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Membrane translocation of Bruton kinase in multiple myeloma cells is associated with osteoclastogenic phenotype in bone metastatic lesions

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Abstract

Using bone biopsy samples, we examined whether osteolytic cytokine profile is changed in situ in bone samples of metastatic multiple myeloma, and whether this creates an environment of lysis within the bone to which it has spread. This also produces the clinical features of increased circulating plasma calcium, and deleterious effects on the kidney.

Using multiple myeloma biopsy and cell extracts from bone metastatic lesions, Bruton kinase, a tyrosine kinase, was demonstrated to be translocated to the membrane. Several transcription factors were upregulated included activin A, inflammatory transcription activator like such as nuclear factor kappa B, and specific bone lytic factor such as receptor activator of nuclear factor kappa-B ligand that is known to drive osteoclastogenesis as opposed to a osteogenic environment. The transcript for Bruton kinase was also elevated in its expression.

Cytokines that support osteolytic activity such as a proliferation-inducing ligand, RANTES (regulated on activation, normal T cell expressed and secreted), interleukin-8, and activin A were upregulated. Tartrate resistant acid phosphatase (TRAP)-positive osteoclastic enzymatic activity was significantly elevated in the bone microenvironment in metastatic multiple myeloma. Several tyrosine kinase inhibitors, including inhibitors for Bruton kinase such as ibrutinib have been developed. The results of the present study provide evidence that multiple myeloma possess signal transduction mechanisms to support a bone lytic environment.

The results provide a preliminary molecular basis to design specific inhibitors for management of bone metastasis of multiple myeloma.

Abbreviations: APRIL = a proliferation-inducing ligand, DPBS = Dulbecco's phosphate-buffered saline, DPBS-Ca-Mg = Dulbecco's phosphate-buffered saline supplemented with calcium chloride and magnesium chloride, DTT = dithiothreitol, GAPDH = glyceraldehyde-3-phosphate dehydrogenase, IL-8 = interleukin-8, MM = multiple myeloma, NF-κB = nuclear factor kappa B, NHS-SS-biotin = succinimidyl-2-(biotinamido)-ethyl-1,3-dithiopropionate, RANKL = receptor activator of nuclear factor kappa-B ligand, RANTES = regulated on activation, normal T cell expressed and secreted, RIPA = radioimmunoprecipitation assay, SEM = standard error of mean, SH2 = Src homology 2, TRAP = tartrate resistant acid phosphatase.

Keywords: multiple myeloma, osteoclastogenesis, src homology, tyrosine phosphorylation

1. Introduction

There are several mechanisms that cluster proteins that regulate functions, survival, and spread of different varieties of blood cancers including multiple myeloma.[1–3] One recurring theme in blood cancers is the occurrence of proteins that have the modular domain Src homology 2 (SH2), which has the ability to bring together a multitude of proteins that regulate cell function in a polydimensional fashion.[4–7] SH2 proteins bind to tyrosine phosphorylated proteins, and they also undergo tyrosine phosphorylation.[6] Incipient reports suggest that a particular kind of tyrosine kinase, Bruton Kinase, the same enzyme that is deficient in X-linked agammaglobulinemia, is upregulated in multiple myeloma.[8] In the present study, we had access to patient samples of multiple myeloma obtained during bone biopsy. We designed the present study to examine the changes in cellular location of this protein Bruton Kinase with reference to bone biopsy samples obtained from patients with non-tumorigenic conditions.

In particular, the major rationale for examining the expression of Bruton kinase are the previous reports that this tyrosine kinase has the ability to sustain bone lysis by the process of increased osteoclastic activity.[9] In the present study, we specifically

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The authors report no conflicts of interest.

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examined this aspect. In fact, using the bone biopsy samples, we examined whether osteolytic cytokine profile is changed in situ in bone samples of metastatic multiple myeloma. In fact, it is well-known that one of the most aggressive features of multiple myeloma is its ability to metastasize to the bone.[10] This also produces the metabolic features of increased circulating plasma calcium, and deleterious effects on the kidney.[11] Thus, our study provides a molecular basis for the sustenance of osteoclastogenic activity in the metastatic variants of multiple myeloma.

