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A Novel Noninvasive Method for Evaluating Experimental Lung Metastasis in Mice

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Metastasis remains the most significant problem in the field of cancer. The biologic complexity that characterizes metastasis requires relevant in vivo models. When using murine models for pulmonary metastasis, longitudinal studies are valuable for following the progression of metastatic burden. Currently, the progression of pulmonary metastatic burden in experimental mice over time is monitored through advanced imaging approaches or the clinical assessment of morbidity. Because clinical signs of morbidity are often vague and unpredictable, an inexpensive and reproducible method to detect advanced metastatic burden—before the development of mortality—is needed. We have developed a noninvasive technique for assessing pulmonary metastatic burden in laboratory mice. The pulmonary assessment of advanced metastasis (PAAM) test is performed by restraining an awake mouse and gently applying pressure with the index finger under the xiphoid process. This pressure reduces the diaphragmatic component to respiration. Mice with advanced lung metastases show transient signs of respiratory distress within 3 s of the application of this pressure. Using PAAM in 4 distinct models (including sarcoma and mammary carcinoma histologies) of experimental (tail vein) pulmonary metastasis (n = 114 mice), among 3 independent evaluators yielded 94% positive and negative predictive values, which were validated by histologic assessment of postmortem lung tissue. PAAM is a simple, reproducible, and efficient method to assist in the detection of advanced pulmonary metastasis in mice and contributes to their humane care during longitudinal studies.

Abbreviation: PAAM, pulmonary assessment of advanced metastases.

Metastasis is the most common cause of death in cancer patients. To improve treatment outcomes for patients, advances in our understanding of metastasis biology are crucial. The lungs are a common site of metastasis in a wide range of cancers.[9] The use of murine models is an important and necessary means to study the biologic complexity that characterizes metastasis to the lungs. Longitudinal observation of animals during metastatic progression is a valuable use of such murine models and provides experimental endpoints of cancer progression that are similar to those used in human clinical trials (for example, Kaplan–Meier survival analysis). Such studies can use either genetically engineered mice or transplantable models of metastasis, such as spontaneous metastasis from primary tumors or experimental metastasis using intravenous tail vein injection of metastatic cells.[6]

During these longitudinal experiments, it is necessary to designate endpoints for euthanasia of experimental animals. Endpoints optimally should allow the development and maturation of advanced metastatic burden without requiring the use of death from metastasis as an experimental endpoint. In some models, the development of metastatic lesions can be unpredictable and vary considerably between individual mice and animal groups.[4,7] Current methods for evaluating pulmonary metastatic burden in mice include advanced imaging techniques such as bioluminescence imaging, to detect and quantify luciferase-expressing cells in live mice.[5] Alternatively, MRI, microCT, and X-ray imaging techniques[3,8] can be used to evaluate metastatic progression in the lung. However, such techniques are expensive and time-consuming. In addition, these techniques require anesthetization of mice, which increases the risk of morbidity and mortality in animals with potential respiratory compromise. For this reason, investigators often use physical indications of morbidity to evaluate experimental endpoints for metastatic progression. Clinical indications such as rough hair coat, hunched posture, anorexia, dehydration, decreased activity, decreased grooming behavior, and dyspnea may represent endpoints suggestive of advanced metastasis.[4,7] These clinical signs can be vague and unpredictable; at times, mice with high metastatic burden do not show symptoms until they are found dead or they may rapidly develop advanced clinical signs. Improved clinical approaches to consistently define advanced pulmonary metastasis before the development of significant morbidity or mortality due to metastasis in mice are needed. These methods should be simple, inexpensive, accurate, and reproducible.

For this reason, we have used the pulmonary assessment of advanced metastasis (PAAM) technique as a simple yet effective evaluation of metastatic burden. The technique has aided investigators in the assessment of unmasked subclinical respiratory distress during the progression of pulmonary metastatic disease in longitudinal experiments. Because of the anecdotal success of this technique, we here describe the PAAM technique and its validation.

Materials and Methods

PAAM technique. The PAAM technique is schematically described in Figure 1.

