Differences between epithelial and mesenchymal human tongue cancer cell lines in experimental metastasis

WEI-XIN CAI[1,2], RU QING YU[3], LI MA[4], HONG ZHANG HUANG[1,2], LI WU ZHENG[3] and ROGER ZWAHLEN[5]

[1]Department of Oral and Maxillofacial Surgery, Guanghua School of Stomatology, Hospital of Stomatology; [2]Guangdong Provincial Key Laboratory of Stomatology, Sun Yat-Sen University, Guangzhou, Guangdong 510055; Departments of [3]Oral Diagnosis and Polyclinics, [4]Oral Rehabilitation and [5]Oral and Maxillofacial Surgery, Faculty of Dentistry, The University of Hong Kong, Prince Philip Dental Hospital, Hong Kong, SAR, P.R. China

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Abstract. Distant metastasis represents the outcome with the worst prognosis for various types of malignant tumors, but little is known regarding the impact of interacting epithelial and mesenchymal phenotypic cancer cells within its etiopathogenesis. In a novel animal model, 48 male athymic Balb/c nude mice underwent subcutaneous and intravenous injection of human tongue cancer cell lines of green fluorescent mesenchymal and red fluorescent epithelial phenotypes, in order to visualize and monitor eventual phenotypic interaction in lung metastasis as well as experimental metastasis in *in vivo, ex vivo* and histopathological analyses. While the epithelial, but not the mesenchymal, phenotypic human tongue cancer cell line led to direct metastasis in the lungs when injected intravenously, neither of them, even when injected in combination, were able to establish distant metastasis. The results of the present study provide evidence regarding the role of epithelial phenotypic cancer cells in the release of experimental metastasis following tail vein injection in male athymic Balb/c nude mice, in addition to proving fluorescent human tongue cancer cells may be reliably detected under a fluorescence microscope even 8 weeks after the two injection types.

Introduction

Distant metastasis represents the outcome with the worst prognosis for various types of malignant tumors (1-5), but little is known regarding the impact of interacting epithelial and mesenchymal phenotypic cancer cells within its etiopathogenesis. Oral squamous cell carcinoma, particularly when localized at the tongue base, with >5 cm of tumor thickness and histologically confirmed angiogenesis has been reported to exhibit regional and/or distant metastasis (6) more frequently than those located at other tongue sites.

Metastasis is defined as the breakaway of cancer cells from a primary tumor site and their spread via blood or lymphatic fluid to other parts or organs in the body (7). This process includes the following steps: i) Penetration of cancer cells through the epithelial basement membrane and invasion of surrounding tissues, ii) intravasation of blood and/or lymphatic vessels, iii) spread through circulation, and iv) extravasation and growth in distant sites or organs (8).

Based on a hamster cheek pouch carcinoma (HCPC) model, Tsuji *et al* (9) proposed a 'cooperation theory', suggesting that cancer cells undergo a phenotypic change to accomplish the various steps of metastasis (9,10). It was proposed that only coaction of epithelial and mesenchymal cells may lead to spontaneous metastasis. Mesenchymal phenotypic cancer cells degrade the extracellular matrix, thereby enabling cancer cell invasion, intravasation and transport, epithelial phenotypic cancer cells eventually reestablish colonies at distant sites. Although this animal trial provided insight into certain cancer metastasis phases, particularly emphasizing the different roles of two cancer cell phenotypes, its predictive power is limited due to an inherent 5% genomic difference between rodent and human cells (11).

In order to investigate the interaction of human mesenchymal and epithelial tongue cancer cell lines in cancer metastasis, such phenotypic cancer cell lines stably labeled with two different fluorescent proteins (12) were injected into 48 male athymic Balb/c nude mice (13). *In vivo* and *ex vivo* analyses were performed to investigate whether or not lung metastasis occurred following subcutaneous injections due to phenotypic interaction, and whether or not metastasis after intravenous injection into the tail vein could be observed under a fluorescence microscope and in histopathological analyses.

Materials and methods

Animal care. A total of 48 male athymic Balb/c nude mice (4 weeks old; 12-14 g; Charles River Lab, Wilmington, USA) were supplied by the Laboratory Animal Unit (LAU) of The

Correspondence to: Professor Roger Zwahlen, Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, The University of Hong Kong, Prince Philip Dental Hospital, 34 Hospital Road, Hong Kong, SAR, P.R. China
E-mail: zwahlen@hku.hk

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University of Hong Kong. LAU also provided daily animal care with a standard rodent diet (a complete life cycle diet; LabDiet, St. Louis, MO, USA) and autoclaved water. The animals were housed in a 12 h light/dark cycle at a temperature between 16 and 22˚C in a 5% carbon dioxide, 80% oxygen, 15% nitrogen humidified atmosphere. All mice were acclimatized to their new surroundings for two days prior to the start of the experiment.