2. Materials and methods

2.1. Human bone biopsies from patients with multiple myeloma

All our patients were male and age ranges between 49 and 69 years. Age-matched samples were incorporated for control studies. Experiments were performed by pooling samples and performing the studies in triplicates for each condition, with a total of 3 patients in each group. Bone biopsies were obtained from multiple myeloma patients for the diagnosis of metastatic lesions. Control biopsies were obtained from healthy male volunteers with nonmetastatic disease at age ranges between 49 and 69 years. Biopsies from patients with multiple myeloma nonmetastatic disease were obtained at age ranges between 49 and 69 years. Explicit permission was obtained from the institutional review board of Traditional Chinese Medical Hospital of Xinjiang Uygur Autonomous Region and all experiments were performed according to Helsinki guidelines and with informed patient consent. Bone biopsies were stored at negative 20C until further studies. Uniform protocols were followed for all experimental procedures. A total of 15 patients and control subjects' specimens were obtained and pooled blindly and randomly in three groups and quantitative experiments performed in triplicates. Each of the samples was also individually run for the experiments, without knowing the identity of the sample that went into each pool. This ensured that the variance was not because of skewed expression. Because of paucity of patient samples, we undertook this approach.

2.2. Antibodies and chemicals

All antibodies were obtained from Santa Cruz Biotech and Abcam, enzyme-linked immunosorbent assay (ELISA) kits from R&D Biosystems, RayBiotech, Biosensis, and MyBioSource and chemicals from Aldrich.

2.3. Assay for tartrate-resistant acid phosphatase

Tartrate-resistant acid phosphatase (TRAP) is a glycoprotein metalloprotein enzyme selectively expressed in the osteoclasts. This enzyme is widely distributed in monocytes/macrophages, including in the osteoclasts. The enzyme resembles several proteins, including uteroferrin, an iron-containing protein found in pig uterus, as well as osteoclast-enriched proteins such as receptor activator of nuclear factor kappa-B ligand (RANKL). Antisera to uteroferrin have been used as an alternate approach to immunoassay TRAP. In similar way, antibodies to RANKL shall also complementarily bind to the acid phosphatase and provide a means to sample the concentration of the enzyme present in the lysate by acting as an internal control. To confirm whether osteoclasts were upregulated, TRAP was assayed. The bone tissues (100 mg) were homogenized in 0.5-mL 0.4 mol/L sodium acetate, pH 5.6 containing 1% w/v Triton X-100. Immunoabsorption was performed with fixed antibodies to receptor activator of nuclear factor kappa B (NF-κB) ligand after adding neutralizing supernatant tissue extracts to pH 7.5 with Tris base. For controls, immobilized protein A was mixed to the extract instead of using the antibody. TRAP activity was finally determined spectrophotometrically before and after immuneabsorption with the substrate sodium tartrate and 10 mmol/L 4-nitrophenyl phosphate to develop a colored product, as previously described by Hayman et al.[12–13] Nonaddition of the dye did not result in development of the colored product. The final enzymatic activity was expressed as nmol/min/mg lysate incubated.

2.4. ELISA for assays of soluble cytokines

Because of increased sensitivity, solid-phase enzyme linked assay was utilized to identify specific expression of soluble factors that drive osteoclast formation and suppresses osteoblast/osteocytic activities. Comparisons were made between control sample microenvironments and those obtained from bone biopsies of patients with diagnosed and pathologically confirmed multiple myeloma. Readings were obtained in duplicates on a plate-reader and averaged to obtain the final results. The kits were obtained from Thermofisher Scientific, R&D Quantikine kit and Abcam. Inter-kit variability was the minimum.