Cell culture. Mouse mammary carcinoma cell line 4T1 (obtained from Dr Fred Miller, Barbara Ann Karmanos Cancer Institute, Detroit, MI),[1] mouse osteosarcoma K7M2 cells,[7] and

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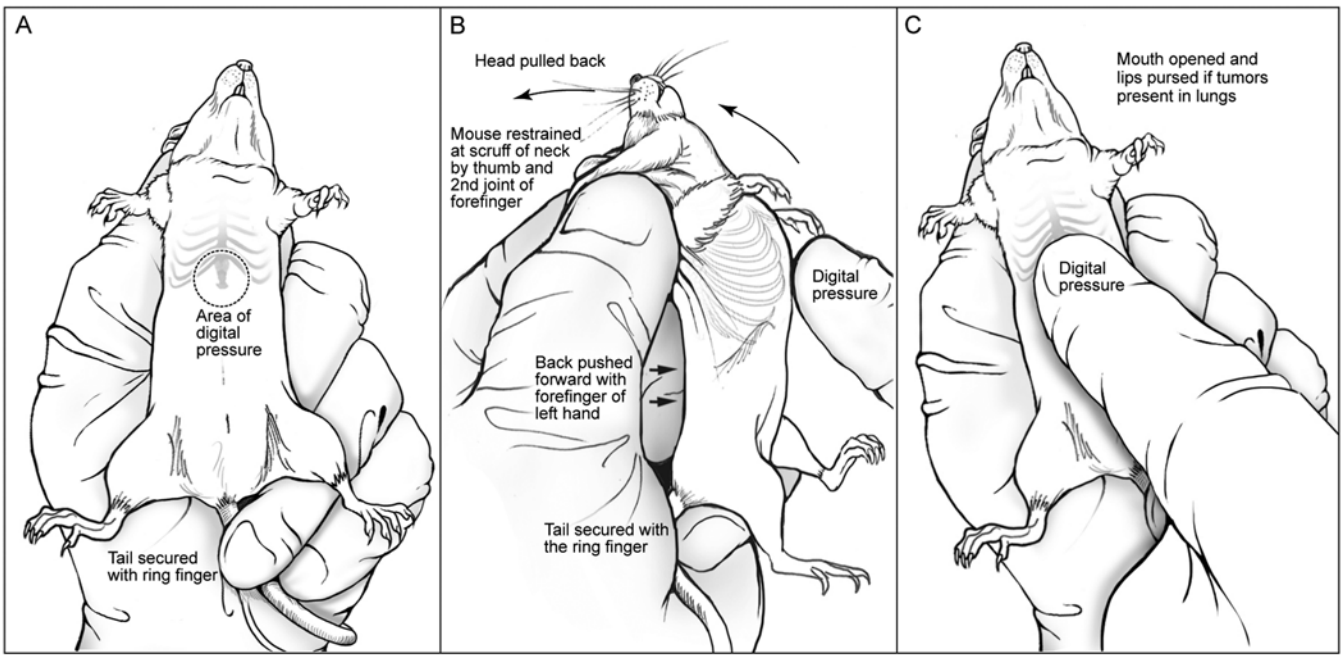

Figure 1. Step-by-step schematic description of the PAAM technique for detecting advanced pulmonary metastatic burden. (A) Mouse is restrained by using the thumb and forefinger of the nondominant hand, and the ring finger is used to secure tail. The mouse is aligned parallel to the forearm of the evaluator, with the head pulled back slightly. (B) Digital pressure is applied gently just caudal to the xiphoid sternum by using the forefinger of dominant hand. (C) Gentle to moderate pressure is applied for 3 s. In normal mice or mice without advanced pulmonary metastasis, this digital pressure results in either no response or only a mild increase in respiratory rate (negative PAAM). In mice with advanced pulmonary metastasis, the described 3 s of digital pressure results in a pronounced increase in chest excursion during respiration or agonal breathing (that is, open-mouth breathing, gasping; positive PAAM).

human MG63.2 osteosarcoma (provided by Dr Hue Luu[10]) and HOS-MNNG (ATCC, Manassas, VA) cells were maintained in DMEM containing 10% FBS, L-glutamine (2 mmoL/L), penicillin (100 U/mL), and streptomycin (100 U/mL, BioSource International, Camarillo, CA) at 37 °C in a humidified CO_2 incubator.

