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Ultrastructural and Immunohistochemical Studies of Transplanted Canine Lung Carcinoma Cell to Severe Combined Immunodeficiency Mice

(*STUDI ULTRASTRUKTUR DAN IMUNOHISTOKIMIA TRANSPLANTASI SEL KANKER PARU-PARU ANJING PADA MENCIT SEVERE COMBINED IMMUNODEFFICIENCY*)

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ABSTRACT

Primary lung cancers, or tumors originating in the lung, are relatively uncommon in dogs. The objective of this study was to describe the canine lung carcinoma that serially transplanted into severe combined immunodeficiency (SCID) mice, in order to established cell line from this tumor cell. Morphology and characteristic of this canine lung carcinoma in SCID mice by histopathological and ultrastructural examinations with metastatic lesion in lung were also examined. Histopathologically, the tumor mass were consisted of cuboidal to columnar cells with papillary pattern, uniform in size, the nuclei were often variable in size, and some cells have vacuole on their cytoplasms. Glandular forms were predominant with lobulated pattern, ductal pattern with papillary injected into tube-like structure were also encountered. Mitotic figures commonly found with inflammatory reaction were sometimes present in the interstitium and lumen gland. Ultrastructural analysis of the tumor cells showed round to oval cells with one or more prominent nucleoli. The cells possessed numerous mitochondria, smooth endoplasmic reticulum, and individual cells which were interconnected via desmosomes. Tonofilament characterize by long cytoplasmic material was encountered. Positive reaction of the round to oval tumor cells to anti keratin antibody confirmed that their epithelial cell nature. Lung metastatic lesions were found in SCID mice after transplantation and this phenomenon indicated that canine lung carcinoma is tumorigenic to SCID mice.

Keywords: canine lung carcinoma, cell culture, SCID mice, metastasis, immunohistochemistry

ABSTRAK

Tumor paru-paru atau tumor yang berasal dari paru-paru jarang ditemukan pada anjing. Untuk mengetahui tentang tumor paru-paru pada anjing maka penelitian ini dilakukan. Penelitian ini bertujuan untuk mempelajari sel kanker paru-paru anjing yang ditransplantasikan secara serial ke mencit *severe combined immunodeficiency* (SCID) untuk mengembangkan sel lestari (*cell line*) dari tumor tersebut. Morfologi dan karakteristik tumor paru-paru aniing pada mencit SCID diamati dengan metode histopatologi dan ultrastruktural. Selain itu, lesi metastatis di paru-paru juga diamati. Secara histopatologi, massa tumor terdiri dari sel kubus dan sel kolumner dengan bentuk papila yang sama ukurannya. Ukuran inti bervariasi dan beberapa sel mempunyai vakuola pada sitoplasma. Formasi glanduler mendominasi dengan bentuk lobulasi, duktus dengan papila yang membentuk struktur seperti tubuler juga ditemukan. Gambaran mitotik umumnya ditemukan dengan reaksi inflamasi yang kadang-kadang ditemukan di daerah interstisium dan lumen kelenjar. Struktur analisis dari sel tumor menunjukkan bahwa sel berbentuk bulat hingga oval dengan satu atau beberapa nukleolus. Sel memiliki banyak mitokondria, retikulum endoplasmik serta antar sel dihubungkan dengan desmosom. Karakteristik tonofilamen ditunjukkan dengan adanya material sitoplasmik. Reaksi positif pada sel tumor dengan antibodi antikeratin menunjukkan bahwa sel tumor tersebut berasal dari sel epitel. Lesi metastasis ditemukan pada mencit SCID setelah transplantasi, menunjukkan bahwa sel kanker paru-paru anjing bersifat tumorigenik pada mencit SCID.

