SHORT COMMUNICATION

Model Validation of Breast Cancer Induced to Bone Using ^{99m}Tc-MDP

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ABSTRACT

Metastasis is a process of tumour cells escaping from the primary site and form a new lesion in other organs. It is a common phenomenon where bone is the frequent metastatic site. Bone scan using 99m-Techetium-Methylenediphosphate (^{99m}Tc-MDP) is used to diagnose bone pathologies such as bone metastases. ^{99m}Tc-MDP specifically binds to calcium which present in bone. This study aimed to validate ^{99m}Tc-MDP in breast cancer-induced to bone rat model and to determine calcium presence in the progression of metastasis. The rats were divided into two groups (normal and cancer-induced groups). For cancer-induced group, the left femur was induced with breast cancer cell line, MDA-MB-231. After 21 days, all rats were subjected to SPECT-CT scan. Our finding suggests that the kidney uptake of ^{99m}Tc-MDP is due to the calcium crystal presence caused by hypercalcemia. This is only a preliminary data, and further analysis will be conducted.

Keywords: Metastasis ; ^{99m}Tc-MDP ; SPECT-CT

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INTRODUCTION

It is estimated that 231,840 invasive carcinoma cases and 40,000 deaths due to breast cancer were reported in 2015 and make up for almost 7% of cancer mortality annually (1,2). While cancer is still confined to the breast ducts, chance of survival is up to 100%, however as the cancer migrated and enters lymphatic system and targeting other organs, the chance of survival is reduced. Invasive breast cancer treatment is unfortunately unassured and considered incurable. Treatments now focus on hormonal therapies to slow down the tumour progression and to increase the quality of life and helping patients to live longer (3). While cancer is being labelled as the deadliest disease by the society, the presence of metastasis worsened the condition.

Metastasis is a process of tumour cells escaping from the primary site and form a new lesion in other organs (4). It is a common phenomenon of malignant disease where the most frequent site of metastasis occurs in bone (5), nevertheless the effect of bone metastasis to other organs are poorly understood. Studies on bone metastasis have been conducted using animal models, by the implantation of cancer cells injected into orthotopic locations such as bones or heart (6). However, this artificial bone metastasis requires validation to confirm the model development.

Bone scan using 99m-Techetium-Methylenediphosphate (^{99m}Tc-MDP) is used to diagnose bone related pathologies such as osteomyelitis, bone metastases and occult fracture. The uptake is bone specific, however traces of ^{99m}Tc-MDP can also be found in the urinary tract due to excretion of ^{99m}Tc-MDP (7). Other than excretion, nonosseous uptake of ^{99m}Tc-MDP is an indicator of tissue abnormalities. Hypercalcemia is also the cause of nonosseous uptake of ^{99m}Tc-MDP (8).

Model validation of bone metastasis in animal model is essential to extrapolate the findings to human disease (6). The aim of this project is to validate the animal model using ^{99m}Tc-MDP. Validation methods used in this study is imaging of hybrid single-photon emission computed tomography (SPECT) - computed tomography (CT) and further confirmed by histology with hematoxylin and eosin staining.

MATERIALS AND METHODS

Breast Cancer-induced Bone Metastasis animal model

Female Sprague-Dawley (SD) rats obtained from the Animal Research Centre, Advanced Medical and Dental Institute (IPPT), Universiti Sains Malaysia. Ethical approval obtained from USM Animal Institutional Animal Care and Use Committee (Ref no. USM/IACUC/2017/(106)(851). The study was conducted in

Animal Research Center, Advanced Medical and Dental Institute, USM. Animals were placed in room with maintained temperature of 17°C to 21°C, and 12 hour light and dark cycle. Standard food pellet and water was supplied ad libitum throughout the study. Cleanliness of cage was maintained twice a week.

MDA-MB-231, breast cancer cell line was prepared by culturing the cells in complete medium consists of 90% RPMI 1640 supplemented with 10% Foetal Bovine Serum, and 1% Penicillin-Streptomycin. The cells were trypsinized and centrifuged at 1500 rpm for 10 minutes, at room temperature. 10 million cells in 10µl complete media were used in the study to induced bone metastasis. Rats (n=12) were divided into two groups: (i) Sham (control) and, (ii) breast cancer-induced. Breast cancer cells (for breast cancer-induced rats) or culture media (for control rats) were injected in the left femur and the injection site were covered with bone wax. Rats were anaesthetized intraperitoneally with a cocktail of ketamine (100mg/ml) and xylazil (20 mg/ml) with ratio of 1:1 before the injection. Antibiotic (Baytril 5mg/ml) were given intramuscularly to the rats 5 days post operational procedure and observations were done daily to assess the surgery site recovery. The rats received same post-operational care and after 21 days, the rats were sacrificed.