2.5. Cell surface biotinylation to detect membrane-bound Bruton Kinase

Thawed samples were placed on ice and washed thrice with ice-cold Hank's buffer supplemented with calcium chloride and magnesium chloride. These were incubated with 1 mg% succinimidyl-2-(biotinamido)-ethyl-1,3-dithiopropionate (NHS-SS-biotin) reagent (Pierce) in Dulbecco phosphate-buffered saline supplemented with calcium chloride and magnesium chloride (DPBS-Ca-Mg) for an hour in cold on a vibrating stage. After accomplishment of the biotinylation reaction, the lysates were rinsed and washed with 75 mmol/L of glycine in DPBS-Ca-Mg to terminate the biotinylation reaction. The lysates were solubilized with radioimmunoprecipitation assay (RIPA) buffer optimally supplemented with protease inhibitors. The lysates were centrifuged at 25,000*g* for 7 minutes in the cold. The supernatants were carefully siphoned off and incubated with 200 μL streptavidin-agarose beads (Pierce). Complete preparation of biotinylated protein was made possible by repeating the extraction process over a period of 24 hours. After repeated washing with RIPA buffer, Laemmli sample buffer (Bio-Rad) containing dithiothreitol was incubated at 37°C for 3 hours to leach out the biotinylated protein. The eluates were identified for Western blot analysis using specific antibody for Bruton kinase (Abcam). The membrane signals were normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signals obtained by western blotting of the RIPA solubilized lysates. The relative signal intensities were computed and compared. Negative controls were performed by omitting the biotin from the initial incubation step, which did not yield any detectable signal.

2.6. RNA extraction and reverse transcription

Total RNA was extracted from the prepared lysates with the help of RNeasy Mini kit (Qiagen). All reactions were performed under cold conditions. cDNA was obtained using SuperScript RNase H

Reverse Transcriptase kit (Life Technologies). The experiments were performed in a nuclease-free environment to ensure integrity of the nucleic acid materials.

2.7. Real-time quantitative polymerase chain reaction to detect expression levels of mRNA

Real-time quantitative polymerase chain reaction (RT-PCR) was operated using ABI 7000 SDS incorporating Taqman probe mixture (Applied Biosystems). Probes/primes for the genes of interest related to osteoclast differentiation were designed with Primer Express 2.0 software. The oligonucleotide probes were bound with the reporter dye 6-carboxyfluorescein at its 5′ end and the quencher dye tetramethylrhodamine at 3′ terminus. The internal control was the housekeeping gene GAPDH. The PCR reaction was the routine 45 cycles. The expression levels of the altered genes were identified with reference to the changes in comparison to the absolute expression levels of mRNA in the control bone tissues. RT-PCR results were finally computed using the comparative Ct method normalized against the internal housekeeping gene.

Primer sequences were as follows: Bruton Kinase: forward, 5′-GAAGCTGGTGCAGTTGTATG-3′ and reverse, 5′-TATACCC TCTCTGATGCCAG-3′; RANK: forward, 5′-TTAAGCCAGT GCTTCACGGG-3 and reverse, 5′-ACGTAGACCACGATGA TGTCGC-3′; NFATc1: forward, 5′- GGAAGGGCGGCTT CTGCGAC-3′ and reverse, 5′- AGGCGTGCGGGCGCAG CAG-3′; NF-κB: forward, 5’-TTTTCGACTACGCAGTGACG-3′ and reverse 5’-GTCCAGAAGGCTCAGGTCAG-3′; Activin A: forward, 5′- CTCGGAGATCATCACGTTTG-3′ and reverse 5-CCTTGGAAATCTCGAAGTGC-3′; GAPDH: forward, 5′-CGACCACTTTGTCAAGCTCA-3′ and reverse, 5′-TTACT CCTTGGAGGCCATGT-3′

2.8. Statistics

Data were expressed as means ± standard error of mean (SEM). Comparisons between paired groups were performed by student *t* test.