Kata kunci: tumor paru-paru anjing, kultur sel, mencit SCID, metastasis, immunohistokimia

INTRODUCTION

Primary lung tumors are rare in dogs and cats (Theilen and Madewell, 1987; Moulton *et al*., 1981; Mori *et al*., 1991) although metastatic lesions in the lung are relatively common because of the vulnerability of tumor emboli. In some case, the rarity of primary neoplasm tumors in domestic animals is in contrast to their frequency in human (Leopold *et al*., 1974; Moulton *et al*., 1981). However, the reported incidence of lung carcinomas has increased at least 100% during the last 20 year. This is attributed to an increased average life span, better detection and awareness, or, possibly, increasing exposure to environmental carcinogens (Kann and Line, 2010). Primary neoplasm of the lungs arises from cell, which normally present in the pulmonary tissue and it can be epithelial or mesenchymal, although the later is rare (Maxie, 2007; Ogilvie *et al*, 1989). Lung carcinomas were encountered more often in dogs and cats than in other species. Primary lung cancer, or tumors originating in the lung, are relatively uncommon in dogs (less than 1% of all cancers in dogs). In Europe and North America, lung carcinoma occurs in approximately 0.5% of dogs and cat that die from all causes examined at necropsy (Moulton *et al*., 1981). Incidence for lung carcinoma is 5.6 cases per 100,000 dogs in the population per year. In dogs, the overall frequency of lung carcinoma examined at necropsy in various veterinary facilities throughout the world from 1928 to 1984 is 1.24% (Stunzi *et al*., 1974; Meuten, 2002).

Classification of epithelial tumors of the lung is complicated by the recognition that was looked on as specific cells types can undergo metaplasia (transdifferentiation) in both inflammatory and neoplastic lesions. Classification of lung carcinoma consists of bronchogenic carcinoma, bronchiolar carcinoma, epidermoid carcinoma, bronchilia gland carcinoma, and anaplastic carcinoma (Maxie, 2007). The five most frequent types of lung cancer as defined by World Health Organization (WHO) criteria are squamous cell carcinoma, adenocarcinoma, large cell carcinoma, small cell carcinoma and carcinoid tumor (Wagenaar and Tazelaar, 1994) . Most of pulmonary tumors in the domestic animals are adenocarcinoma of bronchogenic or bronchiolar origin; adenosquamous carcinomas are the next common, at least in dog. Squamous cell carcinomas are occasionally found. Other varieties are extremely rare (Moulton *et al*., 1981). However, according to Anderson *et al*., (1992); Charan and Katiyaar, (1996), pulmonary carcinomas in animals seen to arise generally from the bronchioalveolar region (Clara cells or type II pneumonocytes), in contrast to those in human beings, which are mostly bronchogenic (Stenberg, 1989).

The purpose of this study was to describe the canine lung carcinoma that serially transplanted into severe combined immunodeficiency (SCID) mice, in order to established cell line from this tumor cell. Morphology and characteristic of this canine lung carcinoma in SCID mice were also examined by histopathogical and ultrastructural examination with the possibility of metastatic lesion.

REASEARCH METHODS

Original Tumor.

A tumor mass from biopsy case was obtained surgically from the lung of 10 years old female Japanese dog. Histologically, the tumor mass was composed of epithelial cells, which lined by cuboidal and columnar epithelium, often with small papillary projection into the alveolar lumina. The differentiated tumor have well formed glandular structures, lack epithelial stratification, uniform in size, infrequent mitotic figures, minimal stromal fibrosis, and single layered columnar epithelium with uniform basal nuclei. From these findings, the case was diagnosed as a bronchioalveolar carcinoma of canine lung (BAC) according to the classification of Moulton *et al*., (1981) and Maxie (2007).

Transplantation Techniques.

A suspension of canine lung carcinoma (dose volume: 1 mL) was inoculated subcutaneously into four male SCID mice aged 4-5 weeks. The mice were maintained in a specific pathogenic-free condition with temperature of 25 + 3°C. Animals were fed with sterilized pellets and water *ad.lib*. Five weeks after inoculation, the mice were sacrificed with 10% chloroform inhalation and around 1 cm^2 from each tumor was minced, resuspended in Minimum Essential Medium (MEM) and re-inoculated into three to four male SCID mice (six times passages).