Mice. SPF female (age, 4 to 8 wk) BALB/c and Fox Chase SCID Beige CB17.B6-*PrkdcscidLyst bg*/Crl were obtained from Charles River Laboratories International (Wilmington, MA). In addition, SPF female and male (age, 4 to 10 wk) mice of various strains (C57BL/6NCr, C3H/HeNCr MTV, FVB/NCr, Swiss Webster Crl:CFW, and BALB/cAnNCr) were obtained from Frederick National Laboratory for Cancer Research (Frederick, MD. Housing included ventilated microisolation cages with ad libitum autoclaved food (autoclaved NIH31) and chlorinated water for immunocompetent strains but autoclaved water for immunodeficient strains. A 12:12-h light:dark cycle (lights on, 0600) was provided, with temperature and humidity monitored digitally continuously.

In vivo experimental metastasis assay. In the pilot assessment and subsequent validation study, mice underwent intravenous delivery of tumor cells and were followed by PAAM assessment 3 times weekly by 3 independent evaluators, starting 7 d after injection. Each evaluator performed and interpreted his or her own PAAM tests on individual mice without knowledge of the other evaluators' assessments. The 3 distinct evaluations were all conducted with at least 30 min of rest between PAAM assessments and within a 12-h period. When the majority of the 3 evaluators agreed on a positive PAAM result, the mouse was euthanized and pulmonary metastatic burden was assessed visually. Those mice not yielding a positive PAAM result by 60 d after injection were euthanized at 60 d. For these experiments, 4- to 6-wk-old female Balb/C mice (Charles River Laboratories) were inoculated with 1×10^6 K7M2 cells via the tail vein (pilot, $n = 8$; validation, $n = 10$). Similarly, 4- to 6-wk-old female Fox Chase SCID Beige CB17.B6-*PrkdcscidLyst bg*/Crl (Charles River Laboratories) were inoculated with 5×10^5 HOS MNNG-GFP (pilot, $n = 6$; validation, $n = 13$) or 2.5×10^5 MG63.2 (pilot, $n = 6$; validation, $n = 8$) cells per mouse via the tail vein.

To define the sensitivity and specificity of the PAAM technique, a third experiment was conducted, by using tail-vein injection of osteosarcoma and mammary carcinoma tumor cells. SPF 4- to 8-wk-old female BALB/c mice (Charles River Laboratories) were inoculated with 1×10^6 K7M2 cells or 1×10^6 4T1 cells per mouse via the tail vein. Furthermore, 4- to 6-wk-old Fox Chase SCID Beige CB17.B6-*PrkdcscidLyst bg*/Crl (Charles River Laboratories) were inoculated with 5×10^5 HOS MNNG-GFP, or 2.5×10^5 MG63.2 cells per mouse via the tail vein. Mice were monitored daily until one mouse in each cohort for each tumor cell line showed signs of advanced metastasis-related morbidity (hunched posture, poor hair coat, weight loss, respiratory distress at rest). Confirmation of advanced metastasis in this 'sentinel' mouse was obtained through necropsy examination. When a sentinel mouse was confirmed to have advanced pulmonary metastasis, all the other mice from that experimental group were evaluated by 3 independent evaluators using the PAAM technique. After PAAM assessment, all mice underwent complete necropsy for evaluation and histologic quantification of pulmonary metastases. To provide a context for a PAAM-positive result in regard to pulmonary metastatic burden, we obtained lungs from mice that were found dead due to pulmonary metastases. These mice were taken from studies that were conducted independent of this PAAM validation and used conventional measures of metastasis-associated morbidity. Lungs from these mice were evaluated by using imaging analysis of lungs and histologic examination.

All animal work was done with the approval of the Animal Care and Use Committee of the National Cancer Institute in an SPF animal facility.

Lung imaging. A dissecting microscope (MZ FLIII, Leica Microsystems, Wetzlar, Germany) and camera (Color Chilled 3CCD camera, Hamamatsu Photonics, Bridgewater, NJ) were used to capture green fluorescent and bright-field images from formalin-fixed lungs.

Lung histopathology. Lung tissue was fixed in 10% formalin and embedded in paraffin. A single 5-µm paraffin cross-section was taken per mouse lung at the midpoint through the lung block depth. Sections were stained with hematoxylin and eosin.

