Journal of BUON **17:** 585-590, 2012

ORIGINAL ARTICLE

A new semiquantitative method for evaluation of metastasis progression

A. Volarevic, B. Ljujic, V. Volarevic, M. Milovanovic, T. Kanjevac, A. Lukic, N. Arsenijevic

Centre for Molecular Medicine and Stem Cell Research, Faculty of Medicine, University of Kragujevac, Kragujevac, Serbia

Summary

Purpose: *Although recent technical advancements are directed toward developing novel assays and methods for detection of micro and macro metastasis, there are still no reports of reliable, simple to use imaging software that could be used for the detection and quantification of metastasis in tissue sections. We herein report a new semiquantitative method for evaluation of metastasis progression in a well established 4T1 orthotopic mouse model of breast cancer metastasis.*

Methods: *The new semiquantitative method presented here was implemented by using the Autodesk AutoCAD 2012 program, a computer-aided design program used primarily for preparing technical drawings in 2 dimensions.*

Results: *By using the Autodesk AutoCAD 2012 software-aided graphical evaluation we managed to detect each metastatic lesion and we precisely calculated the average percentage of lung and liver tissue parenchyma with metastasis in 4T1 tumor-bearing mice. The data were highly specific and relevant to descriptive histological analysis, confirming reliability and accuracy of the AutoCAD 2012 software as new method for quantification of metastatic lesions.*

Conclusion: *The new semiquantitative method using AutoCAD 2012 software provides a novel approach for the estimation of metastatic progression in histological tissue sections.*

Key words: cancer, metastasis, method, quantification

Introduction

Metastasis is defined as the spread of cancer cells from a primary site resulting in the establishment of secondary tumors in distant locations [1,2]. The metastatic process is comprised of a series of complex and sequential steps which cancer cells have to successfully complete in order to give rise to a metastatic tumor [1-4]. These steps are: i) intravasation (escape from the primary tumor); ii) dissemination (via the blood or lymphatic system) and survival within the circulation; iii) arrest and extravasation into a secondary site; iv) initiation of growth into micrometastases; and v) maintenance of growth as vascularized, clinically detectable macrometastases [1-4]. Metastasis is the primary cause of death from breast cancer, the most common form of cancer affecting women in the United States, and the second leading cause of cancer-related deaths in women around the world [1]. Certain types of cancers have organ-specific preferences for metastatic growth [1]. However, most of cancer cells usually metastasize to the lungs and liver, making these metastases the leading cause of death for cancer patients [5-7]. In particular, breast cancer cells display a predilection for metastasis to lungs, liver, bone, brain and regional lymph nodes [1,4,8-11].

Opportunities to improve outcomes of cancer patients require a greater understanding of the biology of the metastatic process. *In vitro* analysis of the metastatic process is not sufficient to mimic the complex interaction between cancer cells and the surrounding microenvironment that is crucial for metastasis in humans [7]. On the contrary, *in vivo* models of metastasis, largely in mice, are developed with an aim to provide end points of the metastatic outcome (i.e., presence or absence of metastasis) and time to late-stage metastatic events [7].

The 4T1 mammary carcinoma cell line, originally isolated by Fred Miller and coworkers at the Karmanos Cancer Institute [12,13], when introduced orthotopically is an useful experimental model for the evaluation of breast cancer metastasis [14]. The 4T1 tumor cell line

Correspondence to: Nebojsa Arsenijevic, MD, PhD. Centre for Molecular Medicine and Stem Cell Research, Faculty of Medicine, University of Kragujevac, 69 Svetozara Markovica Street, 34000 Kragujevac, Serbia. Tel: +381 34306800, Fax: +381 34306800, ext.112, E-mail: arne@medf.kg.ac.rs

Received 30-12-2011; Accepted 24-01-2012

has the capacity to metastasize to all organs affected in human breast cancer, including lungs, liver, brain and bone [15-24]. Cell surface proteoglycans are major P selectin ligands that are responsible for the metastatic potential of 4T1 cells [25]. These molecules, expressed on the surface of 4T1 cells, are involved in prometastatic heterotypic adhesion of 4T1 tumor cells to platelets and endothelial cells, enabling 4T1 metastasis to the lungs, liver, bone and brain [25].