SPECT-CT Imaging

At day 20, the animals were injected with ± 37MBq of technetium (^{99m}Tc) medronic acid (MDP) through tail vein and the activity in the post injection syringe was measured. Imaging was initiated 2 hours after injection. The animals were sedated with ketamine-xylazil cocktail intraperitoneally half an hour before imaging. The animals were placed on the table and the emission scan were performed using GE 670 (GE Healthcare, USA)SPECT/CT. SPECT acquisition were performed with 60 steps in step and shoot mode, 20 seconds per step.

Histology

Rats were euthanized at day 21 using carbon dioxide (CO2), and kidneys were collected and fixed in 10% neutral-buffered formalin. Fixed kidney tissues were cut into several sections and placed on a histological cassette and processed in automated Excelsior TM AS Tissue Processor for 16 hours. The processing of tissues includes dehydration, clearing, and infiltrating process. Sections were then embedded to form tissue blocks and sectioned into 3 µm thick slices. The sections were then transferred into water bath at 37 C and then mounted on a glass slide. Hematoxylin and Eosin (H&E) staining were performed on the section for the analysis of calcium crystal deposition. The slides were then assessed blindly by a histopathologist.

RESULTS AND DISCUSSION

Histological analysis and Polymerase Chain Reaction result showed an uptake of ^{99m}Tc-MDP in the bone and kidney (Table I) by SPEC-CT, and the presence of calcium crystal in the kidney were observed by H&E staining. (Fig. 1, Fig. 2) This is a preliminary study which only consists of qualitative data, thus no statistical analysis was performed.

Result showed three out of six cancer-induced rats showed ^{99m}Tc-MDP kidney uptake and calcium crystal deposition in kidney. This postulated an incidence rate of 50% in animals. A study in 2005 by Samuel and Dhuri reported 26 kidney uptake cases out of from 1300 bone scan which makes up probability of 2% in humans. To date, our study is the only study focusing in animal with

Table. I: Summary of SPECT-CT and histology.

Group	SPECT-CT	Histology Kidney
Sham (Normal)	No uptake on kidney	Normal
Cancer Induced	Uptake on Kidney	Presence of calcium

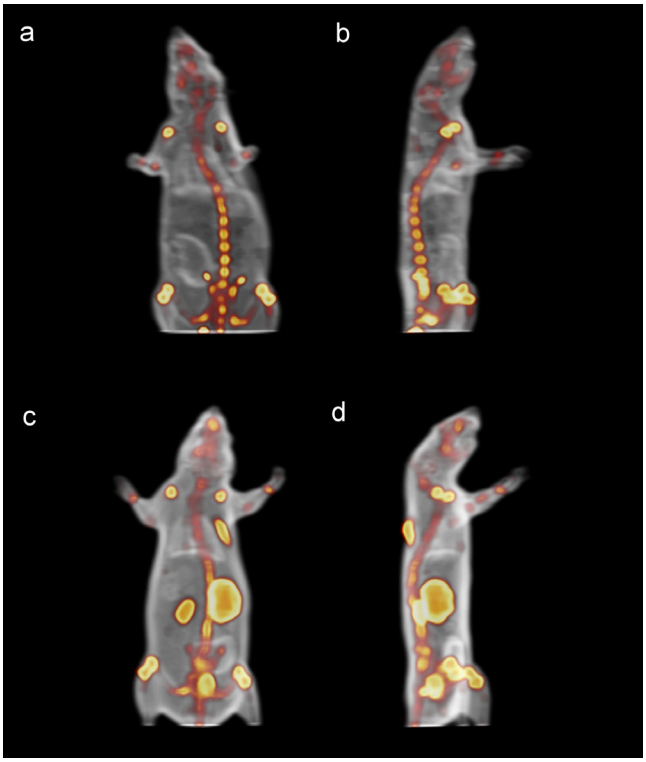

Figure 1: SPECT-CT image of SD rats using 99mTc-MDP. (a) Anterior view of normal rat. (b) Lateral view of normal rat. (c) Anterior view of ^{99m}Tc-MDP kidney uptake in cancer-induced rat. (d) Lateral view of ^{99m}Tc-MDP kidney uptake in cancer-induced rat. These figures are the representatives of one rat from both groups.