Quantification of metastatic burden. Digital image data files were created for each specimen by optically' scanning individual slides (ScanScope XT, Aperio, Vista, CA) at 20× magnification (resolution, 0.5 µm/pixel). Aperio Genie pattern-recognition software, a 'teachable" image-analysis program, was used to identify tumor tissue in each section of lung and to determine the percentage of the lung section analysis area that was occupied by tumor. This process involved establishing histologic features as diagnostically meaningful classes to create an image analysis montage based on one or more tissue sections for training (training set), followed by analyzing the series of specimens as unknowns (testing set) by using the established parameters (algorithm). Tissue classes identified were lung, tumor, and background (glass). Tumors were characterized by expansion or effacement of the alveolar septa by dense aggregates of pleomorphic, neoplastic cells. Two algorithms were developed to accommodate varying tumor morphologies, including the formation of mucinous extracellular matrix in the case of MG63.2 osteosarcoma cells. Heart, esophagus, skeletal muscle, lymphoid tissue, and cartilage were excluded from analysis areas. As a quality-control measure, after automated image analysis, mark-up images of all specimens were reviewed by the operator for accurate segmentation. Area segmentation errors greater than 4% of the total tissue area were adjusted by using manual image segmentation. This method has previously been described previously.[11,12] One hematoxylin- and eosin-stained cross-section at the midsection through the block was analyzed for each mouse assessed.

Statistical analysis. A Mann–Whitney nonparametric statistical hypothesis test was used to compare pulmonary metastasis that was quantified by histologic burden in individual mice. The sensitivity, specificity, and predictive values of PAAM were calculated as commonly defined[2] by using Prism version 4.0c for Macintosh (GraphPad Software, San Diego, CA). Statistical significance was defined as a P less than or equal to 0.05.

Results

PAAM clinical assessment technique for advanced pulmonary metastasis. We here report a clinical method—the PAAM technique—that enables rapid, inexpensive assessment of advanced pulmonary metastatic burden in unanesthetized mice (Figure 1). Our pilot experiment involved using 3 distinct transplantable osteosarcoma cell lines (murine K7M2 and human MG63.2 and HOS-MNNG) in immune-intact or -compromised mice; we use these 3 tumor cell lines frequently in our laboratory studies of metastasis. After tail-vein injection, mice were serially assessed using the PAAM technique and were euthanized when their PAAM assessment was positive. Necropsy was performed to confirm pulmonary metastases. All 11 (K7M2, n = 2; MG63.2, n = 5; HOS-MNNG, n = 4) of the mice determined to be PAAM-positive had gross metastatic disease, with multiple nodules