3. Results

3.1. Increased osteoclast protein expression in bone biopsies obtained from multiple myeloma

TRAP was assayed to estimate their activity in expression of osteoclasts in bone biopsies obtained from control, multiple myeloma with nonmetastatic disease and multiple myeloma with metastatic disease. This was significantly increased in multiple myeloma ($P < 0.01$, student *t* test) (Fig. 1).

3.2. Increased membrane localization of Bruton kinase in metastatic multiple myeloma

In comparison to controls, biotin assay showed increased membrane-bound signals when probed for Bruton kinase. Signal intensities were estimated by image analyses without any alteration to the gamma settings. All experiments were performed in pooled samples and in triplicates and membrane signal expression was normalized to GAPDH expression of whole lysates. Representative images of the membrane-localized signals of the Bruton kinase and GAPDH expression of solubilized

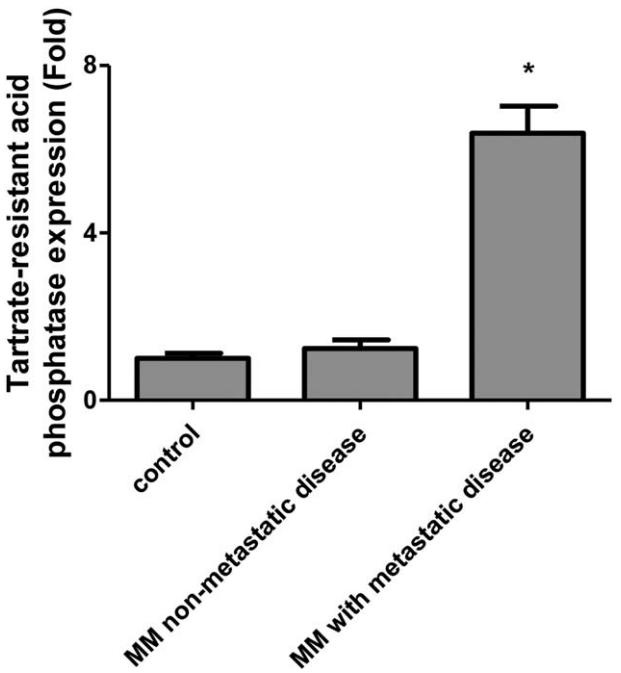

Figure 1. Histogram depicting marked elevation of tartrate-resistant acid phosphatase enzymatic activity in multiple myeloma cells in comparison to controls This elevation is reflective of increased osteoclastic activity in cell extracts in bone metastasis in multiple myeloma. The changes in expression between the 3 groups (control, multiple myeloma with nonmetastatic disease and multiple myeloma with metastatic disease) are significant (comparison of pooled samples in triplicates, *t* test). MM = multiple myeloma.

lysates are shown in Figure 2 that the Bruton kinase expressions in membrane was increased in multiple myeloma with metastatic bone disease compared with controls (upper) and multiple myeloma with nonmetastatic disease (lower). The differences in the signal intensities of the membrane localized signals were significant when compared between groups (normalized mean values 0.87 ± 0.14 vs. 0.19 ± 0.09, $P < .05$, student *t* test).

3.3. Upregulation of mRNA transcripts that promote cellular differentiation to osteoclasts

RT-PCR showed upregulation of mRNA for Bruton kinase, *RANK, NFATc1,* NF-κB, and *activin A*. Three groups' samples were detected: control, multiple myeloma with nonmetastatic disease, and multiple myeloma with metastatic disease. These were significantly increased when compared to controls and multiple myeloma with nonmetastatic disease ($P < .05$, *t* test for each pair of compared transcript) (Fig. 3).

3.4. Upregulation of cytokines in bone microenvironment of multiple myeloma

ELISA was used to compute bone microenvironment cytokines in control, multiple myeloma with nonmetastatic disease, and multiple myeloma with metastatic disease. The assayed cytokines include RANTES (regulated on activation, normal T cell expressed and secreted), APRIL (a proliferation-inducing ligand), interleukin-8, and activin A. This was significantly elevated in multiple myeloma, as compared by paired *t* test for each individual cytokine examined (Fig. 4).

