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Amplification of autocrine motility factor and its receptor in multiple myeloma and other musculoskeletal tumors

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ABSTRACT

Autocrine motility factor (AMF: GPI) and its receptor AMFR (AMF Receptor: gp78) regulate the metastatic process. Here, we have tested the expression levels of AMF, AMFR, and AMF × AMFR in 1348 patients with musculoskeletal tumor. The results depicted here identified that multiple myeloma highly express AMF × AMFR value as compared with normal bone samples ($p < 0.00001$). To visualize the AMF × AMFR autocrine amplification in multiple myeloma microenvironment, we have developed a novel software aimed at analyzing numerous cell-to-cell and ligand-to-receptor interactions, *i.e.*, Environmentome. It has led to the identification that myeloma-associated interactions with normal bone cells including osteoblast, osteoclast, immunological components, and others in a paracrine manner. In conclusion, the data showed that AMF × AMFR amplification is a clinical manifestation in bone microenvironment of multiple myeloma.

1. Introduction

Malignant musculoskeletal tumor originates in bone or soft tissues such as muscle, cartilage, connective tissues and metastatic foci from primary lesion to the skeleton(s) [1,2]. In some cases, they shows wider invasion to the surrounding soft tissues, or metastatic spreading to other parts of the body. Malignant musculoskeletal tumor includes osteosarcoma, Ewing's sarcoma, undifferentiated pleomorphic sarcoma, chondrosarcoma, chordoma, giant cell tumor, multiple myeloma, bone metastasis of prostate cancer, breast cancer or other origins, synovial sarcoma, liposarcoma, rhabdomyosarcoma, leiomyosarcoma, fibrosarcoma, angiosarcoma, hemangiopericytoma, malignant peripheral nerve sheath tumor, alveolar soft part sarcoma, clear cell sarcoma, epithelioid sarcoma, to name but a few [3–6].

To understand the aggressive behavior of malignant musculoskeletal tumors, several factors associated with invasion and metastasis have been identified, including AMF. AMF is secreted by tumor cells and stimulates migration [7,8] proliferation [9], angiogenesis [10], and resistance to apoptosis [11]. AMF is, *a.k.a.* Glucose-6-Phosphate Isomerase (GPI), Neuroleukin (NLK), Maturation factor (MF) [12].

Intracellular AMF/GPI is an essential cytosolic enzyme of the sugar metabolism both in glycolysis and gluconeogenesis pathway, catalyzing the interconversion of glucose 6-phosphate and fructose 6-phosphate [13]. GPI is secreted extracellularly from various tumors, and moonlights as AMF [14]. The upregulated AMF secretion induces the metastasis of sarcomas, while the silencing leads to mesenchymal-to-epithelial transition, and inhibition of metastasis of sarcoma [15]. AMF/GPI is suggested to be a possible predictor of metastasis in bone and soft tissue tumors [16], whereby may be influential to the tumor stage and survival time. The expression of AMFR has been reported to be significantly correlated with more advanced tumor stage and decreased survival rates in a variety of tumors [17–25]. Similarly in sarcoma, an in vivo experiment clearly showed that AMFR expression was associated with shorter survival time [26].