Animal experiment. Experimental metastasis (10) was induced by subcutaneous or intravenous injection of fluorescence-labelled human tongue cancer UM1 and UM2 cell lines and their mixture labelled with a green fluorescent protein (GFP) and a red fluorescent protein (RFP), respectively, to obtain spontaenous, direct metastasis. The cell lines were cultured in a mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium at a ratio of 1:1 with 10% fetal bovine serum and 100 U/ml penicillin-streptomycin. The parental UM1 and UM2 cells were donated by Dr David Wong, School of Dentistry, University of California (LA, USA). The manufacturing process and the validity examination of the tumorigenicity of these two cell lines in nude mice have already been published elsewhere (12,13). The number of injected cancer cells and the volume of phosphate-buffered saline (PBS) added were determined according to the technique described by Nakayama *et al* (14). While the mice in groups A, B and C underwent subcutaneous injection with 0.3 ml PBS (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) containing $1x10^7$ cancer cells (UM1-GFP cells in Group A, UM2-RFP cells in Group B, the 1:1 mixture in Group C), those in groups D, E and F received intravenous injection with 0.2 ml PBS containing $5x10^5$ cancer cells (UM1-GFP cells in Group D, UM2-RFP cells in Group E, the 1:1 mixture in Group F) into the tail vein. Table I provides a survey of the experiment. Injected dosages of cancer cells in each group correspond to those used in previous experiments (14).

Assessment of experimental metastasis. All animals were screened for fluorescent singals one week after the injection and once every week thereafter until their euthanasia in week 8 under anesthesia. The latter was performed using ketamine (100 mg/kg; Alfasan Diergeneesmiddelen BV, Woerden, Netherlands) and xylazine (10 mg/kg; Alfasan Diergeneesmiddelen BV). All mice underwent *in vivo* imaging in a supine position using the IVIS® Spectrum *in vivo* imaging system (PerkinElmer Inc., Waltham, MA, USA). The system captured fluorescent light in targeted organs using GFP and DsRed filter sets with excitation wave lengths of 465 and 535 nm, respectively, and emission wave length widths of 500-580 and 620-680 nm, respectively, once a week following cell injection. Following euthanisation at week 8, the lungs were harvested and physically examined for metastatic tumors. Thereafter, the lungs were immerged in PBS, followed by *ex vivo* imaging using the IVIS® Spectrum *in vivo* imaging system (PerkinElmer Inc.). Imaging data were recorded and analyzed using the Living Image 4.4 software package (PerkinElmer Inc.).

Histology. Subsequently, the lung tissue underwent cryosection and paraffin embedding for histological examination. The specimens were inserted in a 2:1 mixture of 20% sucrose (Electron Microscopy Sciences; EMS, Hatfield, PA, USA) and Tissue-Tek® O.C.T. (EMS) for 30 min. Next, the specimens were submersed in a 1:1 mixture of 20% sucrose (EMS) and Tissue-Tek® O.C.T (EMS) and mixed for another 30 min. Finally, all specimens were embedded in Tissue-Tek® O.C.T. (EMS), laid in a tissue mold (EMS) and cut into slices of 6 μm thickness which were examined under a green or/and red fluorescent light detecting microscope (Nikon Corporation, Tokyo, Japan) at a magnification of x20.

The remaining lung tissue was washed three times for 5 min each time with 1X PBS and fixed in 4% paraformaldehyde overnight at room temperature (Sigma-Aldrich; Merck KGaA). Following abundant rinsing under tap water, the fixed tissue was embedded in paraffin, using a Shandon Excelsior ES® Operator (Thermo Fisher Scientific Inc., Waltham, MA, USA) that automatically performed the paraffin infiltration process. Tissue slices of 6 μm thickness were cut with a microtome (Leica RM2155; Leica Microsystems GmbH, Wetzlar, Germany) and stained with hematoxylin for 3 min and eosin for 1 min (H&E) at room temperature prior to being examined under a light microscope (Leica Microsystems GmbH) at a magnification of x200.

Results

In vivo physical examination of spontaneous metastasis groups. Mice in groups A, B and C that underwent subcutaneous injection with cancer cells at the dorsal site developed progressive tumor growth at the location of injection. Regular physical examination of the mice revealed palpable tumors at the end of the first week after injection (Fig. 1A-C).