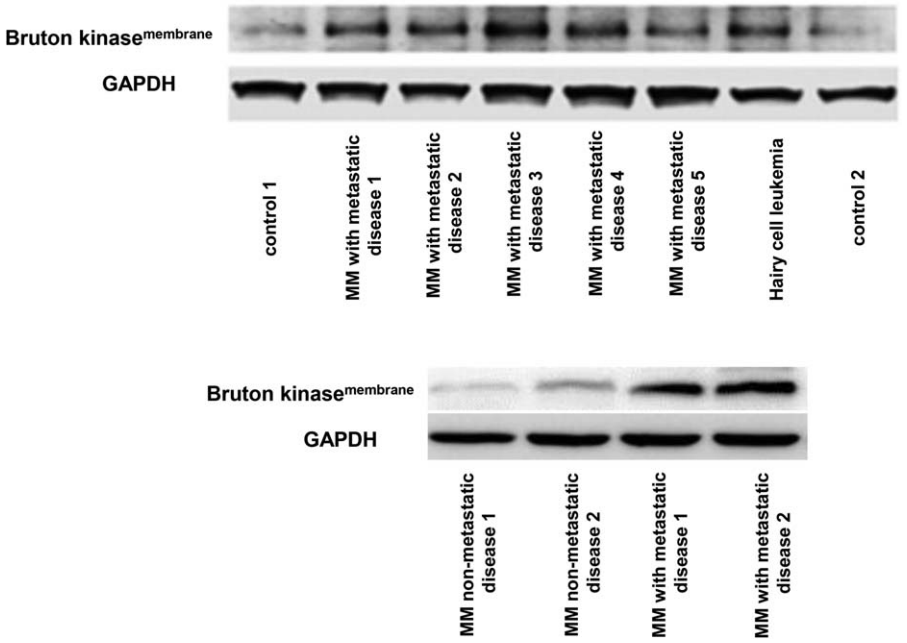

Figure 2. Increased membrane expression of Bruton kinase assayed by biotin switch in metastatic multiple myeloma. Independent patient samples were assayed. Representative western blots of individual patient samples are shown here. Hairy cell leukemia was used as a positive control. When biotin was omitted, membrane signals were not detected (data not shown). The membrane signals were detected by the tyrosine kinase Bruton kinase-specific primary antibody by conventional technique of western blotting (upper and lower). Pooled samples were also used for quantitative detection of membrane-localized signals, normalized to the GAPDH expression. MM = multiple myeloma.

4. Discussion

The results of the present study provide convincing evidence of the role of the multiple myeloma cells in driving its own survival during its metastasis to bone. It is not clear from the results of the present study whether the expression of Bruton kinase drives