Although recent technical advancements are directed toward developing novel assays and methods for detection of micro and macro metastasis [26,27], there are still no reports of reliable, simple to use imaging software that could be used for the detection and quantification of metastasis in tissue sections. We herein report a new semiquantitative method for the evaluation of metastasis progression in a previously described 4T1 orthotopic mouse model of breast cancer metastasis. This semiquantitative method was implemented by using the Autodesk AutoCAD 2012 program, a computer-aided design (CAD) program, used primarily for preparing technical drawings in 2 dimensions (2-D).

Methods

Animals

BALB/c female mice (10 animals per group), 7-9 weeks old, were used in this experiment.

Animals were monitored closely and euthanized when displaying signs of distress or until the experiment was terminated on the 35th day.

Mice were housed in a temperature-controlled environment with a 12-h light/12-h dark cycle and were administered standard laboratory chow and water *ad libitum*. All animals received humanly care and all experiments were approved by and conducted in accordance with the Guidelines of the Animal Ethics Committee of the Faculty of Medicine of Kragujevac, Serbia.

Cell lines and cell culture techniques

4T1 mouse breast cancer cell line was used in this experiment because it represents a good model for the study of breast cancer metastasis [15-24]. The mouse breast tumor cells line 4T1 was purchased from the American Type Culture Collection (ATCC, Manassas, VA). This line is syngeneic to the BALB/c mice. It is derived from the BALB/c mammary tumor and is highly tumorigenic. Mouse breast cancer 4T1 cells spontaneously metastasize to the lungs, liver, bone and brain as evidenced by the formation of visible nodules in these organs.

Mouse breast cancer 4T1 cells were cultured in 25 mL tissues culture flasks (BD Falcon) in high glucose DMEM (Sigma, UK) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma, UK) at 37° C in humidified atmosphere containing 5% CO_2. Mouse breast cancer 4T1 cells used in this study were tested and found free of mycoplasma and viral contamination.

In vivo orthotopic mouse model of breast cancer metastasis

Mouse breast cancer 4T1 cells (5×10^4), previously resuspended in 60 μl of RPMI 1640, were inoculated into the 4th mammary fat pad of BALB/c mice.

Measurement of primary tumor growth

Tumor volume measurements were registered every day and tumor volume was calculated using external caliper [28,29].

Tumor volume based on caliper measurements was calculated by the modified ellipsoidal formula: Tumor volume = 1/2 × (length × width^2) [28,29].

In order to confirm tumor volume measured by external caliper during the experiment, tumors were removed on the 35th day of the experiment, after animals were sacrificed.

The greatest longitudinal diameter (length) and the greatest transverse diameter (width) of tumors were determined and tumor volume was calculated by using the previously described formula.

Histopathological analysis

Because lungs and liver are the most common sites of metastasis in orthotopic mouse model of breast cancer [30], tissue sections of these organs were analyzed and used for implementation of the new semiquantitative method for determination of the metastatic progression.

For histological examinations, the isolated lungs and livers of tumor-bearing mice were fixed in 10% formalin, embedded in paraffin, and consecutive 4-μm sections were mounted on slides. Sections were stained with haematoxylin-eosin (H&E) and examined under low-power light microscopy (Zeiss Axioskop 40, Jena, Germany) equipped with digital camera to evaluate the presence of metastatic lesions.

To avoid missing micrometastasis, stained sections from at least 3 different levels were examined for the presence of lung and liver metastasis.

The new semiquantitative method for evaluation of metastasis progression

First, lung and liver tissue sections were photographed using light microscopy and a digital camera. The image file was opened in the Autodesk AutoCAD 2012 program and the following procedures were performed (Figure 1):

1. Each photo of the tissue sample was imported into a newly-created Autodesk AutoCAD 2012.dwg file.
2. "Polyline" regions were drawn around the whole sample (marked with A) and around each of the metastatic lesions (marked with B) using the option: "polyline" tool.
3. The surface areas of the drawn regions were determined. First, the whole surface area (A region) of tissue section was determined and then the surface areas of each of the metastatic lesions (B regions) were measured (one by one).
4. The surface area of each drawn region was calculated and presented as a unitless number in the Autodesk AutoCAD program.
5. After examination of all the photos from a whole tissue section, the percentage of the area with metastasis was measured by using the formula:

$$N = Bt \times 100 / At$$

where:

N is the percentage (%) of area with metastasis in the whole tissue section,

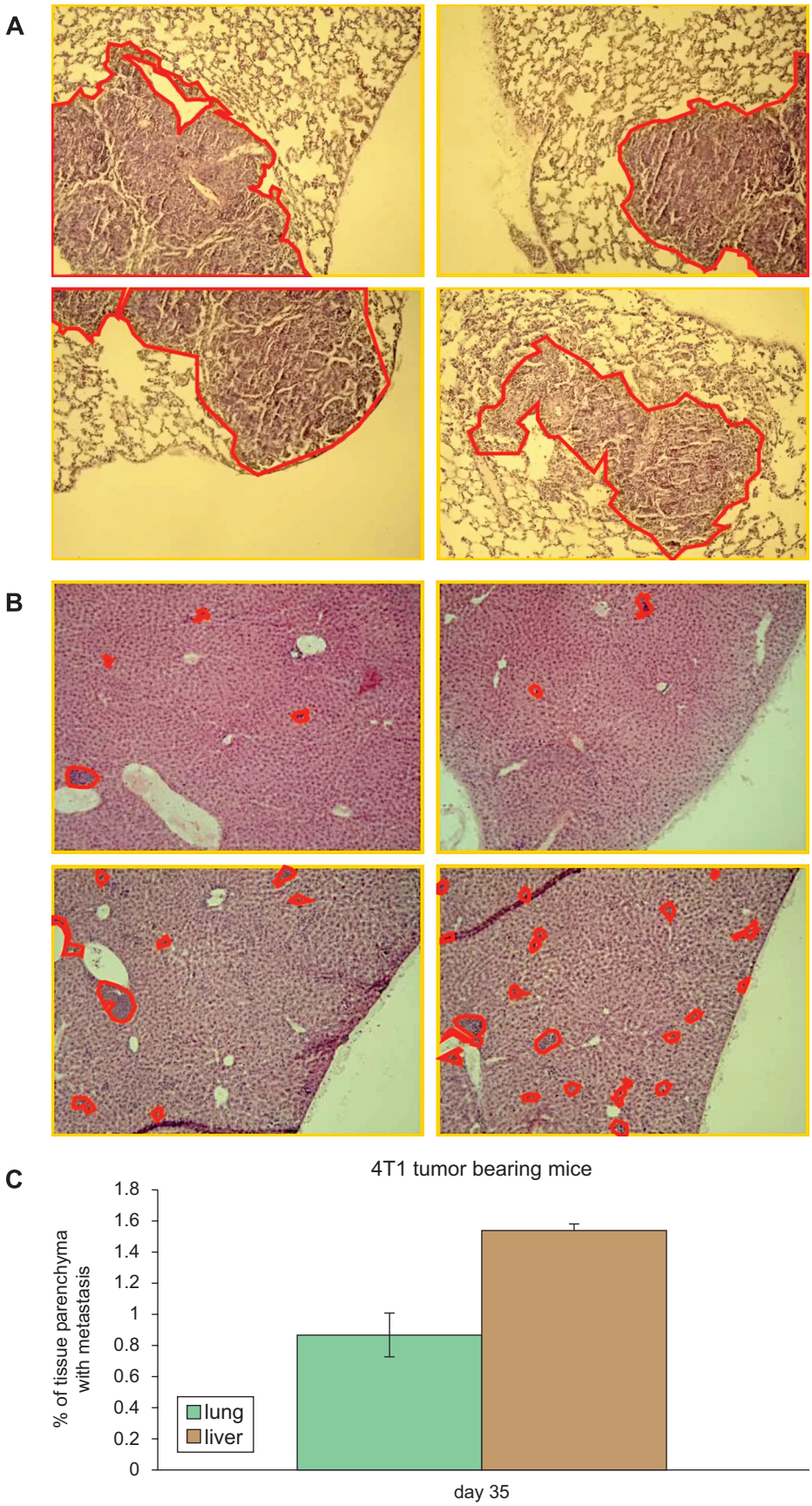

Figure 1. Detection and quantification of metastases in lung **(A)** and liver **(B)** by using the Autodesk AutoCAD 2012 software. Metastatic lesions in representative lung **(A)** and liver **(B)** tissue samples are marked with red polyline. All marked metastatic fields are analyzed by software calculating the percent of tissue parenchyma with metastasis in 4T1 tumor bearing mice **(C)**.