Ex vivo lung examination in all groups. Macroscopical lung metastasis was only detected in the mice in groups E (5/8; 63%) and F (6/8; 75%), but not in any of the other groups (Fig. 2).

In vivo and ex vivo fluorescent imaging. While *in vivo* imaging did not detect any fluorescent signals except in those animals with the primary dorsal tumors (Fig. 1A-C), *ex vivo* imaging examination revealed red fluorescent signals in lungs of the mice in groups E and F (Fig. 3). Any harvested lung tissue, however, revealed green fluorescence signals.

Histological analysis. While no cryosection specimen harvested from the animals of the spontaneous metastasis groups A, B and C revealed any fluorescent signals other than the primary tumor, red fluorescent signals were observed in the harvested specimens of animals from the direct metastasis groups E and F (Fig. 4). By contrast, no specimen from any animal in the direct metastasis groups displayed green fluorescent signals. The H&E stained lung tissue slices (Fig. 5) were inconspicuous, except for in the animals in groups E and F where metastatic lung foci could be observed.

Discussion

While the epithelial phenotypic human tongue cancer UM2-RFP cell line is able to release direct metastasis in lungs when injected into the tail vein of athymic Balb/c nude mice,

Table I. Experimental metastasis cancer cell line injection scheme.

	Animals	Age, weeks	Cells	Injection site	Sacrifice time
A	8	4	UM1-GFP	Dorsal site	Week 8
B	8	4	UM2-RFP	Dorsal site	Week 8
C	8	4	Mixture	Dorsal site	Week 8
D	8	4	UM1-GFP	Tail vein	Week 8
E	8	4	UM2-RFP	Tail vein	Week 8
F	8	4	Mixture	Tail vein	Week 8

GFP, green fluorescent protein; RFP, red fluorescent protein.

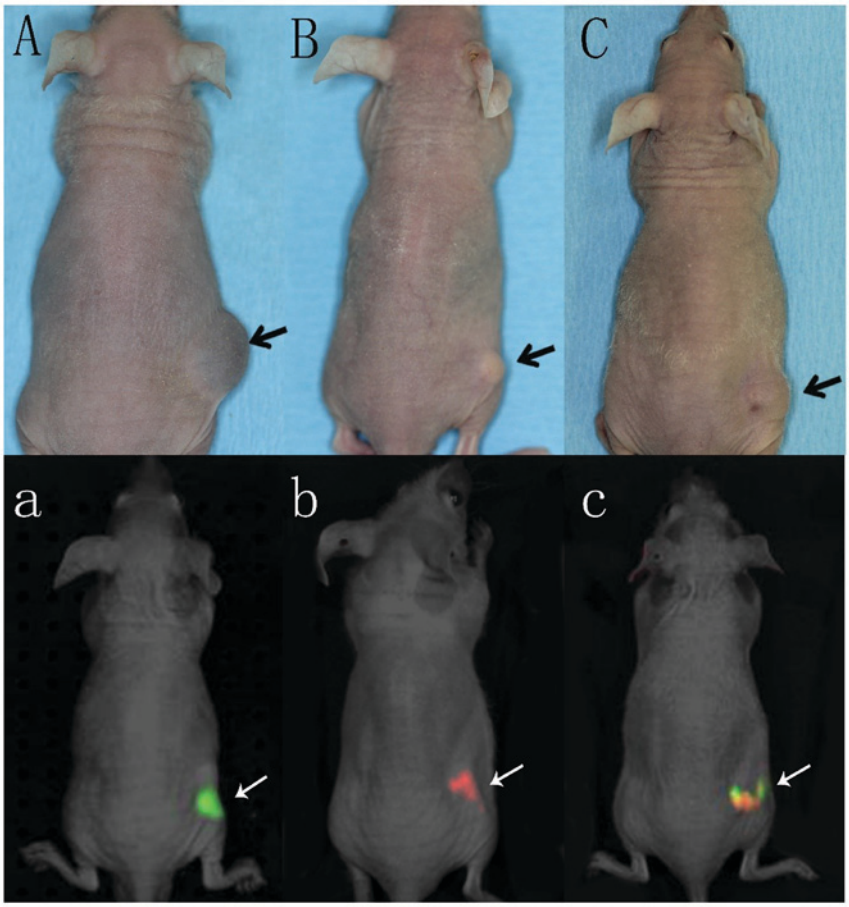

Figure 1. (A-C) Physical examination and (a-c) *in vivo* fluorescent imaging of the subcutaneous tumors (arrow). The injected cancer cells in groups A, B and C were UM1-GFP, UM2-RFP and a 1:1 mixture, respectively. GFP, green fluorescent protein; RFP, red fluorescent protein.

the mesenchymal phenotypic UM1-GFP cell line did not lead to metastasis following intravenous injection. Epithelial and mesenchymal phenotypic cancer cell lines were unable to establish distant metastasis once injected via the subcutaneous pathway.