Tissue Culture.

The remaining cell solution from transplantable tumor mass in SCID mice at 1st passage was culture in 50 mm diameter plastic

dishes in MEM containing 10% fetal calf serum (FCS), 100 iu mL^{-1} penicillin and 100 mg mL^{-1} streptomycin as described by Priosoeryanto *et al*., (1995[a]). The isolated cells were cultured in plastic dishes together with 18x18 mm coverslips for further light microscopy and immunohistochemistry studies. The culture dishes were maintained in 5% carbon dioxide in air at 36.5°C and observed daily with a phase-contrast microscope (Nikon, DIAPHOT-TMD, Tokyo Japan).

Histopathology (HP) and Electron Microscopy (EM).

The remaining tumor mass and a selected part of other organs including the metastatic lesions in the SCID mice were processed for routine histological and ultrastructural examination.

Tissues were fixed in 10% neutral buffered formalin and processed to paraffin embedded. Sections were cut at 4 mm, stained with hematoxylin eosin (HE) or special stain if required, and examined by a light microscope. Established cultured cells from 30th passage were fixed with formalin gas for five minutes, stained with Wright's and HE.

Another part was processed for electron microscopical examination. Briefly, small portions of the tumor were removed and fixed with 1.5% glutaraldehide in 0.1 M cacodylate buffer, pH 7.4 for overnight and then with 1.5% osmium tetroxide. After dehydration in graded alcohol and propylene oxide, the tissues were embedded in quethol mixtures (Nishin EM Co., Ltd., Tokyo, Japan). Ultrathin sections were cut on an automatic ultramicrotome (Reichert/ Leica, Germany), stained with uranyl acetate and lead cytrate and also examined using a transmission electron microscope (TEM) (Hitachi-800 MU, Japan).

Immunohistochemistry.

The 4 mm-sections were placed on slide glasses coated with 2% neophren in toluen. For cultured cells, the cells were fixed with cold acetone for 5 minutes. The sections and cultured cells were subjected immunohistochemically by avidin-biotin complex (ABC) using a commercially available kit (Vectastain Elite ABC kit, Vector Laboratories, USA). The following primary antibodies were used: mouse monoclonal antibodies (moAb) against human vimentin (Dakopatts, Denmark), human a-desmin (Dako Corporation, California, USA) and rabbits antiserum against keratin (Dako).

RESULTS AND DISCUSSION

A palpable tumor mass was growth slowly in the first week after inoculation, but after three to five weeks the tumor growth was increase rapidly in each mouse (Figure 1). The tumor masses were compact, multilobulated, well circumscribed, varied in size from 0.5 to 2 cm in diameter, discrete demarcated and freely movable over the underlying tissue/subcutaneously (Figure 2A). Cut surface was soft, greyish to yellow white, multilobulated. Haemorrhagic and necrotic lesions in the central masses were sometimes found in some tumor masses.

In the first and second passage, the tumor were composed of cuboidal to columnar cells with papillary pattern arranged on delicate fibrous (Figure 2B). The cells uniform in size, numerous mitotic figures and large fibrous stroma were observed. The nuclei were often variable in size, containing abundant chromatin. In the third to five passages (Figure 2C), glandular form were predominant with lobulated pattern, ductal pattern with papillary injected into tube-like structure. Some cells with a vacuol on their cytoplasm were also encountered. Mitotic figures were commonly found. Inflammatory reaction was sometimes present in the interstitium and lumen gland. The central part of the tumor mass was frequently became necrotic and haemorrhagic.

