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Development of a New Focal Mouse Model of Bone Metastasis in Renal Cell Carcinoma

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Abstract. *Background/Aim: Developing animal models of bone metastasis in renal cell carcinoma (RCC) is challenging as immunodeficient mice are required. The aim of this study was to develop a simple immune model of RCC bone metastasis. Materials and Methods: RENCA tumor cells were injected into the right femurs of BALB/c mice. Sixty mice were grouped into each twenty-mouse group according to the tumor cell concentration, and the presence or absence and extent of bone metastasis in the total length of the femur were compared using hematoxylin and eosin staining of the excised tissues. Results: Bone metastasis was significantly higher in the high concentration group than in the other groups ($p<0.05$), with 10 mice developing bone metastasis at two weeks and nine mice developing bone metastasis at three weeks. The extent of bone metastasis was significantly greater in the high concentration group than in the other groups ($p<0.05$). Multiple logistic regression analysis was performed to examine the factors influencing bone metastasis, and only the high concentration was a significant factor ($p<0.05$). Conclusion: We developed a normal immunity mouse model of local bone metastasis from RCC. This model could prove valuable for research into the treatment of bone metastases in RCC.*

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Renal cell carcinoma (RCC) comprises approximately 3% of all human cancers. and although there are regional differences, with a higher frequency in North America and Europe than in South America, Asia, and Africa, it is increasing worldwide due to smoking and lifestyle-related factors (1). By 2020, more than 400,000 new patients were diagnosed and there were approximately 180,000 deaths (2). Patients with RCC are prone to bone metastasis, which is experienced in a reported 29.5% of patients (3), and 85% of those patients having previously experienced skeletal-related events (4). In addition, 36.9% of patients with bone metastasis required some form of surgical treatment (5); however, surgery for bone metastasis carries a high risk of bleeding due to abundant blood flow (6). The number of patients with bone metastasis is expected to increase owing to the development of new therapies that have extended survival times (4). Consequently, there is also an anticipated increase in the number of patients who will require surgical interventions.

Research into the mechanisms and treatment strategies for bone metastasis in RCC warrants increased focus and the use of animal models for comprehensive investigation. Previous studies have reported RCC bone metastasis models established through intracardiac and intraosseous injections (7, 8); however, these methods can exhibit a low probability of bone metastasis, may lead to metastasis in other organs, and require the use of immunodeficient mice (7-9). Breeding immunodeficient mice presents challenges due to infection risks, thereby complicating the development of reliable bone metastasis models. In the present study, we aimed to develop a reproducible model of RCC bone metastasis using mice with normal immunity.

Materials and Methods

Cell culture. RENCA is a spontaneous RCC cell line originated from BALB/c mice (10). RENCA cells (CRL-2947; ATCC, Manassas, VA,

USA) were cultured in RPMI-1640 (30-2001; ATCC), 10% fetal bovine serum (Mediatech, Manassas, VA, USA), 1% non-essential amino acid solution (Fuji Filum Wako Pure Chemical Corporation, Osaka, Japan), 1% sodium pyruvate solution (Fuji Filum Wako Pure Chemical Corporation), 1% L-glutamine solution (Fuji Filum Wako Pure Chemical Corporation), and 0.5% penicillin streptomycin (Life Technologies Corporation, Grand Island, NY, USA). The cell cultures were maintained in a humidified environment of 5% CO_2 and 37˚C. The cells were diluted in phosphate-buffered saline (PBS) so that the final number of cells was either 1.0×10^4/10 μl, 1.0×10^5/10 μl, or 1.0×10^6/10 μl. The survival rate of the tumor cells was evaluated using the trypan blue dye exclusion method with a hemocytometer (Kayagaki, Tokyo, Japan) under an optical microscope (Olympus BH-210, Tokyo, Japan; 400×).