Previous studies in nude mice made use of rodent cancer cell lines to investigate various roles of cell phenotypes in metastasis (9,10). The present study used human tongue cancer mesenchymal (UM1) and epithelial (UM2) cell lines that originated from a Japanese male patient who suffered from a histopathologically confirmed tongue squamous cell cancer (14). The labelling method and the phenotypic stability of the labelled cells *in vitro* and *in vivo* were reported previously (12,13). Xenograft models making use of human derived cancer cell lines are considered more suitable for translational research, due to the human nature of the injected cells and the fact that the majority of human cancer cell lines form subcutaneous nodules that may serve to measure cancer progression or regression (15).

It was unexpected to discover that the two subcutaneously injected cancer cell lines did not lead to distant metastasis. The following two explanations from previously published literature may explain this: i) Compared with other types of human solid cancer, including lung cancer, oral squamous

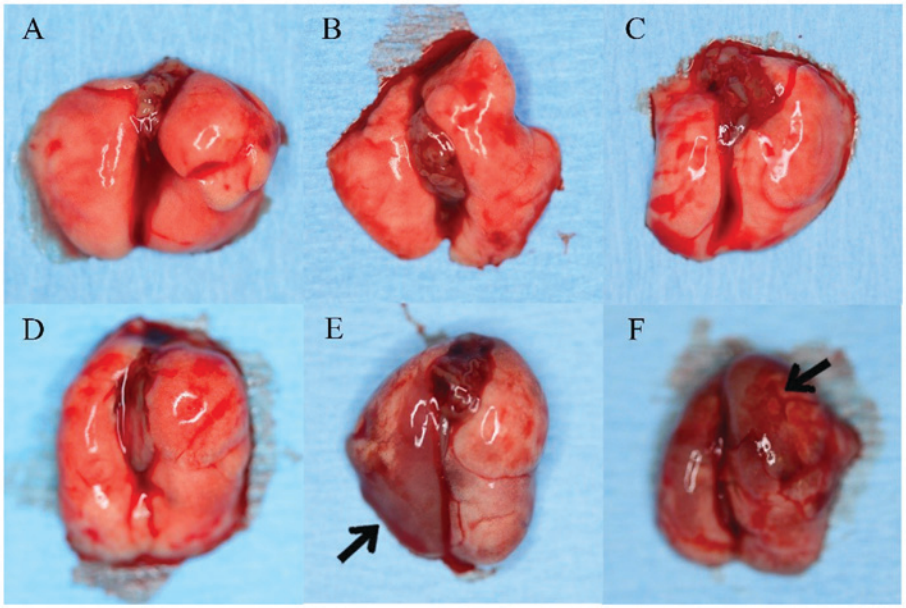

Figure 2. *Ex vivo* examination of the lungs. Group A, UM1-GFP s.c.; Group B, UM2-RFP s.c.; Group C, 1:1 mixture s.c.; Group D, UM1-GFP t.v.; Group E, UM2-RFP t.v.; and Group F, 1:1 mixture t.v.; GFP, green fluorescent protein; RFP, red fluorescent protein; s.c., subcutaneous; t.v., tail vein.