Ultrastructural analysis of the tumor cells showed round to oval cells revealed large nuclei with one or more prominent nucleoli (Figure 3A). The cells possessed numerous mitochondria, smooth endoplasmic reticulum, and individual cells were intercountered via desmosomes (Figure 3B). Tonofilament characterize by long cytoplasmic material and well develop microvili was encountered (Figure 3C)

In the cultured cells, the small and large rounds to oval cells were strongly stained with moAb for antibodies against keratin (Figure 4A) and vimentin (Figure 4B), but was negative to anti-a-desmin. The original (Figure 4C) and transplanted tumors (Figure 4D) showed an identical immunostaining pattern for both antibodies, which was positive to anti-keratin but were negative for vimentin and desmin antibodies. The immunostaining of the original and transplantable tumor are summarised in Table 1.

A few days after primary culture, some cells clumps began to attach to the plastic dishes, from small colonies progressively became larger

Table 1. Immunostaining of the original tumor, transplantable tumor and cultured cells

Antibodies	Original Dog Lung Tumor	In Vivo Passage						TimeCulture Cells					
		1st	2nd	3rd	4th	5th	6th	1st	2nd	3rd	4th	5th	6th
Keratin	+	+	+	+	+	+	+	+	+	+	NT	NT	NT
Vimentin	-	-	-	-	-	-	-	+	+	+	NT	NT	NT
Desmin	-	-	-	-	-	-	-	+	+	+	NT	NT	NT

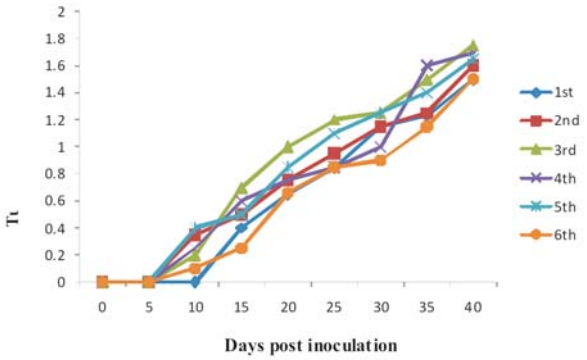
Note:
NT: Not Tested

when allowed to age of few days after confluence and tendency to unite forming large aggregate and some colonies develop individual large cells.

The cultured cells from transplant mass in SCID mice showed several cells morphologies as described above, while the cultured cells derived from the original tissue of canine lung carcinoma showed monotonous appearance of the colonies representing small and large round to oval cells and showed elongated shaped cells with some cells containing many vacuoles in their cytoplasm. These cells have round nuclei with some of them has a large nuclei and one or more prominent nucleoli, and also demonstrating mitotic figure in the culture.

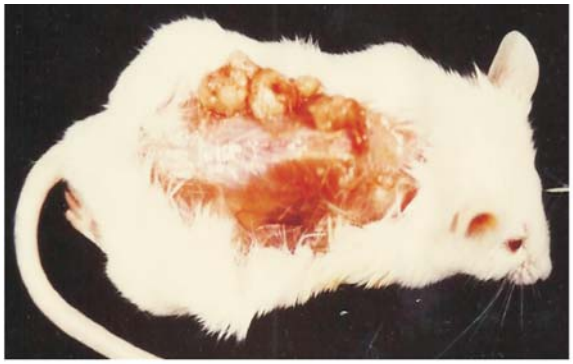
Six months after inoculation, metastatic lesion in the lung of SCID mice transplanted with canine lung carcinoma was detected as small white nodules. The metastatics cells that grow in the lung were round to oval cells with large nuclei (Figure 5).

