samples estimated (43.33%) revealed weak DKK 1 expression. Simultaneously, more frequent occurrence of the weak antigen expression (63.91%) was demonstrated in the samples without osteolytic lesions. The levels of moderate and strong DKK 1 expression were equally distributed — 27.78% and 25.56%, respectively, when evaluating the samples obtained from the 1st group. By contrast, the strong expression of the antigen was very low in the 2nd group and comprised 1.5% only. Furthermore, we found statistically significant differences between DKK 1 an-

tigen expression levels in histologically graded types ($p < 0.001$). Almost two-thirds (70.66%) and 40.78% of the regions of interest evaluated were presented with weak DKK 1 expression in the low-grade and intermediate-grade MM, respectively. Simultaneously, the moderate, and, especially, strong expression of the antigen was common in intermediate and high-grade MM — 23.30 and 59.26%, respectively (Table 3).

Table 2. Distribution of DKK 1 expression levels in MM groups

Groups	Absence expression	Weak expression	Moderate expression	Strong expression
The 1st group	1 (3.33%)	13 (43.33%)	9 (27.78%)	8 (25.56%)
The 2nd group	3 (15.79%)	12 (63.91%)	2 (18.80%)	1 (1.50%)

Table 3. Distribution of DKK 1 expression levels in plasma cell malignancy grade groups

Plasma cell malignancy grade	Absence expression	Weak expression	Moderate expression	Strong expression
Low	2 (8.38%)	20 (70.66%)	5 (16.17%)	1 (4.79%)
Intermediate	0	6 (40.78%)	5 (35.92%)	3 (23.30%)
High	0	0	3 (40.74%)	4 (59.26%)

In the case of intermediate-grade MM, represented by three morphological patterns, we found heterogeneity of DKK 1 expression displayed by malignant plasma cells reflecting the extent of secretory function and osteolytic bone lesions (Figure, *a–d*). Finally, we did not find any statistically significant differences in DKK 1 expression when clinically defined MM stages were evaluated.

DISCUSSION

MM being severe and largely incurable B-cells malignancy accumulating primarily in the bone marrow represents about 1.5% of all cancers and about 10% of hematological neoplasia [14]. Osteolytic bone lesions resulting from the upraised activity of osteoclasts and decreased rates of osteogenesis by osteoblasts commonly develop at an advanced stage of the disease [15, 16]. It has been shown that more than 80% of MM patients develop destructive bone lesions and 60% are presented with fractures during the disease course [17, 18]. Previous studies have reported the success of MM treatment and normalization of bone remodeling, reducing serum DKK 1 levels [19]. DKK1, which is expressed by osteoblasts and bone marrow stromal cells, has been shown to act as an antagonist of the Wnt signaling pathway resulting in inhibition of osteoblasts differentiation and new bone formation [6, 20–23]. Furthermore, the sustained high levels of DKK 1 in the bone marrow of MM subjects have been shown to cause a loss in the viability of osteoblast precursors as well [24]. The Wnt signal is diminished when DKK 1 protein binds to the LPR5/6 receptor and coreceptor, Kremen-1/2, thus promoting internalization of the receptor complex at the cell surface [25]. The authors have demonstrated that DKK 1 impairs local bone formation along with the regulation of systemic bone mass through Wnt signaling.

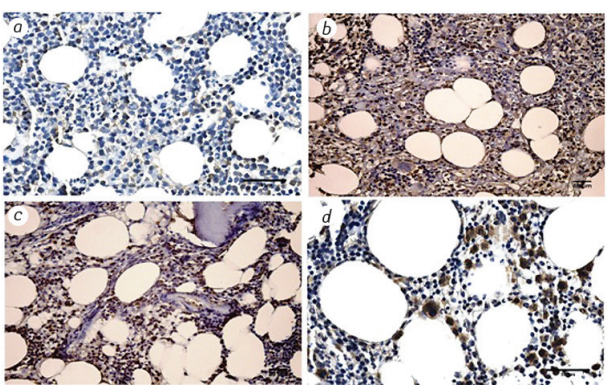

Figure. The greatly varying expression of DKK 1 is demonstrated in intermediate-grade MM and evaluated as weak (panel *a*), and moderate to strong (panel *b–d*). The malignant cells demonstrate notched, cleaved, or even convoluted nuclei of variable size, nucleoli, often prominent and centrally located. Marked cellular polymorphism and multinuclearity, some giant plasma cells are present as well. Magnification: *a*: × 250; b: × 200; *c*: × 200; *d*: × 250

In the present study, we have estimated the levels of the DKK 1 expression displayed in different malignancy grades of the disease suggesting on the contribution of DKK 1 to bone damage demonstrated in MM, assessed morphological patterns of plasma cells using bone marrow trephine biopsies obtained from 49 MM subjects presented with and without osteolytic lesions; investigated the fine structure of malignant plasma cells.

Our observations revealed moderate and strong DKK 1 expression appearing in the samples of osteolytic patients with osteolytic lesions significantly more often when compared to the samples obtained from patients without osteolytic bone lesions. These results are in agreement with those by Kristensen *et al.* [8] who demonstrated significantly higher levels of DKK1 mRNAs expressed in MM samples with advanced osteolytic lesions. Simultaneously, our results show that the levels of DKK 1 expression differ significantly when low, intermediate, and high plasma cell malignancy grades are compared.

Other authors have demonstrated that anti-DKK 1 treatment increased bone formation rate by 25% and mineralizing surface by 28% and have suggested that restoring osteoblast differentiation can prevent the development of osteolytic bone lesions in MM, offering an effective therapeutic approach to treating this clinically important aspect of the disease [12]. Furthermore, clinically tested neutralizing anti-DKK 1 monoclonal antibody BHQ880 has demonstrated beneficial effect resulted in an increase in bone mass found in MM subjects [2].

The present study provides immunohistochemical evidence that inhibition in Wnt signaling may lead to bone damage observed in the advanced stage of MM.

Concluding, we may suggest that the assessment of bone marrow biopsy findings remains an essential part of the diagnostic evaluation of MM. Osteolytic bone lesions are the most common complication of MM. Estimation of DKK 1 in routinely obtained biopsies may offer an additional approach to interpreting this clinically important aspect of the disease.

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