Animal experiments. Six-week-old male BALB/c mice (Charles River Laboratory, Inc., Kanagawa, Japan) were housed in a specific pathogen-free environment. An anesthetic mixture of 0.3 mg/kg medetomidine, 4.0 mg/kg midazolam, and 5.0 mg/kg butorphanol was prepared and administered subcutaneously to achieve good anesthetic depth. An incision in the midline of the knee exposes the white patellar tendon. Another incision was made at the medial border of the patellar tendon, and the patella was laterally dislocated to expose the femoral condyle. A bony hole was created in the femur with a 26 G needle. To assess appropriate tumor cell concentrations, different concentrations of RENCA cells were suspended in PBS and injected by inserting half of the total length of a Hamilton syringe into the bony hole to ensure intramedullary injection beyond the epiphyseal line (Figure 1). The bony hole was closed using bone wax, the patella was repositioned, and the incision was sutured closed. The mice were divided into three groups: i) the low concentration group was injected with 1.0×10^4/10 μl tumor cells (n=20), ii) the medium concentration group was injected with 1.0×10^5/10 μl tumor cells (n=20), and iii) the high concentration group was injected with 1.0×10^6/10 μl tumor cells (n=20). The mice were sacrificed at 2 and 3 weeks post-tumor cell administration. Ten mice from each group were euthanized at each time point (Figure 2) before the right femurs were removed, fixed in neutral formalin, embedded in paraffin, and midsagittal 5 μm sections were obtained from each mouse. Histological evaluation was performed using hematoxylin and eosin (H&E) staining to assess the presence of bone metastasis and measure the percentage of the areas with tumor cells in the total length of the femur. An all-in-one BZ-X800 fluorescence microscope (KEYENCE, Osaka, Japan) was used for this evaluation.

The Animal Experiment Committee of the Akita University School of Medicine approved the protocol for animal experiments in advance, and all subsequent animal experiments were conducted in accordance with the "Animal Experiment Guidelines" of Akita University.

Statistical analysis. Differences in the occurrence of bone metastasis among the three groups were evaluated using Fisher's exact test and Bonferroni's correction. The extent of bone metastasis in the total length of the femur was expressed as a mean value and evaluated using Tukey's multiple comparison test. Factors affecting the occurrence of bone metastasis were analyzed using multiple logistic regression analysis. The value of the variance inflation factors for each factor was less than 2, confirming the absence of multicollinearity. All statistical analyses were performed using Easy R software (26). Statistical significance was established at $p<0.05$.

Results

At 2 weeks, three of the ten mice (30%) in the low concentration group, one of the ten mice (10%) in the medium concentration group, and ten of the ten mice (100%) in the high concentration group had bone metastasis, with the high concentration group having a significantly higher occurrence of bone metastasis than the other groups ($p<0.05$). At 3 weeks, five of the ten (50%), seven of the ten (70%), and nine of the ten (90%) mice in the low, medium, and high concentration groups, respectively, showed bone metastasis; however, no significant difference in bone metastasis occurrence was observed among the three groups (Table I). At 2 weeks, the extent of bone metastasis in the total length of the femur was 31.1%, 10.0%, and 80.2% in the low, medium, and high concentration groups, respectively, with the high concentration group exhibiting a significantly greater extent of bone metastasis than the other groups ($p<0.05$). At 3 weeks, the extent of bone metastasis in the total length of the femur was 24.1%, 51.6%, and 89.3% in the low, medium, and high concentration groups, respectively, with the high concentration group exhibiting a significantly greater extent of bone metastasis than the other groups ($p<0.05$; Table I). Multiple logistic regression analysis of the factors affecting the occurrence of bone metastasis showed that only high concentrations of tumor cell injection was a significant factor ($p=0.0022$; Table II).

Discussion

Novel treatments for RCC have been developed, with recent case reports having demonstrated their therapeutic efficacy (12, 13). However, research on the treatment of bone metastases in RCC remains insufficient (12). As spontaneous bone metastasis in mice is rare, tumor cells must be injected *in vivo* to induce bone metastasis. Mouse models of bone metastasis using intracardiac administration of tumor cells can imitate hematogenous metastasis (7, 14). Studies have reported that RCC bone metastasis can be induced by intracardiac injection of 786-O cells into SCID mice (15, 16), as well as by intracardiac injection of ACHN cells into nude mice (17). However, these procedures are challenging as they fail to induce bone metastasis at a specific site, exhibit limited reproducibility, and may result in hematogenous metastasis in other organs (7, 9, 14). Conversely, direct administration of tumor cells or fragments into the femur or tibia is a simple technique that can induce bone metastasis at specific sites (7, 8, 15, 18). In RCC, 786-O cells injected into the femur of SCID mice (15) and the injection of human RCC tissue fragments into the tibia of RAG2/γc double knockout mice (18) have been reported to induce bone metastasis. However, the use of immunodeficient mice and human tumor cells has drawbacks, including the fact that they do not imitate