There have been many reports on the lung carcinoma in dogs and cats (Moulton *et al*.,



Note:
cm: tumor size in diameter

Figure 1. Growth kinetics of the transplanted tumor in SCID mice



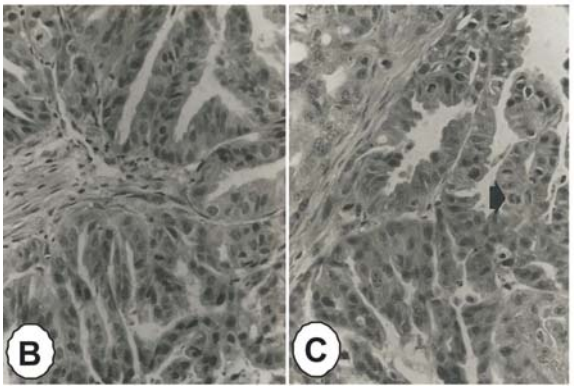


Figure 2. Tumor mass of the transplantable canine lung carcinoma in SCID mice. The tumor masses were compact, multilobulated, well circumscribed, discrete demarcated and freely movable over underlying tissue (subcutaneously) (A). Histological appearance of the canine lung carcinoma serially transplanted into SCID mice. (B) In the first to second passaged, the tumor were composed of cuboidal to columnar cells with papillary pattern. (C) In the third to fifth passaged, glandular form were predominant with lobulated pattern. Mitotic figures were common found (arrow). Hematoxylin-eosin, x 100

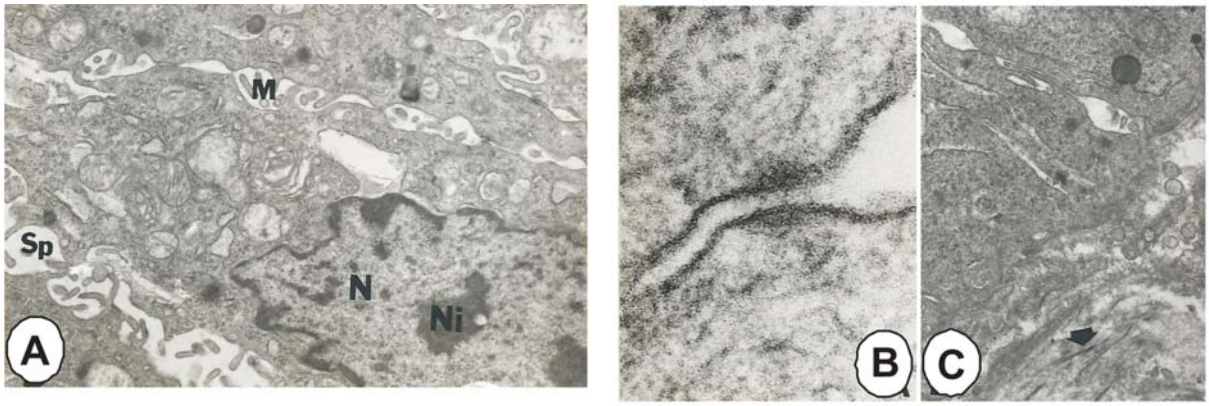


Figure 3. Electron micrograph of the transplantable canine lung carcinoma in SCID mice showing nucleus (N), nucleolus (Ni), microvili (M) and intercellular space (Sp), desmosome (B) and tonofilament (C) (A, x 8,000, B, x 30.000 and C, x 7,000)