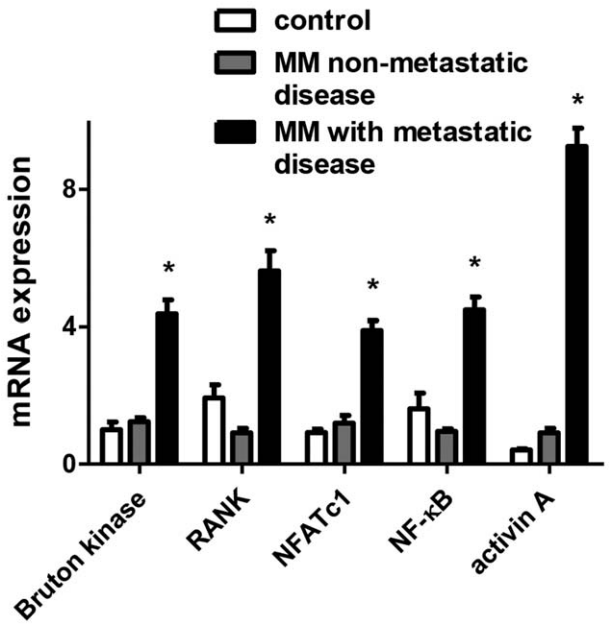

Figure 3. Histogram depicting marked elevation of mRNA transcripts that support osteoclast formation and lytic bone environment. Note that the mRNA for Bruton kinase was elevated. The increase in expression, as well as location to membrane, suggests the specific role of Bruton kinase in signal transduction including osteoclastogenesis. Three groups' samples were measured: control, multiple myeloma with nonmetastatic disease and multiple myeloma with metastatic disease. The fold changes reported in the Y axis represents the fold change ($2^{-\Delta\Delta CT}$, GAPDH normalized). Type 1 isoform for NF-κB was assayed. *Significant difference ($P < .05$). MM = multiple myeloma.

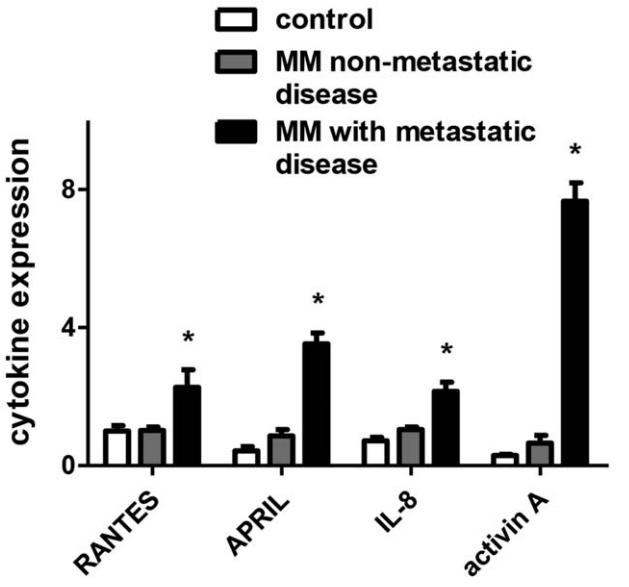

Figure 4. Histogram depicting marked elevation of cytokines that support osteolytic bone environment. The cytokines expression was measured in 3 groups' samples: control, multiple myeloma with nonmetastatic disease, and multiple myeloma with metastatic disease. Note that activin A is elevated, which is a vigorous modulator of osteoclast activity. *significant difference ($P < .05$). MM = multiple myeloma.

metastasis to bone. First of all, because of proximity to bone marrow, it is not known whether locally operant mechanisms drive progression of multiple myeloma cells to a bone environment.[14–17] Previous studies using cell lines have shown the potential role of multiple myeloma in creating a lytic environment.[9–10]

In the present study, we have utilized bone biopsies from patients with metastatic multiple myeloma and demonstrated that tyrosine phosphorylation by Bruton kinase may be a key event. First, Bruton kinase remained translocated to the membrane. This is also coupled with upregulation of transcripts of several factors like activins A that is known to play a major role in osteoclastogenesis. Finally, there is also increased expression of numerous cytokines that support osteolytic activity. Finally, TRAP-positive osteoclastic signals were significantly elevated in the bone microenvironment in metastatic multiple myeloma.

The results of the present study provide incipient, but rigorous evidence that multiple myeloma cells themselves support the osteolytic lesions during metastasis of multiple myeloma to the bone tissues. The results of the present study however do not provide direct evidence for the kind of stimulatory environment that organize the secretion of the cytokines that support survival of multiple myeloma cells in the bone tissues, or provide a sustained lytic environment. Some of these proteins such as APRIL may also support of survival of the multiple myeloma cells when they have metastasized to the bone tissues. Multiple bone metastasis is a hallmark of the disease of monoclonal gammopathy including multiple myeloma.[10] Furthermore, preferential spread to bone tissues results in the major clinical manifestations of hypercalcemia and associated renal injury and renal failure.[11]

Several tyrosine kinase inhibitors, including inhibitors for Bruton kinase like ibrutinib, have been developed.[18–20] The results of the present study provide the molecular basis to design specific inhibitors for preventing bone metastasis of multiple myeloma.