AMF signaling is induced by binding to its receptor AMFR, a cell surface glycoprotein of 78 kDa, (gp78). The AMF-AMFR interaction activates a pertussis toxin sensitive G protein, upregulating GDI-β, which triggers small Rho-like GTPase, Rac1 and RhoA activation. The upregulated signaling of JNK1 and JNK2, resulting in actin fiber rearrangement, that modulates to tumor cell motility, invasion, and
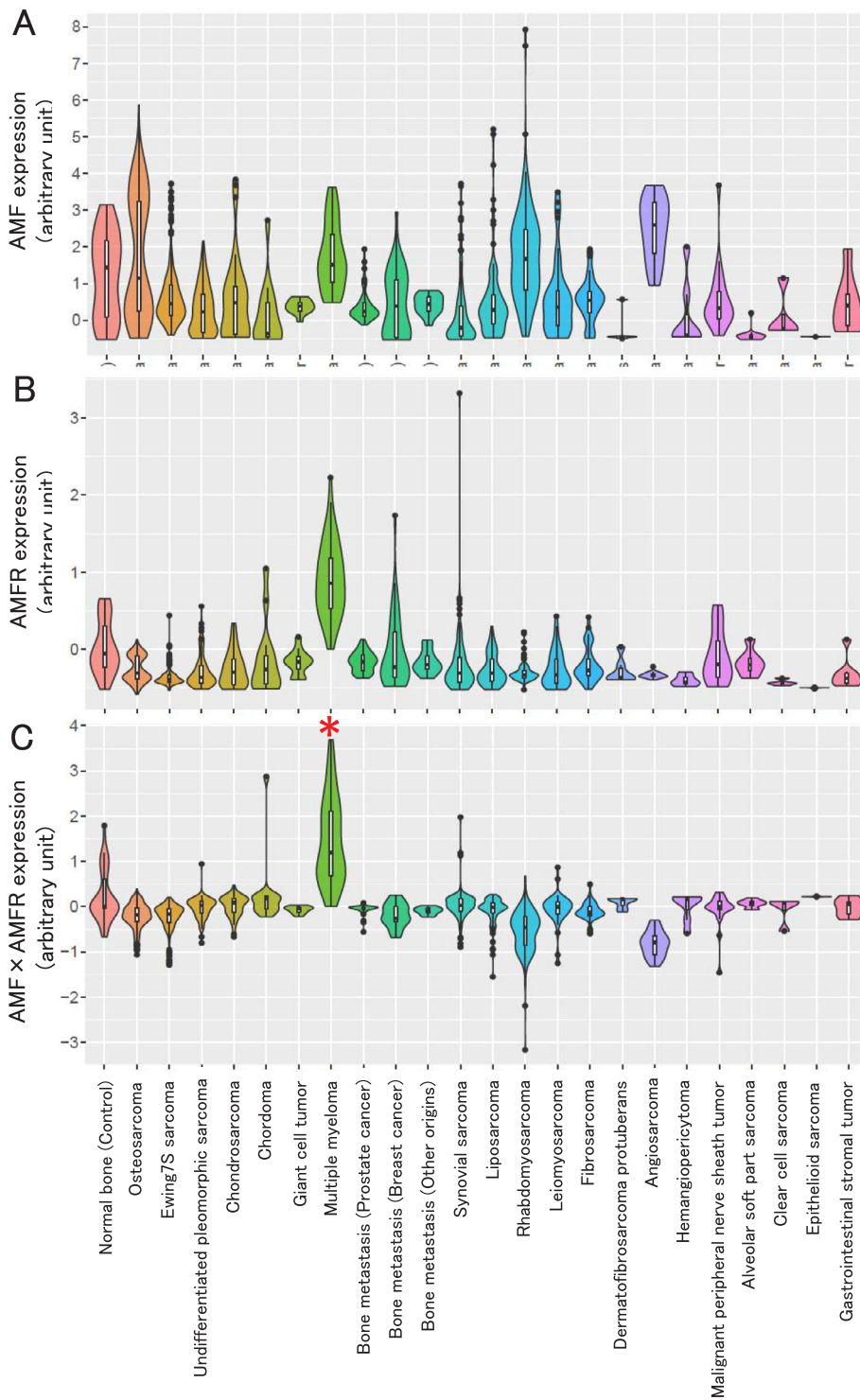
Fig. 1. (A) AMF, (B) AMFR, (C) AMF × AMFR expressions in musculoskeletal tumors. Normalized expression values were summarized using violin plot in each musculoskeletal tumor. Black bar in the white box indicate median values. Considering a nature of case-control study, a nonparametric analysis of variance test, Mann–Whitney test, was used to evaluate the statistical significance for comparison of two categorical groups, *i.e.* normal bone and each musculoskeletal tumor. Differences at $p < 0.001$ were considered statistically significant. *Red asterisk indicates a higher expression compared to control sample with statistical significance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

metastasis [27,28]. In addition, AMFR also may acts as E3 ubiquitin-protein ligase, enhancing the polyubiquitination of a variety of proteins for endoplasmic reticulum-associated degradation (ERAD) in proteasomes [29]. In the current clinical settings, proteasome inhibitors are utilized to suppress ERAD and induce ER stress, consequently activating programmed cell death in tumor cells [30]. In addition, the ubiquitin ligase AMFR promotes sarcoma metastasis by targeting KAI1, a metastasis suppressor, in the process of ERAD [26]. Thus, it was suggested that suppressing AMF-AMFR interaction should be considered as treatment modality for malignant musculoskeletal tumors [7].

However, the expression profiles of AMF and AMFR remain a conundrum due to the variety of histological types in malignant musculoskeletal tumors.