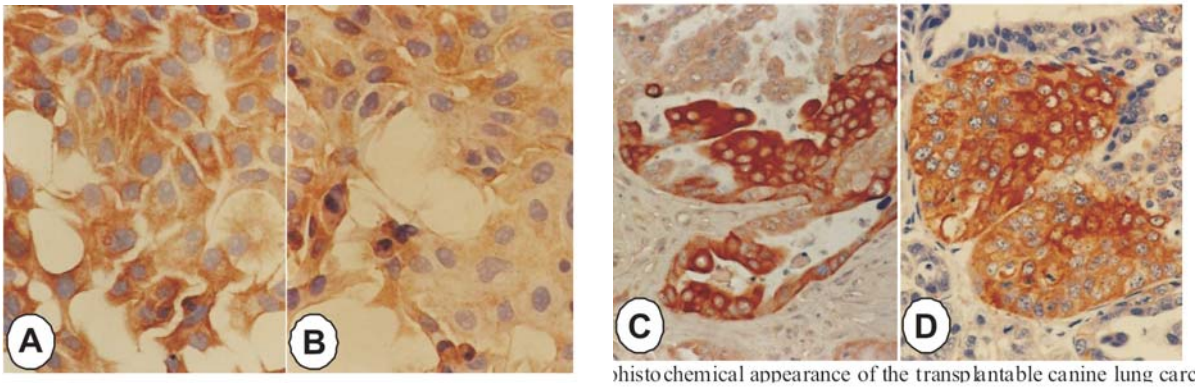


Figure 4. Immunohistochemical appearance of the transplantable canine lung carcinoma cells into SCID mice at 3th passage showing strongly positive to anti-keratin (A) and to anti-vimentin (B). Immunohistochemical examination of the original lung carcinoma (C) and transplantable canine lung carcinoma (D) showing strongly positive to anti-keratin (brown color) Avidin-biotin-peroxidase complex method (ABC), Mayer's hematoxylin counterstained, x 100.

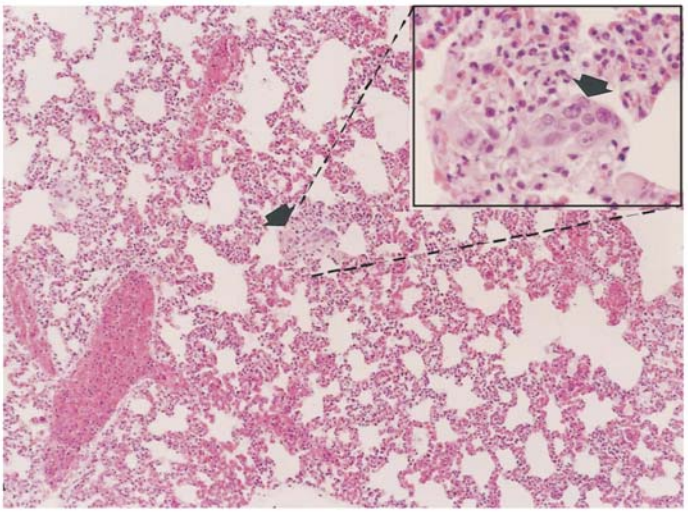


Figure 5. Histological appearance of the metastatic cells in the lung of SCID mice of the transplantable canine lung carcinoma, showing round to oval cells with large nuclei (arrow) (insert), Hematoxylin-eosin, x 100

1981). Dogs and cats are breathe in the same polluted air as man, exposed to same radiation, and eat similar food often containing the same additive. Unlike livestock which are rent to slaughter before reach the cancer age, dogs and cats are allowed to live a full lifespan, thus are more risk for comparative studies of lung carcinoma. Lung carcinoma occurs in dogs at an average age about 11 years and no sex predilection (Moulton *et al*., 1981). Most reviews indicated no breed prevalence for lung carcinomas in dogs and cats. Classification for carcinoma of the lung based entirely on topographical nomenclature (e.g., bronchial, bronchiolar, bronchiolar-alveolar, or alveolar carcinoma) are sometimes difficult to use in veterinary field because the carcinoma are usually so far advanced by time the animal is examined that is difficult to determine the exact site of tumor origin (Stunzi *et al*., 1974; Moulton *et al*, 1981). The rapid growth of the tumor in the inoculated mice after innoculation showed that the canine lung carcinoma is tumorigenic in SCID mice.

Light microscopy of canine lung carcinoma transplanted in SCID mice from the first to sixth passages showed several morphologies that may reflect the composition of the original canine primary tumor. From the first to sixth passage, the tumor were composed of cuboidal to columnar cells with papillary growth pattern, uniform in size, numerous mitotic figures, with some cells has a vacuol on their cytoplasm. The nuclei were often variable in size. The fact that the later passage of tumors in SCID mice were predominantly composed of glandular form with lobulated, ductal pattern and some papillary projection into tubule like structure confirmed that this tumor is classified as bronchioalveolar carcinoma.