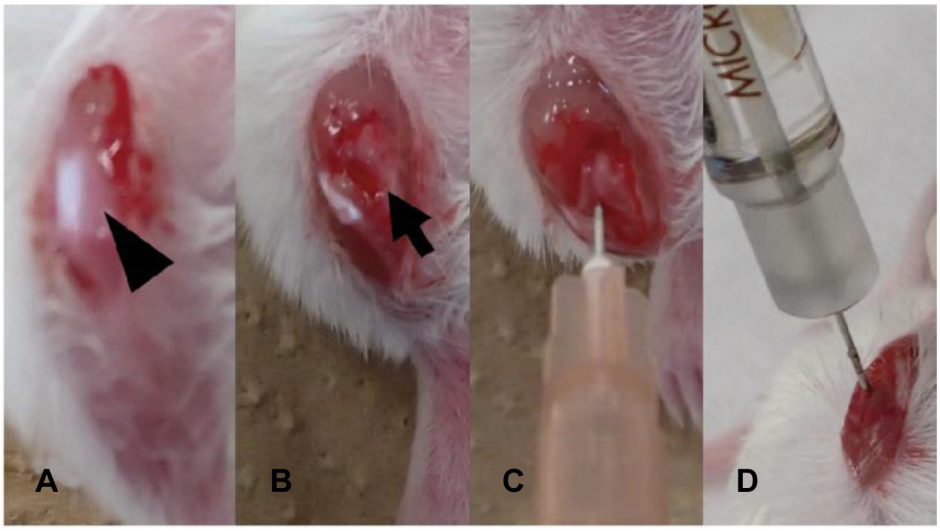

Figure 1. *Method of intrafemoral injection of tumor cells into mouse knees to develop an RCC bone metastasis model. (A) An incision in the midline of the knee exposes the white patellar tendon (arrowhead). (B) Another incision is made at the medial border of the patellar tendon and the patella is laterally dislocated to expose the femoral condyle (arrow). (C) A 26 G needle is used to create a bone socket and (D) tumor cells are injected into the femur using a Hamilton syringe.*

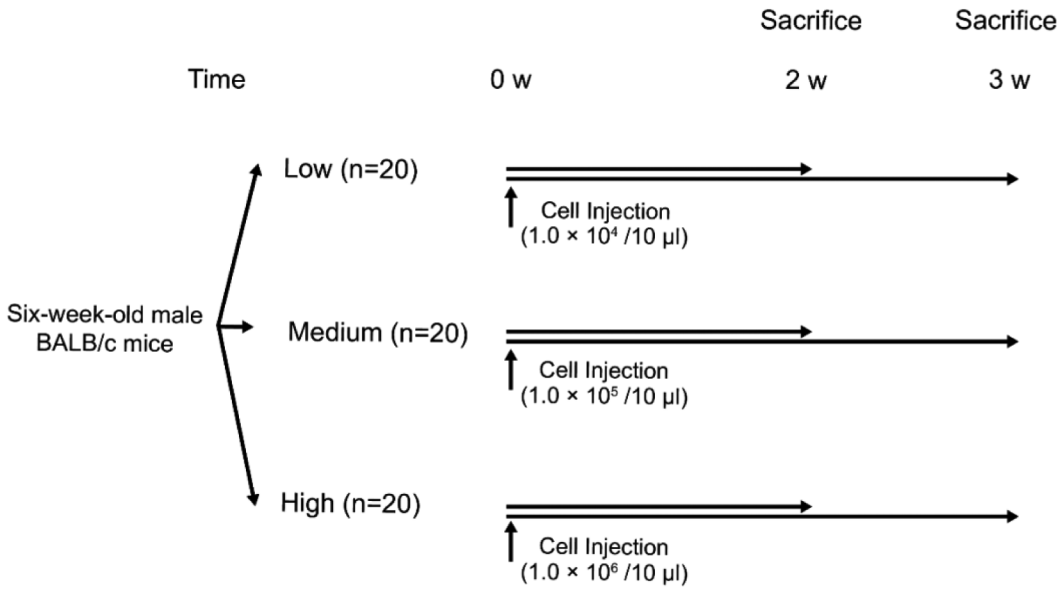

Figure 2. *Experimental groups and time schedule used to develop an RCC bone metastasis mouse model. The mice were divided into i) low (1.0×10^4/10 µl), ii) medium (1.0×10^5/10 µl), and iii) high (1.0×10^6/10 µl) tumor cell concentration groups (n=20 mice in each group). The tumor cell suspensions were injected into the right femur and then ten mice from each group were euthanized at 2 and 3 weeks after tumor cell administration.*

physiological human tumor development and do not enable therapeutic studies of related immune responses (9).