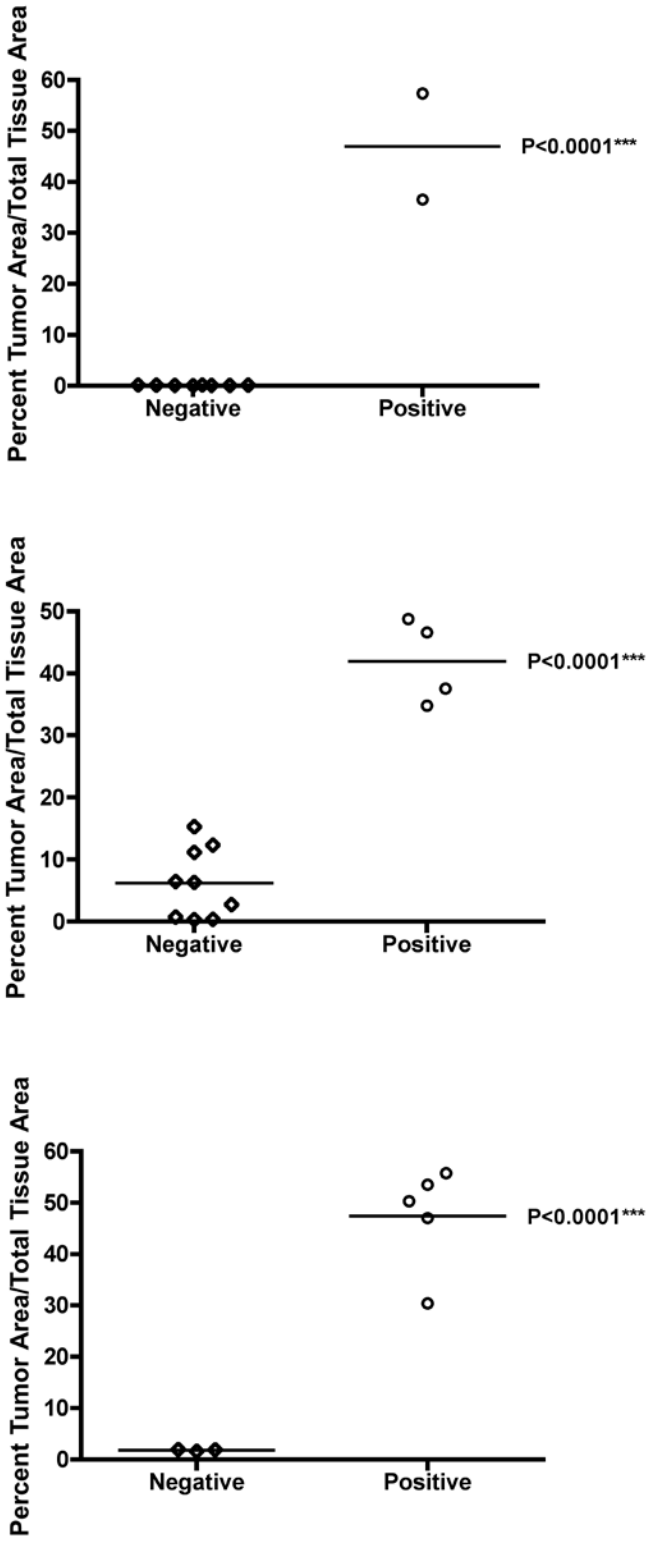

Figure 2. The PAAM technique identifies mice with advanced pulmonary metastatic burden. (A) K7M2, (B) HOS-MNNG, and (C) MG63.3 osteosarcoma cells were delivered by tail-vein injection to BALB/c (K7M2) and Fox Chase SCID Beige CB17.B6-*PrkdcscidLyst bg*/Crl (HOS-MNNG and MG63.3) mice, which then were followed for signs of metastatic disease by using the PAAM technique. Mice were euthanized when their PAAM assessment became positive. Mice that did not yield a positive PAAM result within 60 d were euthanized at that point. Lungs from all mice were processed for quantitative histologic assessment of tumor-per-lung burden. For all 3 cell lines assessed, mice determined to be positive by the PAAM method had a significantly (P < 0.0001) higher histologic metastatic burden than did those that were PAAM negative.