References

[1] López-Corral L, Corchete LA, Sarasquete ME, et al. Transcriptome analysis reveals molecular profiles associated with evolving steps of monoclonal gammopathies. Haematologica 2014;99:1365–72.

[2] Ferrero S, Capello D, Svaldi M, et al. Multiple myeloma shows no intra-disease clustering of immunoglobulin heavy chain genes. Haematologica 2012;97:849–53.

[3] Fux L, Ilan N, Sanderson RD, et al. Heparanase: busy at the cell surface. Trends Biochem Sci 2009;34:511–9.

[4] Gery S, Koeffler HP. Role of the adaptor protein LNK in normal and malignant hematopoiesis. Oncogene 2013;32:3111–8.

[5] Jin LL, Wybenga-Groot LE, Tong J, et al. Tyrosine phosphorylation of the Lyn Src homology 2 (SH2) domain modulates its binding affinity and specificity. Mol Cell Proteomics 2015;14:695–706.

[6] Roskoski RJr. Src protein-tyrosine kinase structure, mechanism, and small molecule inhibitors. Pharmacol Res 2015;94:9–25.

[7] Wybenga-Groot LE, McGlade CJ. RTK SLAP down: the emerging role of Src-like adaptor protein as a key player in receptor tyrosine kinase signaling. Cell Signal 2015;27:267–74.

[8] Yang Y, Shi J, Gu Z, et al. Bruton tyrosine kinase is a therapeutic target in stem-like cells from multiple myeloma. Cancer Res 2015;75:594–604.

[9] Tai YT, Chang BY, Kong SY, et al. Bruton tyrosine kinase inhibition is a novel therapeutic strategy targeting tumor in the bone marrow microenvironment in multiple myeloma. Blood 2012;120:1877–87.

[10] Hameed A, Brady JJ, Dowling P, et al. Bone disease in multiple myeloma: pathophysiology and management. Cancer Growth Metastasis 2014;7:33–42.

[11] Kapoulas S, Raptis V, Papaioannou M. New aspects on the pathogenesis of renal disorders related to monoclonal gammopathies. Nephrol Ther 2015;11:135–43.

[12] Hayman AR. Tartrate-resistant acid phosphatase (TRAP) and the osteoclast/immune cell dichotomy. Autoimmunity 2008;41:218–23.

[13] Hayman AR, Macary P, Lehner PJ, et al. Tartrate-resistant acid phosphatase (Acp 5): identification in diverse human tissues and dendritic cells. J Histochem Cytochem 2001;49:675–84.

[14] Chandra H, Chandra S, Verma SK. Multiple myeloma or metastatic carcinoma breast: diagnostic dilemma in a case presenting with lytic bony lesion. Breast Dis 2015;35:199–201.

[15] Swami A, Reagan MR, Basto P, et al. Engineered nanomedicine for myeloma and bone microenvironment targeting. Proc Natl Acad Sci U S A 2014;111:10287–92.

[16] De Veirman K, Rao L, De Bruyne E, et al. Cancer associated fibroblasts and tumor growth: focus on multiple myeloma. Cancers (Basel) 2014;6:1363–81.

[17] Sawant A, Ponnazhagan S. Myeloid-derived suppressor cells as osteoclast progenitors: a novel target for controlling osteolytic bone metastasis. Cancer Res 2013;73:4606–10.

[18] Bhatt V, Alejandro L, Michael A, et al. The promising impact of ibrutinib, a Bruton's tyrosine kinase inhibitor, for the management of lymphoid malignancies. Pharmacotherapy 2014;34:303–14.

[19] Murray MY, Zaitseva L, Auger MJ, et al. Ibrutinib inhibits BTK-driven NF-(B p65 activity to overcome bortezomib-resistance in multiple myeloma. Cell Cycle 2015;14:2367–75.

[20] Vargas L, Hamasy A, Nore BF, et al. Inhibitors of BTK and ITK: state of the new drugs for cancer, autoimmunity and inflammatory diseases. Scand J Immunol 2013;78:130–9.