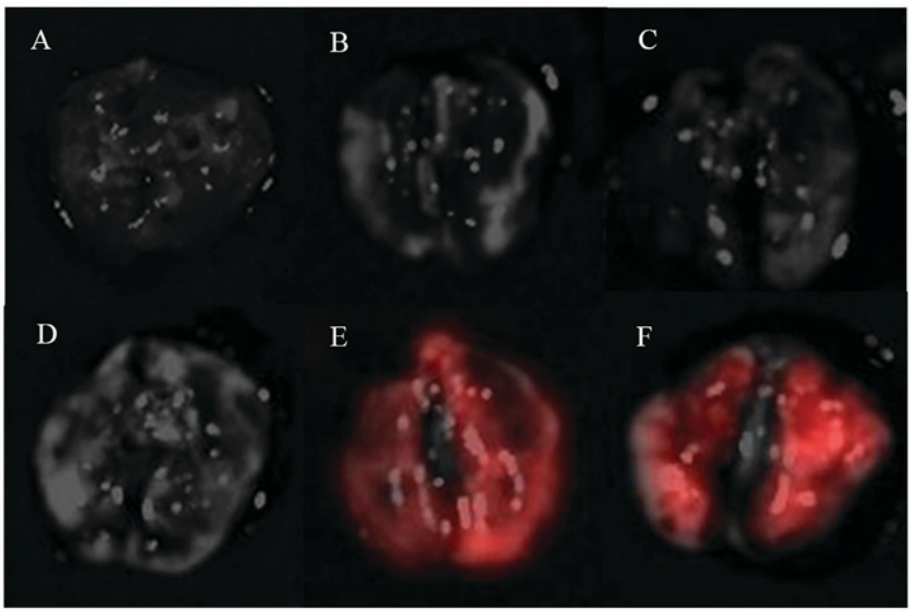

Figure 3. *Ex vivo* fluorescent imaging of the lungs. Group A, UM1-GFP s.c.; Group B, UM2-RFP s.c.; Group C, 1:1 mixture s.c.; Group D, UM1-GFP t.v.; Group E, UM2-RFP t.v.; and Group F, 1:1 mixture t.v.; GFP, green fluorescent protein; RFP, red fluorescent protein; s.c., subcutaneous; t.v., tail vein.

cell cancer is not notorious for distant metastases (16), and ii) innate and humeral adaptive immunity may prevent local oral squamous cell cancer invasion and metastasis (17). The fluorescent proteins GFP and RFP may be integrated into the genetic information of the human tongue cancer cell lines and became hereditary, as previously described (12). Eventual assumptions that these fluorescent proteins within the human cancer cell line genomes may be responsible for generating an immune response inhibiting secondary cancer growth in distant organs (18), warrants further attention in future research. Furthermore, this animal model may be useful in future cancer cell studies investigating the involvement of phenotypes in metastasis (9), as both fluorescence-labelling and cell line phenotypes maintained stable *in vitro* and *in vivo* following several cell cycles (13).

At present, anticancer drugs are in clinical use with the aim of promoting epithelial phenotypes of cancer cells. In this animal trial, lung metastasis was only detected following intravenous injection with the epithelial RFP-labelled phenotypic cancer cell line into the tail vein, irrespective of whether combined or not with mesenchymal GPF-labelled phenotypic cancer cell lines. The intravenous inoculation of cancer cells is no longer accepted as the ideal approach to evaluate metastasis (19). However, the cancer cell survival within the circulation and at secondary sites, including the pulmonary niches, represent crucial steps for cancer metastasis (20).

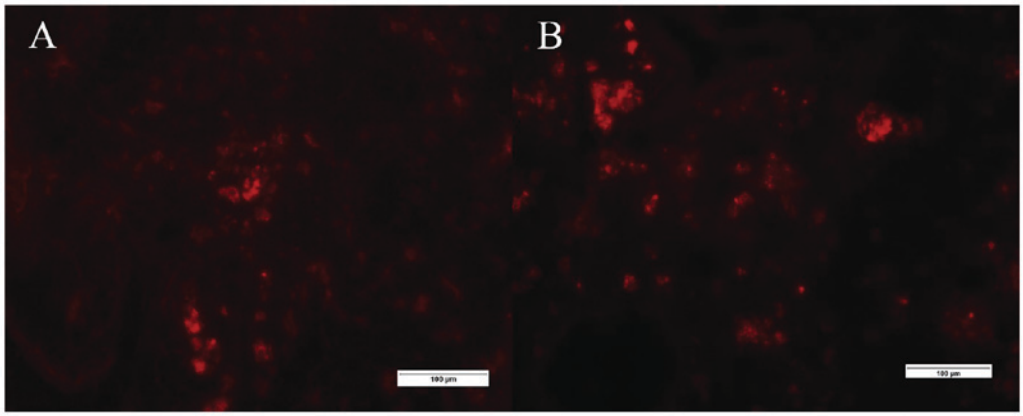

Figure 4. Cryosections of lung tissue harvested from mice in groups E and F. (A) Mice in group E were injected with UM2-RFP. (B) Mice in group F were injected with a 1:1 mixture of UM1-GFP and UM2-RFP. Magnification, x20. GFP, green fluorescent protein; RFP, red fluorescent protein.