At (A total) is the sum of the sample surface areas in the whole tissue section (At=A1+A2+….An, where n is the number of photos),

Bt (B total) is the sum of the areas with metastasis in the whole tissue section (Bt=B1+B2+…. Bm, where m is the number of marked fields with metastatic lesions).

Results

Autodesk AutoCAD 2012 software enables calculation of metastatic lesion in histological tissue sections of lungs and liver

When introduced orthotopically, the 4T1 line grew rapidly at the primary site and formed metastases in the lungs and liver over a period of 5 weeks.

By using the Autodesk AutoCAD 2012 software-aided graphical evaluation (Table 1), we managed to detect and calculate the diameters of each metastatic lesion in histological tissue sections of the lungs and liver, proposing a novel and original method for semiquantitative determination of metastatic progression in tissue sections (Figure 1).

Multiple metastases were found in the lungs of most experimental animals (9/10; 90%) 5 weeks postinjection of 4T1 cells (Figure 1A).

Liver metastases were found in most animals (8/10; 80%) 5 weeks after orthotopic injection of 4T1 cells (Figure 1B). Tumor cells appeared heterogeneous in size and were easily differentiated from hepatocytes as predominately larger cells with an elevated nuclear to cytoplasm ratio. Increased number of circulating neutrophils and lymphocytes were also found in tumor-bearing animals.

Semiquantitative analysis of lung and liver tissue sections obtained via Autodesk AutoCAD 2012 software, estimated the average percentage of lung and liver tissue parenchyma with metastasis in 4T1 tumor-bearing mice (Figure 1C). The data were precise, highly specific and relevant to descriptive histological analysis, confirming the reliability and accuracy of the AutoCAD 2012 software for quantification of metastatic lesions.

Table 1. The consecutive steps needed to realize the new semiquantitative method for evaluation of metastasis progression by using the Autodesk AutoCAD 2012 software.

A. Importing an image of tissue sample.
B. Drawing a rectangle or polyline on the perimeter of the whole image of the sample.
C. Drawing a polyline around the metastatic lesion.
D. Reading of the marked area of an image in properties panel of a rectangle or polyline, and entering that value below the image.
E. Reading of the marked metastatic lesion area in properties panel of a polyline, and entering that value below the image.
F. The calculated result is entered below the image.

Discussion

External caliper is currently the standard method for determination of tumor volume due to the low cost and high throughput of this simple method [28,29]. Compared with measurement of primary tumor growth, there is no standardized and generally accepted method for quantification of metastatic lesions in tissue sections. Because of that, we presented herein a new method for quantifying metastatic lesions in tissue sections using histological examination and the Autodesk AutoCAD 2012 software application for design and drafting. This software is a computer-aided design program that is traditionally used for calculations and design in architecture and engineering. However, we used the Autodesk AutoCAD 2012 software- aided graphical evaluation for calculating the diameters of metastatic lesions in histological tissue sections, proposing a novel and original method for semiquantitative determination of metastatic progression in tissue sections. The results presented herein were obtained from an experimental study in which BALB/c mice were used for investigation of the metastatic potential of 4T1 cells. A 4T1 orthotopic mouse model of breast cancer metastasis helped us to establish and describe an appropriate methodology and confirmed the reliability and accuracy of the AutoCAD 2012 software for quantification of metastatic lesions.

The use of orthotopic systems gives the most precise information about the efficacy of tested, newly synthesized anticancer drugs to prevent and/or to stop progression of primary tumor growth [31]. Thus, our semiquantitative method for the evaluation of metastasis progression using the Autodesk AutoCAD 2012 software provides what we believe is a novel approach to specifically evaluate the efficacy of anticancer agents in the context of metastatic progression at a secondary site. Furthermore, our method allows the evaluation of a new drug candidate against either single metastatic cells or advanced metastatic lesions because this software easily detected and quantitatively determined each tumor cell in tissue sections. In line with these observations, the new semiquantitative method for the evaluation of metastasis progression that we described herein may be useful in testing the efficacy of novel anticancer agents which addresses the nature of metastatic disease at the site of metastasis and provides precise analysis of their therapeutic potential and should therefore accelerate the development of new treatments for patients with metastasis.