(PCR) amplification are two widely used methods to detect bone metastasis in animals using removed tissues, but has limitation in number of sampling. Meanwhile, the use of radiography in in-vivo detection of bone metastases only detects large osteolytic lesion but does not detect micrometastatic lesion (6). In this study, we employed SPECT-CT imaging technique which allows the detection of micrometastasis through the distribution of highly specific radionuclide, ^{99m}Tc-MDP (9). Our

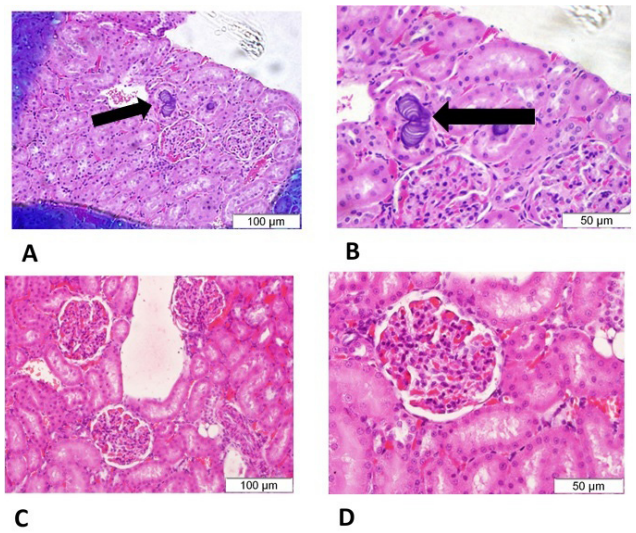

Figure 2: Hematoxylin and Eosin staining of rat kidney (A) Cancer-induced group at 20x magnification. Calcium crystal shown in arrow. (B) Cancer-induced group at 40x magnification. (C) Normal kidney at 20x magnification. (D) Normal kidney at 40x magnification. This figure is a representative of one from the total of three rats. These figures are the representatives of one rat from both groups.

bone metastasis breast cancer-induced animal model using ^{99m}Tc-MDP.

Previous studies focus in human found a significant connection between hypercalcemia and ^{99m}Tc-MDP renal uptake. They suggested that ^{99m}Tc-MDP kidney uptake is caused by the presence of calcium in kidney (10), despite no histopathology data provided by the studies to confirm the presence of calcium in kidney.

Hypercalcemia or increased calcium level in blood is caused by primary hyperparathyroidism or malignancy. In cancer-induced hypercalcemia, the cancer cells caused bone destruction, which in turn released the calcium into the blood (11) which leads to metastatic calcification (12). Saturation of calcium alone is insufficient to form crystal in kidney tubules. The binding of calcium to the cells mediated by proteins such as nucleolic-related protein annexin-II, hyaluronan and osteopontin and the accumulation of the calcium crystal leads to nephrocalcinosis (13).

Osteopontin is a protein involved with cell surface receptors that contributes to cell migration and invasion. In normal conditions, it is expressed at low level. Overexpression of osteopontin is related to tumor progression and metastasis (14). Osteopontin expression in kidney under normal condition is insufficient to form crystal, therefore we postulated the presence osteopontin is due to the cancer cells in bone.

However, in 2005, Samuel and colleague reported normal calcium level in cancer patients with ^{99m}Tc-MDP kidney uptake. Primarily, ^{99m}Tc-MDP bone scan is done for metastatic carcinoma detection but they were unable to find any relationship between the uptake of ^{99m}Tc-MDP with metastasis (15). Zuckier et al., (2010) later proposed the potential mechanisms involved in the ^{99m}Tc-MDP nonosseous uptake which include tumour necrosis with or without calcification, metastatic calcification in renal failure, increased tissue calcium concentration and improper labelling of the radionuclide (16).

To further confirm hypercalcemia in the animals, we will conduct a test to find out the calcium level in blood. The presence of osteopontin at protein and mRNA level will be confirmed using western blot and RT-PCR.

CONCLUSION

^{99m}Tc-MDP is a tool to validate the model and supported by the histopathology. Our finding suggest the uptake of ^{99m}Tc-MDP in kidney is due to calcium deposition. We hypothesized that cancer cells induced bone metastasis caused hypercalcemia and deposition of crystal in kidney. However, further studies need to be conducted to confirm the mechanisms involved.

ACKNOWLEDGEMENTS

This work was supported by RU grant (RUI 1001/CIPPT/8012227) and Short Term Grant (304/CIPPT/6315160) from Universiti Sains Malaysia. We gratefully acknowledge the assistance of Mr. Safri Zainal Abidin, Animal Research FacilityAMDI USM and Mr. Khairul Nizam Jaafar, Medical Lab Technologist at Nuclear Medicine department, AMDI, USM

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