Here, we present a molecular pattern of AMF, AMFR, and AMF × AMFR in 23 types of musculoskeletal tumors, and visualized the bone tumor microenvironment using a novel bioinformatics approach.

2. Materials and methods

2.1. Patient samples and microarray

The patient sample data were downloaded from Gene Expression Omnibus (GEO). In total, following 34 series of dataset were analyzed in this study. In detail, "GSE41619", "GSE43045", "GSE44115", "GSE49327", "GSE50137", "GSE52392", "GSE56183", "GSE6481", "GSE8406", "GSE90592", "GSE9508", "GSE92689", "GSE94321", "GSE102193", "GSE108089", "GSE12532", "GSE12475", "GSE116294", "GSE14020", "GSE14325", "GSE14359", "GSE16102", "GSE16091", "GSE17679", "GSE2553", "GSE2719", "GSE30699", "GSE30835", "GSE4303", "GSE43861", "GSE51588", "GSE19276", "GSE9059", "GSE92689". Consequently, in these datasets, 1348 patient samples were analyzed. From these diverse microarray platforms, 2386 commonly shared genes were selected, and standardized and transformed to Z-scores using the scale function, followed by quantile normalization to minimize datamining errors such as the ranking of expressional values. Following quantile normalization, AMF, AMFR, and AMF × AMFR expressional value, and ratio between AMF and AMFR were shown in Figures using violin plot and box plots, descriptive statistics in Supplementary Tables with respect to each musculoskeletal tumor (Dynacom, Chiba, Japan). The histological classifications were defined at the time points when the samples were extracted.

2.2. Tumor microenvironment analysis software

An online open software to visualize novel cell-to-cell interaction networks using CAGE database including 144 human primary noncancerous cell types was previously reported [31]. We further newly extracted CAGE data (Dnaform, Kanagawa, Japan) from primary cultured cells, and added to the software together with more than 1500 CAGE data including tumor cells in FANTOM5 database. Subsequently, it improved to analyze any tumor microenvironment with high scalability which enable to add further CAGE data of interest, we thus termed 'Environmentome' (Amelieff, Tokyo, Japan). Since this study focused on bone tumor microenvironment, we have analyzed the intercellular interactions among the cellular components of bone microenvironment of malignant musculoskeletal tumors such as tumor cell, osteoblast, osteoclast, osteocyte, CD4$^+$ T cell, and CD8$^+$ T cell, *etc.*

2.3. Statistical analysis

For quantified data of microarray, a nonparametric analysis of variance test, Mann–Whitney test was used to evaluate the statistical significance for comparison of two categorical groups, *i.e.* normal bone and each musculoskeletal tumor as a case-control study. Differences at p < 0.001 were considered statistically significant. Statistical analyses were performed using R 3.5.3 software (The R Project for Statistical Computing).

3. Results

3.1. AMF expression in malignant musculoskeletal tumors

We first analyzed the AMF expression levels in musculoskeletal tumors in comparison to normal bone as control samples. For all 1348 patients, and separately for each histological group, and the AMF levels were summarized with descriptive statistics. Patient number, average, standard deviation, median, maximum value, minimum value were shown [Supplementary Table I]. The result showed that the patients with osteosarcoma, multiple myeloma, rhabdomyosarcoma, and angiosarcoma tend to express higher levels of AMF [Fig. 1-A].

3.2. AMFR expression in malignant musculoskeletal tumors

Next, we examined AMFR expressional levels in malignant musculoskeletal tumors. In comparison to normal bone, remarkably, patients suffering from multiple myeloma expressed AMFR (p < 0.000001), indicating multiple myeloma is capable of receiving myeloma-secreted AMF in an autocrine manner, which may lead to tumor malignancy [Fig. 1-B, and Supplementary Table II].

3.3. AMF-AMFR amplification in malignant musculoskeletal tumors

AMF-AMFR interaction results in positive feedback loop in an autocrine manner, transforming tumor cell dynamically into an aggressive phenotype [32]. Therefore, in order to understand the net effect of AMF-AMFR interaction, we have calculated the value of AMF expression × AMFR expression. The result showed that, in comparison to normal bones, the patients with multiple myeloma expressed AMF × AMFR significantly higher value (p < 0.000001), suggesting that AMF-AMFR amplification mechanism plays a crucial role in development of multiple myeloma [Fig. 1-C and Supplementary Table III].