Ultrastructural examination from transplantable tumor in SCID mice revealed the round to oval cells with large nuclei and one or more prominent nucleoli. These cells also showed dense secretory granules in their cytoplasm (Clara cells). Stenberg, (1989); Anderson *et al*., (1992); Maxie (2007) described that bronchioalveolar carcinoma originate from either secretory bronchioalveolar (Clara cells) or alveolar type II epithelial cells.

In this study, we tried to established cell line derived from canine lung carcinoma transplanted into SCID mice. To the present of our knowledge, there is no report of cell line derived from canine lung tumor. The isolation and maintenance of this tumor cell culture was achieved by adapting dissociation techniques to obtain significant improvements in cell cultivation (Schmid *et al*., 1983).

Confirmation of tumor cell type as epithelial cells was supported by general morphological features using light microscopy, phase contrast and electron microscopy such as the formation of pavement like colonies (Priosoeryanto *et al*., 1995[b]) of small and large round to oval cells and elongated shaped cells and the presence of desmosome (Brambilla *et al*., 1989).

Immunostaining for keratin and vimentin indicated the epithelial nature and purity of the culture cells. The cultured cells showed strongly positive reaction with antiserum against keratin which known to be a major intermediate filament protein of epithelial cells (Kataoka *et al*., 1993) and also positively stained with moAb against vimentin, the typical filament protein of mesenchymal cells (Satoh *et al*., 1993; Bouchard *et al*., 1995). Some epithelial cells synthesize vimentin filament and some do not, thus demonstrating that permanent cell grow in vitro and epithelial cells line does not depend on the production of vimentin (Virtanen *et al*., 1981; Priosoeryanto *et al*., 1995[b]).

In addition, the immunostanining of tumor cells from paraffin section of the transplant tumor mass in SCID mice and the original tumor showed that they were strongly positive to antiserum against keratin but negative for vimentin and desmin antibodies. Based on these findings above, we suggest that this tumor cells is an epithelial cell origin (Priosoeryanto *et al*., 1992).

Metastatic lesion was found in the lung of SCID mouse. Lung is the most frequent organ of metastasis, because of the central position of the lung in the vascular system, lymphatic and haematogenous metastasis and transmigration through air space (Stenberg, 1989). The sites of metastatic from lung carcinomas are poorly recorded because animals usually are killed before metastasis developed fully. The reported sites of metastasis are the regional (usually bronchial) lymph nodes, spleen, heart, pericardium, pleura, kidney, skeleton and skeletal muscle (Moulton *et al*., 1981; Rogers, 1993) but in this study, metastatic lesion was occur in the lung of SCID mouse.

Spontaneous metastasis, which result from subcutaneously implanted tumor cells , can be distinguished from artificial metastasis, which result from intravenously injected tumor cells (Cole *et al*., 1986; Shtivelman and Namikawa,

1995). The later are much more commonly reserved. The failure of tumors to spontaneously metastasis in SCID mice and nude mice was not well understood and the subject of much ongoing research (Kerbel *et al*., 1984). The site of implantation may be important for the mechanism of metastasis. Subcutaneous implantation was used in the present study and it would be necessary to compare with orthotopic implantation (Stephenson *et al*., 1992; Furukawa *et al*., 1993). Nevertheless, SCID mice would be useful, at least for studying lung metastasis in canine cancer (Sugimoto *et al*., 1994: Maruo *et al*., 1996), especially canine lung carcinoma.

CONCLUSION

From this study, the canine lung carcinoma has a high possibility to develop as an established cell line. Transplantation study of this canine lung carcinoma into SCID mice may contribute to the understanding of growth, behavior and metastatic development of canine lung carcinoma as well as may contribute to the research on drug therapy for cancer disease in animal and human.

SUGGESTION

From this study, we suggested that need more research about canine lung carcinoma that originated from lung and also study about lung metastatic in canine cancer because the cases in dogs is rare and could be more aggressive in the future.

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