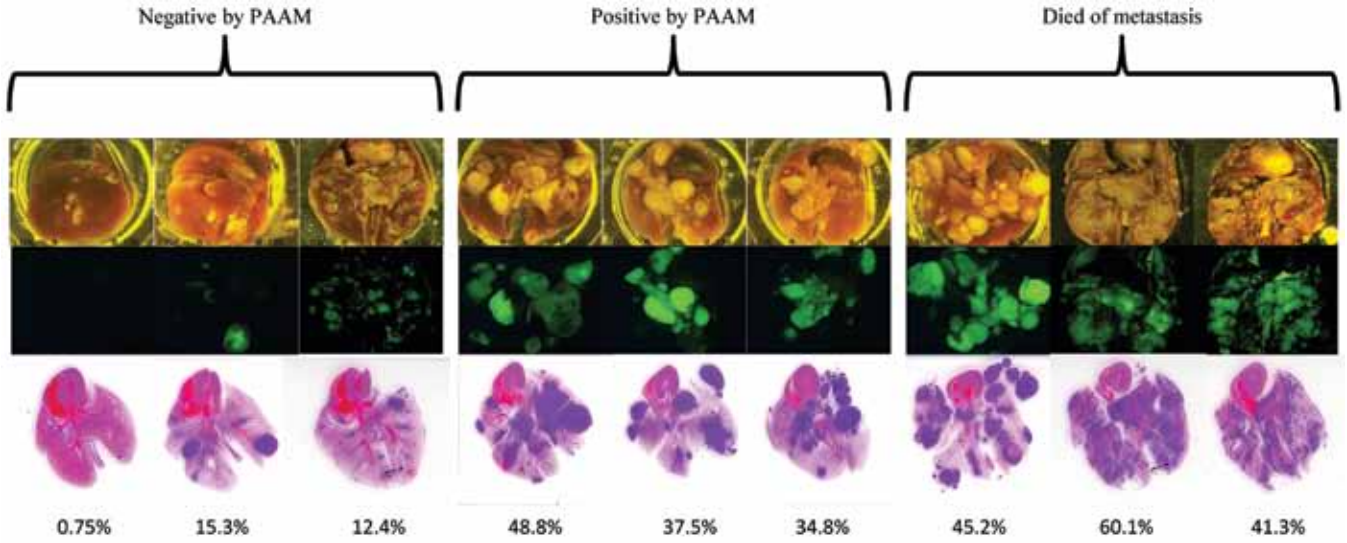

Figure 3. Pulmonary metastatic burden is distinctive in PAAM-positive and PAAM-negative mice. Images presented are Fox Chase SCID Beige CB17.B6-*PrkdcscidLyst bg*/Crl mice intravenously injected with GFP-labeled HOS-MNNG cells into the tail vein. Representative examples of pulmonary metastatic burden are provided for mice that were negative and positive for the PAAM technique. Also included are mice taken from studies unrelated to the PAAM validation that were found dead as a result of pulmonary metastasis. (A) Whole-lung images obtained by bright field stereomicroscopy. Magnification, 10×. (B) Whole-lung images obtained by fluorescent (GFP channel) stereomicroscopy. Magnification, 10×. (C) Histologic sections showing densely basophilic pulmonary metastasis against the eosinophilic lung and heart. Hematoxylin and eosin stain; magnification, 10×. (D) Histologic quantification of percentage tumor area in lungs.

effacing the lungs. In addition, 9 mice (K7M2, $n = 6$; MG63.2, $n = 1$; HOS-MNNG, $n = 2$) did not yield a positive PAAM result within 60 d and therefore were euthanized at this time point. Examination of the lungs of these PAAM negative mice revealed no gross metastatic nodules, thus supporting the value of the PAAM technique. Tolerance to the PAAM technique across mouse strains was assessed by testing healthy, unaffected mice (C57BL/6NCr, $n = 5$; C3H/HeNCr MTV, $n = 5$; FVB/NCr, $n = 4$; Swiss Webster Crl:CFW, $n = 4$; BALB/cAnNCr, $n = 4$). Across these strains, the PAAM technique, as applied by 3 independent evaluators, yielded a negative result in all 22 (100%) of these unaffected and healthy mice.

To further validate the PAAM technique to detect advanced pulmonary metastasis in a longitudinal study of metastasis, we conducted an experiment in which mice again were injected with osteosarcoma cells (K7M2, HOS-MNNG, MG63.3) and followed for signs of metastatic disease by using the PAAM technique. Mice were euthanized when their PAAM assessment became positive; mice that did not yield a positive PAAM result within 60 d were euthanized at that time point (Figures 2 and 3) . Pulmonary metastatic tumor burden was expressed the percentage of tumor divided by percentage of total lung tissue evaluated, as quantified from hematoxylin- and eosin-stained sections of lung. Mice determined to be positive by the PAAM method had a significantly ($P < 0.0001$) higher histologic metastatic burden than did those that were PAAM-negative (Figure 2). Images of lungs from mice determined to be PAAM-positive and -negative in the HOS-MNNG osteosarcoma group (Figure 3) were compared with those of the lungs of mice in other studies that were monitored by using conventional methods of morbidity and were found dead due to advanced metastatic disease. Collectively, these data suggested that the PAAM technique reliably identifies mice with an advanced pulmonary metastatic burden.

The PAAM technique was highly consistent among evaluators, with high concordance of PAAM result among the 3 evaluators. Complete concordance of the 3 evaluators was seen in 88 of 93 (94.6%) PAAM assessments.