Additionally, we further examined the ratio between AMF and AMFR to determine the prioritized expression of the two molecules, and expected to identify tumor types by the ratio. As a result, the ratio of AMF to AMFR, *i.e.* AMF/AMFR, showed that there was no statistical significance in the increased ratio [Supplementary Fig. I-A and B, Supplementary Table IV]. On the other hand, the ratio of AMFR to AMF, *i.e.* AMFR/AMF, was elevated in epithelioid sarcoma and multiple myeloma. The result indicated that the expression of AMFR overcome that of AMF, implying a nature of AMF-sensitive characteristics in these tumors.

3.4. Visualizing AMF-AMFR amplification in bone microenvironment of multiple myeloma

The significance of AMF and AMFR in multiple myeloma was clarified above. Next, in order to visualize the bone tumor microenvironment, we have modified an online open software in order to investigate novel cell-to-cell/ligand-to-receptor interaction networks using the CAGE database [31]. We further extracted CAGE data from primary cultured cells and added them to the software together with more than 1500 CAGE data including tumor cells from the FANTOM5 database in order to analyze tumor microenvironment, *i.e.* 'Environmentome'. First, we confirmed the AMF-AMFR feedback amplification in bone tumor microenvironment of multiple myeloma in an autocrine manner [Fig. 2-A]. Next, since this study mainly focused on a bone microenvironment of multiple myeloma, we have simultaneously analyzed the interactions among the cellular components of the musculoskeletal tumors. The result discovered that multiple myeloma indeed release AMF into the bone microenvironment, while several AMFR-expressing cells were influential in a paracrine manner, including mature osteoclast, osteocyte, bone marrow stromal cell, bone marrow mesenchymal stem cell, CD4$^+$ T cell, CD8$^+$ T cell, and dendritic cells [Fig. 2-B]. Among them, mature osteoclast, osteocyte, and dendritic cell were exceptionally sensitive responders [Table1]. These results suggested that AMF affected to multiple myeloma in an autocrine manner, and that AMF also considerably interacts with normal osteoblasts, osteoclasts, CD4$^+$ T cells, CD8$^+$ T cells and other immunological cellular components in a paracrine mode, resulting in the induction of a dynamic influence in the bones of multiple myeloma.

4. Discussion

Here we depicted the AMF and AMFR level of expression in musculoskeletal tumors, and the ligand-receptor interaction was evident in bone tumor microenvironment of multiple myeloma.

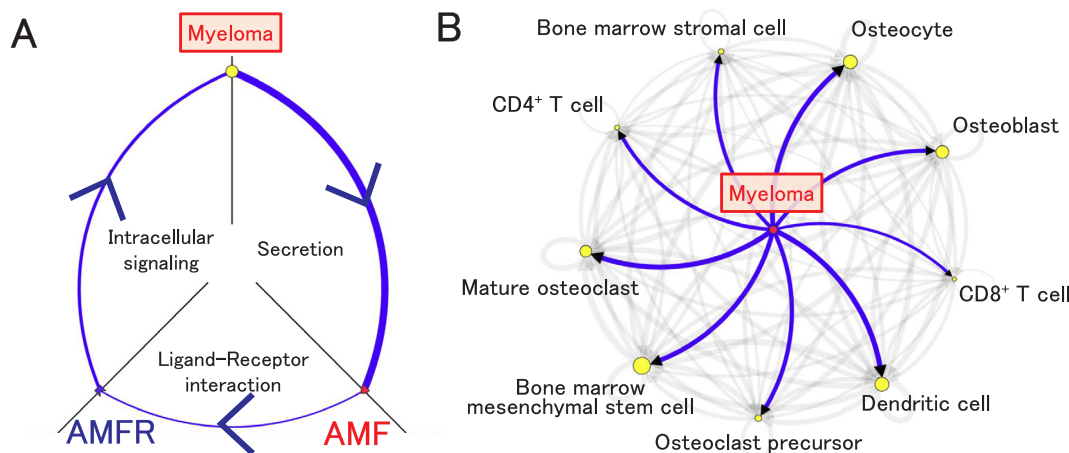Additionally, higher levels of AMF (GPI) was observed in a certain

Fig. 2. (A) Visualizing autocrine AMF-AMFR amplification in bone tumor microenvironment of multiple myeloma. Blue arrows indicate each expression and interaction, showing the feedback mechanism of AMF-AMFR interaction. The strength of the association is represented by the thickness of blue arrows. (B) Visualizing paracrine AMF-AMFR amplification in bone tumor microenvironment of multiple myeloma. Blue arrows indicate AMF interaction to cellular component of bone tumor microenvironment. The strength of the association is represented by the thickness of blue arrows. Gray arrows indicate other numerous interactions of AMF in bone microenvironment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

In order to quantify the interaction of AMF and AMFR in multiple myeloma, we calculated 'expression product', representing the strength of the molecular association. Note that AMF highly interacted with matured osteoclast, osteocyte, and dendritic cell.