In our previous study, we injected the E0771 breast cancer cell line into the femur of C57BL/6 mice with normal immunity to create a local bone metastasis model of breast cancer (19). In contrast, in an orthotopic model of RCC, RENCA cells were injected directly into the kidneys of BALB/c mice (20, 21). Using the local bone metastasis model of breast cancer (19) as a guide, we demonstrated a new local bone metastasis model of RCC with normal immunity by injecting RENCA cells into the

Table I. *Summary of the three groups.*

	Low concentration group	Medium concentration group	High concentration group
Total number of mice in each group	20	20	20
Number of mice who developed tumors after injection (% of group)			
2 weeks	3 (30)	1 (10)	10 (100)[a]
3 weeks	5 (50)	7 (70)	9 (90)
Extent of bone metastasis (%)			
2 weeks	31.1±23.2	10.0±NA	80.2±13.5[b]
3 weeks	24.1±25.0	51.6±29.8	89.3±7.3[b]

[a]$p<0.05$ (Bonferroni correction) *vs.* the low and medium concentration groups. [b]$p<0.05$ (Tukey's *post hoc* test) *vs.* the low- and medium concentration groups. NA: Not available.

femurs of BALB/c mice. The occurrence of bone metastases in the low and medium concentration groups was not high at either the 2-week or 3-week time points, whereas the high concentration group had an occurrence rate of almost 100%, regardless of the time period. The extent of bone metastasis was greater in the high concentration group than in the other groups, regardless of the time period. The sole factor affecting the occurrence of bone metastasis was high concentration. Given the fact that bone metastasis should be treated immediately, the results of this study indicate that focal bone metastasis occurs after 2 weeks of intrafemoral injections of RENCA at a concentration of 1.0×10^6/10 μl.

Our model demonstrates three primary strengths. First, it is possible to establish uniform bone metastasis by administering the correct amount of tumor cells at a defined injection site, thereby reducing individual variability in determining treatment efficacy (19). Second, the outward dislocation of the patella exposes the femoral condyle, which serves as a guide for the injection, rendering the tumor administration technique simple and reproducible. Third, the immune system of BALB/c mice is normal, excluding the rearing requirements of immunodeficient mice. This model also has the potential to be used to study novel immune-related therapeutics, which is not achievable in previous RCC bone metastasis models that use immunodeficient mouse strains.

A limitation of our study is that the mechanism of bone metastasis occurrence did not follow a physiological process. The process of bone metastasis cannot be fully represented by a single model, and different models must be used for each stage of bone metastasis (7). Although our bone metastasis model is not suitable for studying the early stages of bone metastasis, it is valuable for studying the later stages of bone metastasis.

Conclusion

Using BALB/c mice and RENCA cells, we successfully developed a simple and reproducible local model of RCC bone metastasis. This model may be effective for studying the treatment of bone metastasis in RCC. The application of this model could contribute to the development of bone metastasis models for various carcinomas and facilitate research into their treatment.

Table II. *Summary of multiple logistic regression analysis of factors affecting the occurrence of bone metastasis.*

	Odds ratio	95%CI	*p*-Value
Terms (weeks)	3.19	0.903-11.30	0.0715
Medium concentration	1.25	0.338-4.62	0.7390
High concentration	34.10	3.560-327.00	0.0022

For the multiple logistic regression analysis, the low concentration was used as the reference level. CI: Confidence interval; Variance inflation factors (VIF) of concentration: 1.007413; VIF of terms: 1.014880.

Conflicts of Interest

The Authors declare no conflicts of interest directly relevant to the content of this article.

Authors' Contributions

All Authors were involved in the planning and revision of this manuscript. OK, TH, and NH raised the experimental animals and administered drugs. OK analyzed the raw data and wrote the manuscript. HM, KY, KD, SR, KF, KT, WM, TK, and MN reviewed the manuscript.

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