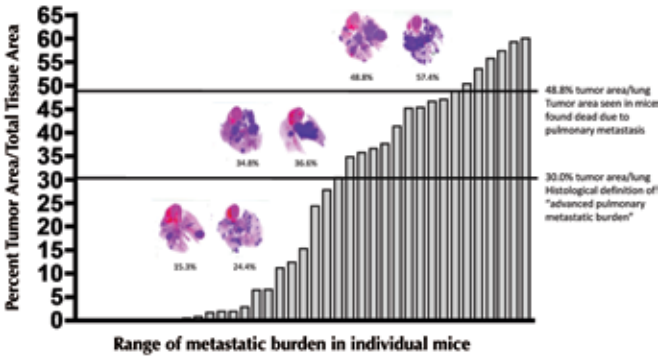

Figure 4. Definition of the histologic threshold for 'advanced pulmonary metastatic burden.' The spectrum of pulmonary metastatic burden quantified by histologic assessment is presented from mice receiving osteosarcoma (K7M2, HOS-MNNG, and MG63.3) and mammary carcinoma (4T1) cells and animals that were found dead due to pulmonary metastasis (these mice were obtained from studies using the same cancer cells but were independent of the PAAM study). Pulmonary metastatic burden was expressed as percentage tumor divided by percentage total lung tissue, quantified from hematoxylin- and eosin-stained sections of lungs by using Genie Pattern Recognition software as outlined in the Methods. Mice that were found dead had a mean histologic pulmonary metastatic burden of 48.8%. By examination of the spectrum of histologic metastatic burden seen in mice and our knowledge that 48.8% burden was seen in mice that died of metastatic disease, we chose to define 'advanced pulmonary metastatic burden' as a histologic burden of greater than 30% tumor area per lung area. Sensitivity, specificity, and positive and negative predictive values for the PAAM technique then were established in comparison to this histologic definition (that is 30% tumor area per lung area). Mice included in this analysis are BALB/c (K7M2 and 4T1 cells) and Fox Chase SCID Beige CB17.B6-*PrkdcscidLyst bg*/Crl (HOS-MNNG and MG63.3 cells).

Accuracy of PAAM. To evaluate and quantitatively validate the spectrum of disease captured by positive and negative PAAM results, we injected mice with osteosarcoma (K7M2, HOS-MNNG, MG63.3) or mammary carcinoma (4T1) cells. Mice were followed until one mouse from each cell-line group

Table 1. Diagnostic validation of the PAAM technique

Tumor model	*n*	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
Overall	114	88 (30/34)[a]	98 (78/80)[a]	94 (30/32)[a]	94 (78/83)[b]
K7M2	27	100 (4/4)	96 (22/23)	80 (4/5)	100 (22/22)
MG63.2	20	100 (13/13)	100 (7/7)	100 (13/13)	100 (7/7)
HOS-MNNG (5×10^5 cells)	16	100 (8/8)	100 (8/8)	100 (8/8)	100 (8/8)
4T1	21	– (0/0)	(1/21)	– (0/0)	100 (21/21)

Quantitative histologic assessment defined 'advanced pulmonary metastasis' as 30% tumor area per lung area (see Figure 4).
[a]No. of mice that were true positives/total no. of mice evaluated.
[b]No. of mice that were true negatives/total no. of mice evaluated.

developed conventional clinical signs of advanced metastasis (hunched posture, rough hair coat, weight loss, respiratory distress at rest). This clinically affected mouse was euthanized and necropsied to confirm substantial pulmonary metastasis. After the confirmation of metastasis in this first ('sentinel') mouse, all remaining mice in the experimental group were examined by PAAM and then euthanized for quantitative histologic evaluation of pulmonary metastatic burden (Figure 4).

Because the goal of the PAAM technique is to identify mice with advanced metastatic burden before they develop significant morbidity or mortality, we examined the histologic burden of mice that were PAAM negative (that had no histologic evidence of pulmonary metastasis) and those from other studies that were found dead due to pulmonary metastasis. Mice that were found dead had a mean histologic pulmonary metastatic burden of 48.8%. By examination of the spectrum of histologic metastatic burden seen in mice and our knowledge that 48.8% burden was seen in mice that died of metastatic disease, we chose to define 'advanced pulmonary metastatic burden' as a histologic burden of greater than 30% tumor area per total lung area (Figure 4). We then established sensitivity, specificity, and positive and negative predictive values for the PAAM technique in light of to this histologic definition of advanced pulmonary metastatic burden. Sensitivity and specificity were 88% and 98%, respectively, and both positive and negative predictive values were 94% (Table 1). No significant differences were seen in the use of PAAM across all cancer types and models assessed.

Discussion

The PAAM technique is a rapid, inexpensive, and accurate method for evaluating metastatic progression in unanesthetized, manually restrained mice. The PAAM technique successfully identifies mice with advanced pulmonary metastatic burden, allowing for the definition of informative and consistent endpoints of metastatic progression in longitudinal studies before significant morbidity or death from metastasis occurs. The PAAM technique may be most useful as an aid to determine endpoints for euthanasia in longitudinal mouse studies, by unmasking subclinical respiratory distress. We experimentally validated the PAAM technique against the 'gold standard' of quantitative histologic assessment of pulmonary metastasis and found our PAAM technique to have high positive and negative predictive values for detection of advanced metastatic burden in mice.