Our method is a newly designed, reliable semiquantitative way to determine areas with metastasis in tissue sections. By using this method, the whole tissue section is analyzed. This is a significant advantage compared with similar, previously published methods that

analyzed metastatic lesions in histological tissue sections. All these methods were either descriptive (signifying presence or absence of metastatic colonies) or calculate areas of metastasis in randomly chosen microscopic fields [32,33]. When metastatic lesions are measured in only few (representative or randomly chosen) microscopic fields, the possibility of an error is high when evaluating the metastatic progression. By using this method the percentage of area with metastasis in the whole tissue section is calculated and precise results are obtained, suggesting that the procedure described here is more accurate compared with earlier methodologies [32,33].

A "black box" exists during which cancer cells spread from a primary site to a metastatic site, resulting in the establishment of gross metastatic lesions in distant sites [34]. Recent attempts to shed light in this process have included highly sophisticated imaging systems that allow some of the steps of metastatic progression to be followed *in vivo* [34,35]. However, these approaches often involve expensive imaging techniques that are time-consuming and do not easily allow serial assessment of early metastatic progression at secondary sites, particularly in the lung and at the single-cell level [34]. Therefore, there is a need in the field of cancer research for a simple method that will be used for study of metastatic progression at a secondary site. Our semiquantitative method for the evaluation of metastasis progression using the Autodesk AutoCAD 2012 software is time-independent, reliable and precisely determines the expansion of metastatic lesions in tissue sections.

In addition, an important advantage of this method is its simplicity and the availability of the Autodesk AutoCAD 2012 software for public use. The description and validation of this method immediately provide researchers an opportunity to explore mechanisms for cancer progression at secondary sites and to optimally develop novel treatment approaches specific to cancer metastasis.

Acknowledgement

This study was supported by the Ministry of Science Republic of Serbia (Grants no. ON175069, ON175071 and ON175103).

The authors are grateful to the architect Igor Zivkovic, BSc and the architect Bojana Petrovic, BSc, for help and technical support.

References

1. Chambers A, Groom A, McDonald I. Dissemination and growth of cancer cells in metastatic sites. Nat Rev Cancer 2002; 2: 563-572.
2. Pantel K, Brakenhoff R. Dissecting the metastatic cascade. Nat Rev Cancer 2004; 4: 448-456.
3. McDonald I, Groom A, Chambers A. Cancer spread and micrometastasis development: quantitative approaches for in vivo models. Bioassays 2002; 24: 885-893.
4. Allan AL, Vantyghem SA, Tuck AB, Chambers AF. Tumor dormancy and cancer stem cells: implications for the biology and treatment of breast cancer metastasis. Breast Dis 2006; 26: 87-98.
5. Abbott A. Cancer: the root of the problem. Nature 2006; 442: 742-743.
6. Bogdanov M, Perisic M, Jurisic V, Stojacic-Djenic S, Kerkez MM. Ultrasonographic examination of the liver circulation in patients with liver metastasis from colorectal cancer. J BUON 2010; 15: 378-381.
7. Eccles SA, Welch DR. Metastasis: recent discoveries and novel treatment strategies. Lancet 2007; 369: 1742-1757.
8. Kocic B, Filipovic S, Petrovic B, Mijalkovic D, Rancic N, Poultsidi A. Clinical and biological characteristics of breast cancer. J BUON 2010; 15: 660-667.
9. Uslu R, Kapkac M, Karaca B et al. Screening for metastasis in primary breast cancer patients having four or more axillary lymph node involvement: is it really necessary? J BUON 2010; 15: 561-567.
10. Zampakis P, Romanos O, Kraniotis P, Solomou EK. Skeletal metastases: an update of the literature with pictorial review. J BUON 2011; 16: 24-37.
11. Dirier A, Burhanedtin-Zincircioglu S, Karadayi B, Isikdogan A, Aksu R. Characteristics and prognosis of breast cancer in younger women. J BUON 2009; 14: 619-623.
12. Miller FR, Miller BE, Heppner GH. Characterization of metastatic heterogeneity among subpopulations of a single mouse mammary tumor: heterogeneity in phenotypic stability. Invasion Metastasis 1983; 3: 22-31.
13. Miller FR. Tumor subpopulation interactions in metastasis. Invasion Metastasis 1983; 3: 234-242.
14. Tao K, Fang M, Alroy J, Sahagian GG. Imageable 4T1 model for the study of late stage breast cancer. BMC Cancer 2008; 8: 228-247.
15. Aslakson CJ, Miller FR. Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. Cancer Res 1992; 52: 1399-1405.
16. Pulaski BA, Ostrand-Rosenberg S. Reduction of established spontaneous mammary carcinoma metastases following immunotherapy with major histocompatibility complex class II and B7.1 cell-based tumor vaccines. Cancer Res 1998; 58: 1486-1493.
17. Eckhardt BL, Parker BS, van Laar RK et al. Genomic analysis of a spontaneous model of breast cancer metastasis to bone reveals a role for the extracellular matrix. Mol Cancer Res 2005; 3: 1-13.
18. Tomasevic Z, Radosevic-Jelic Lj, Jovanovic D. Brain metastases as late breast cancer relapse. Single institution experience and review of the literature. J BUON 2009; 14: 225-228.
19. Wang H, Li X, Xi X et al. Effects of magnetic induction hyperthermia and radiotherapy alone or combined on a murine 4T1 metastatic breast cancer model. Int J Hyperthermia 2011; 27: 563-572.
20. Volarevic V, Milovanovic M, Djekovic A, Petrovic B, Arseni-