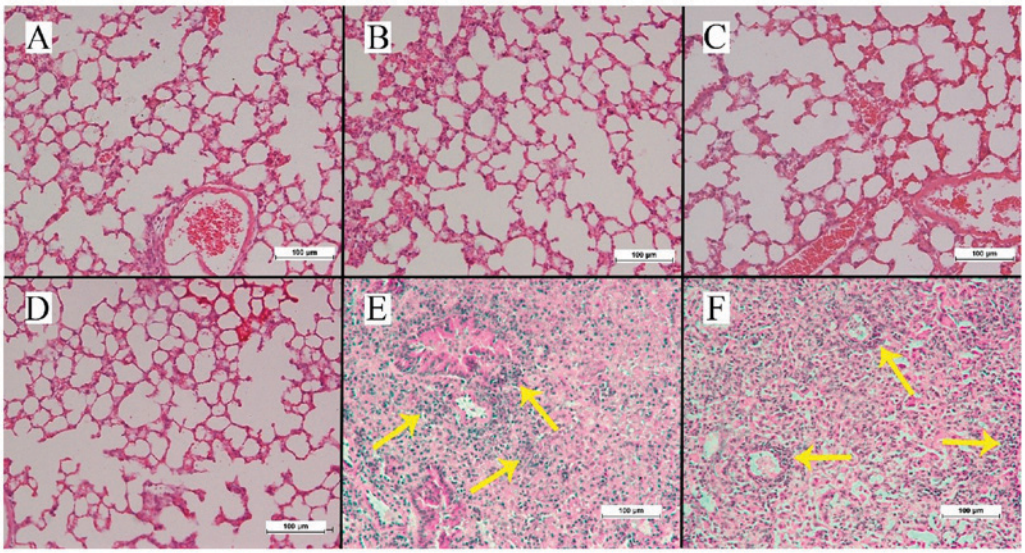

Figure 5. (A-D) H&E staining of the lungs. Metastasis (yellow arrows) was present in lung tissue harvested from mice in groups E and F. Mice in groups E and F underwent injection with UM2-RFP or a 1:1 mixture of UM1-GFP and UM2-RFP, respectively, into the tail vein. Magnification, x200. GFP, green fluorescent protein; RFP, red fluorescent protein.

It may be hypothesized that epithelial phenotypic cancer cells in the circulation, as observed in the present study, contribute to experimental metastasis. This finding may be of importance in designing future anticancer drugs (21). While this finding is opposite to others (14), who detected experimental metastasis into the lung of animals following intravenous injection of mesenchymal phenotype cancer cell lines, it is in line with several other animal experiments (9,10,22). A conclusion that mesenchymal cells do not contribute to cancer metastasis requires to be confirmation in future experiments. In future research, the lineage tracing method (23) may be useful in determining whether the transduction of fluorescent proteins influences the disparity in these observations. Furthermore, a potential cooperation of fluorescent epithelial and mesenchymal phenotypic cancer cells in cancer metastasis (9) may be investigated in this animal model using intravital video-microscopic methods.

One shortcoming of the present study may lie in the subcutaneous application site of the human tongue cancer cell lines, as the microenvironments of the latter location and the tongue tissue are heterogenous (15). The fact that it was not possibile to monitor the metastatic process *in vivo* represents a second shortcoming. Other than in the spontaneous subcutaneous metastasis groups, *in vivo* imaging of fluorescent signals was impossible, likely due to the interference of the chest wall with the signal reception. An eventual combination of similar animal models with intravital video microscopy technique (24) may provide a more promising method for investigating the dynamics of metastatic diseases and angiogenic processes. A third shortcoming is the fact that the liver, which is the first pass organ for the blood flow of the inferior caval vein, was not investigated. Future studies of tail vein injection may study the liver.

The results of the present study contributed evidence to the role of epithelial phenotypic cancer cells in the release of experimental metastasis following tail vein injection in male athymic Balb/c nude mice, as well as proving that fluorescent human tongue cancer cells may be reliably detected under the

fluorescence microscope for as long as 8 weeks after the two types of injection.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WXC, LWZ and RZ contributed to the study design. WXC, RQY and LM performed the animal experiments and examined the specimens. WXC, HZH and LWZ analyzed the data. WXC, LWZ and RZ were major contributors in writing and revising the manuscript. All authors read and approved the final manuscript.

Ethics statement and consent to participate

The present study protocol was approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR 3088-13), Li Ka Shing Faculty of Medicine, The University of Hong Kong (Hong Kong, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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