From a pathophysiologic standpoint, we consider that the PAAM technique reduces the diaphragmatic component to respiration when the user applies pressure to the xiphoid process of the mouse. It is reasonable to assume that mice with normal lung volume and function are able to compensate for this temporary reduction in breathing capacity, but mice with advanced lung nodules immediately (that is, within 3 s) show signs of respiratory distress. In our experience, mice that have positive PAAM responses die or develop advanced signs of morbidity within 1 to 2 d of the positive assessment. The PAAM method has been adopted informally by many investigators and laboratory animal veterinarians at our institution over the past several years and has contributed to the success of pulmonary metastasis studies in mice. This anecdotal success and acceptance of the PAAM technique prompted us to quantify and rigorously evaluate this approach.

In the current study, the PAAM technique was validated by 3 independent users who had varying experience with in vivo mouse handling. The study yielded consistent results independent of past experience and required minimal training of the users. Successful use of the PAAM technique requires some practice and repetition. As a user learns the PAAM technique, it is valuable to practice the approach in mice that do not have pulmonary metastasis, to consistently observe the negative PAAM result. As mentioned previously, the speed of the technique allows for the assessment of many mice in a short period of time, because as digital pressure is applied for only 3 s for PAAM assessment. Most importantly, the PAAM technique can be used serially to monitor the progression of metastatic lesions in mice on a daily basis without the need for anesthesia. Our choice of tumor cell lines in this study was based primarily on our own interest in studying the metastasis of solid tumors, particularly in patients with sarcoma and mammary cancer. Our specific selection of 4 distinct tumor model systems included both human and murine cell lines and the use of both immune-intact and immunocompromised mice. The 4 transplantable tumor systems we used are representative of the growth kinetics of most tumor cell lines used in metastasis research. There is no basis to believe that the utility of the PAAM technique will be restricted to these tumor models, but we have no data to specifically address the use of the PAAM method in other cancer models. In addition, we assessed the use of the PAAM technique in experimental (that is tail vein) models of pulmonary metastasis. In our experience, the development and distribution of advanced metastasis after tail-vein injection is similar to that after orthotopic delivery of cells. As such, we expect the PAAM technique to be successfully applied to orthotopic models of metastasis; nonetheless, additional validation of the PAAM technique is needed for such orthotopic models.

A primary limitation of the PAAM technique is that subtle differences in pulmonary metastatic burden cannot be distinguished, and the technique is meant to detect only advanced metastatic disease. For the most part, the result is interpreted as either positive or negative. Furthermore, the PAAM approach cannot distinguish differences in the pattern or nature of metastatic progression and will not distinguish nonmetastatic from metastatic causes of respiratory distress. For these reasons, the PAAM technique is best used in well-controlled experimental systems using models that have been validated for the use of PAAM against a 'gold standard' for pulmonary metastatic

burden assessment. The use of the PAAM technique likely will compliment other methods of assessing metastatic burden, such as imaging protocols.

Stress from over-handling of mice may contribute to the false-positive rates for PAAM. This risk can be minimized through gentle handling and by allowing sufficient time to elapse between repeated tests. In addition, repeated handling of naïve mice for 'training' before and during early phases of studies may acclimate mice to restraint and subsequently reduce stress. We assessed several but not all strains of mice in the current study and found no strain-dependent response or sensitivity to the PAAM technique. Nonetheless, the innate responses of some strains to handling-associated stress may be increased compared with those of other mice and therefore may influence the result of the PAAM. An additional limitation of our validation approach was that a single 5-µm cross-section from each mouse was used to represent the total metastatic burden in the lung. Mice that had low levels of metastatic burden in this single section may have had more advanced disease that accounted for the respiratory distress that was unmasked by the PAAM assessment. As such, these false-negative results may have been over-reported.

Ultimately, the goal of the PAAM technique is to identify mice with advanced metastatic burden. In our experience with most models of pulmonary metastasis, morbidity associated with significant metastasis or death (or both) occurs within 1 or 2 d of a positive PAAM result. This test likely will be most useful during longitudinal studies of metastatic progression and can be used similarly to survival studies, without requiring advanced morbidity or death as endpoints and thereby maximizing the humane care of mice in such studies. Our description and validation of the PAAM technique provides investigators with an immediate opportunity to reduce the costs and time involved in conducting in vivo pulmonary metastasis studies, opening the door for increasingly accurate, informative, and humane mouse studies.

Acknowledgments

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