jevic N, Bugarcic ZD. The cytotoxic effects of some selected gold (III) complexes on 4T1 cells and their role in the prevention of breast tumor growth in BALB/c mice. J BUON 2010; 15: 768-773.
21. Yoneda T, Michigami T, Yi B, Williams PJ, Niewolna M, Hiraga T. Actions of bisphosphonate on bone metastasis in animal models of breast carcinoma. Cancer 2000; 88: 2979-2988.
22. Chavdarova L, Piperkova E, Tsonevska A, Timcheva K, Dimitrova M. Bone scintigraphy in the monitoring of treatment effect of bisphosphonates in bone metastatic breast cancer. J BUON 2006; 11: 499-504.
23. Opoku-Darko M, Yuen C, Gratton K, Sampson E, Bathe OF. Tumor endothelial marker 8 overexpression in breast cancer cells enhances tumor growth and metastasis. Cancer Invest 2011; 29: 676-682.
24. Alexandrova E, Sergieva S, Nikolova V, Danon S. Bone marrow micrometastases as a prognostic factor in early breast cancer patients. J BUON 2003; 8: 133-137.
25. Monzavi-Karbassi B, Stanley JS, Hennings L et al. Chondroitin sulfate glycosaminoglycans as major P-selectin ligands on metastatic breast cancer cell lines. Int J Cancer 2007; 120: 1179-1191.
26. Lianidou ES, Markou A. Circulating tumor cells in breast cancer: detection systems, molecular characterization, and future challenges. Clin Chem 2011; 57: 1242-1255.
27. Graham KC, Detombe SA, MacKenzie LT et al. Contrast-enhanced microcomputed tomography using intraperitoneal contrast injection for the assessment of tumor-burden in liver metastasis models. Invest Radiol 2008; 43: 488-495.
28. Euhus DM, Hudd C, LaRegina MC, Johnson FE. Tumor measurement in the nude mouse. J Surg Oncol 1986; 31: 229-234.
29. Tomayko MM, Reynolds CP. Determination of subcutaneous tumor size in athymic (nude) mice. Cancer Chemother Pharmacol 1989; 24: 148-154.
30. Aslakson CJ, FR Miller. Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. Cancer Res 1992; 52: 1399-405.
31. Bibby MC. Orthotopic models of cancer for preclinical drug evaluation: advantages and disadvantages. Eur J Cancer 2004; 40: 852-857.
32. Radosavljevic G, Jovanovic I, Majstorovic I et al. Deletion of galectin-3 in the host attenuates metastasis of murine melanoma by modulating tumor adhesion and NK cell activity. Clin Exp Metastasis 2011; 28: 451-462.
33. Jovanovic I, Radosavljevic G, Mitrovic M et al. ST2 Deletion Enhances Innate and Acquired Immunity to Murine Mammary Carcinoma. Eur J Immunol 2011; 41: 1902-1912.
34. Mendoza A, Hong SH, Osborne T et al. Modeling metastasis biology and therapy in real time in the mouse lung. J Clin Invest 2010; 120: 2979-2988.
35. Henriquez NV, van Overveld PG, Que I et al. Advances in optical imaging and novel model systems for cancer metastasis research. Clin Exp Metastasis 2007; 24: 699-705.