Cells in bone microenvironment	AMFR expression value (TPM)	Total expression value* (TPM)
Matured osteoclast	53.2	11715.4
Osteoclast precursor	33.3	7329.3
Osteocyte	48.7	10714.0
Osteoblast	29.7	6537.0
Bone marrow stromal cell	24.5	5381.2
Bone marrow mesenchymal stem cell	42.8	9419.9
CD4$^+$ T cell	24.9	5469.2
CD8$^+$ T cell	15.2	3341.0
Dendritic cell	54.8	12067.5

* Total expression value indicates 'AMF of myeloma (220.1 TPM) × AMFR of cellular components in tumor bone microenvironment'.

patient cohort of osteosarcoma, rhabdomyosarcoma, and angiosarcoma. As reported, these tumors may enhance the malignant potentials such as glycolysis and gluconeogenesis by expressing GPI, whereas invasion, metastasis by secreting AMF [7,8,13–16]. To inhibit GPI expression and AMF secretions, we have previously reported that hyperthermia treatment exhibited the efficacy [7]. Consistently, a randomized phase III trial proved that regional hyperthermia combined with neo-adjuvant chemotherapy for the sarcomas resulted in better local progression-free survival than chemotherapy alone [33]. Considering these results, sarcoma patients with upregulated AMF (GPI) may be candidate of hyperthermia therapy.

The result showed that higher expression of AMFR (gp78) was significantly characterized by multiple myeloma among 23 types of musculoskeletal tumors. The data is supported by a previous report suggesting that AMFR may be a possible candidate as a biomarker of multiple myeloma [34]. The result indicated that gp78 function as ubiquitin ligase activity also enhanced in multiple myeloma, which leads to higher protein degradation. Taken together, AMF (GPI) and AMFR (gp78) contribute to higher metabolic turnover of protein and glucose besides the ligand-receptor interactions.

In addition to autocrine amplification of AMF-AMFR expression levels in multiple myeloma, we have noted interactions to normal cells in the bone tumor microenvironment. Myeloma-secreted AMF

interacted with osteoblasts, which subsequently induces RANKL, an osteoclast differentiation regulator, leading to osteoclastogenesis [35]. This axis may play a crucial role in osteolytic bone remodeling in multiple myeloma. Similarly, osteoclast-expressing AMFR was reported to be involved in osteoclastogenesis [36] and that myeloma-secreting AMF have a possibility to induce alkaline phosphatase activity and mineralization, indicating that AMF is an enhancer of osteoblast differentiation [37]. Of note, myeloma-derived AMF affects monocyte/ macrophage induced phagocytic capacity, and adherence morphology [38]. Further, myeloma-secreted AMF evokes immunoglobulin secretion in T cells [39], and systemic administration of AMF induces T cell-dependent skeletal degradation [40]. With regards to bone marrow mesenchymal stem cell, bone marrow stromal cells, and dendritic cells, the proposed new technology detected novel AMF-AMFR interactions in the bone tumor microenvironment.

The data could be translated to a novel clinical therapeutic approach with clinical value and understanding tumor progression and metastasis. It should be emphasized that Bortezomib is currently considered to be a first line therapeutic treatment for multiple myeloma, and one of the proteasome degradation inhibitor to a variety of ubiquitinated proteins through the ERAD, in which AMFR is a key player in its early step [30]. Thus, we conclude that a specific inhibition of AMF-AMFR interaction may induce ER stress and apoptosis in multiple myeloma, and the data could be interoperated as a key proof of principle providing the mode of therapeutic action of Bortezomib.

In conclusion, the results provide a novel outlook for understanding of multiple myeloma progression, and suggesting a new diagnostic and therapeutic target while providing an insight, not previously considered, to patients afflicted with malignant musculoskeletal tumors.

CRediT authorship contribution statement

Kosei Nakajima: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization. **Avraham Raz:** Supervision, Writing - review & editing.

Declaration of Competing Interest

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbo.2020.100308.

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