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Full Length Research Paper

Non-cell metastasis of malignant tumor: A preliminary experiment

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The present study aimed to investigate the non-cell metastasis of malignant tumor (metastasis at the molecular level). Primers for the specific SPY gene of male mouse were designed and used to detect the SPY gene in the Lewis lung cancer (LLC) cells by PCR; LLC cells were inoculated into females C57BL/6 mice. H and E staining was performed to detect the subcutaneous primary tumor and metastatic tumors in the lungs and liver, and polymerase chain reaction (PCR) and *In situ* hybridization (ISH) were employed to detect the sex determining region of the Y (SRY) gene in the primary and metastatic tumors. After inoculation of LLC cells carrying SRY gene into female C57BL/6 mice, the SRY gene was detectable in the metastatic tumor in these female mice, which suggests the metastatic cancer cells originate from the primary tumor. Our results demonstrated the metastasis of cancer cells at the cellular level but not the non-cell metastasis. It is very difficult to verify the "hypothesis of non-cell metastasis" by animal experiment for the limit of itself. Further studies with new strategies are required to elucidate our hypothesis due to the presence of limitations in the present study.

Key words: Cancer, metastasis, sex determining region of the Y (SRY) gene.

INTRODUCTION

The metastasis of malignancies is the main cause of death among patients with malignant tumor. An understanding of metastasis at the cellular and molecular levels is an important goal in cancer research because the major barrier to adequate treatment of metastatic lesions is the biologic heterogeneity of cancer cells in both the primary neoplasm and the metastatic tumors. Since Liotta proposed the hypothesis of infiltration of cancer cells in the metastasis of malignant tumors, it has been accepted that the infiltration and migration of cancer cells are the main causes of metastasis of cancer cells. However, the radiotherapy and chemotherapy directly targeting the cancer cells achieve poor efficacy in patients with metastatic malignancies (Fidler, 2003; Katona et al., 2007). It has been found that the micro-environment in the tissues may affect the gene expressions (Sternlicht et al., 1999; Maffini et al., 2004; He et al., 2007; Barcellos-Hoff et al., 2000). Molecular biological studies have demonstrated that cancers can produce a large number of molecules which can be transmitted between cells and influence the homeostasis of micro-environment (Hanahan and Folkman, 1996).

Traditionally, tumors have been considered to be clonal populations originating from a single transformed cell. Studies suggest that several distinct clones may be present in a primary tumor and that more than one of these clones may ultimately metastasize resulting in multiple, genetically independent tumors within the same patient (Goldstein et al., 2005; Morita et al., 1998). In the study on the melanoma of the skin, Katona et al. (2007) found that in some cases, multiple coexisting metastases seem to be derived from different, genetically unrelated

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Abbreviations: LLC, Lewis lung cancer; **H and E,** hematoxylin and eosion; **ISH,** *in situ* hybridization; **SRY,** sex determining region of the Y; **PCR,** polymerase chain reaction; **DMEM,** Dulbecco's modified eagle medium; **PBS,** phosphate buffered saline; **DNA,** deoxyribonucleic acid.

Table 1. Primers for mouse SRY and β-actin.

Gene	Primer	Primer sequence (5'-3')	Product size (bp)
SRY-182	Sry-182-F	TCATCGGAGGGCTAAAGTG	182
	Sry-182-R	TCAACAGGCTGCCAATAAA	
β-actin	β-actin-F	GAGACCTTCAACACCCCAGC	363
	β-actin-R	GCCGTCAGGCAGCTCATA	

F: forward primer R: reverse primer.

tumor clones. Genetic divergence after clonal expansion may lead to the development of several subpopulations of neoplastic cells with only rare cells gaining the capacity to metastasize (Heppner, 1984; Nicolson, 1984).

Carcinogenesis involves multiple genetic changes including the activation of dominantly acting oncogenes that promote cell proliferation and the loss of tumor suppressor genes that negatively regulate cell proliferation. The inactivation of tumor suppressor genes by mutation or by loss or replacement of a chromosomal segment containing the allele provides a selective advantage essential for transformation or progression of a malignancy (Knudson, 1993). In this clinical setting, clearly defining the genetic relationships among the multifocal lesions and assessing the malignant potential of each lesion could have important diagnostic, surgical, and prognostic implications (Jones et al., 2005). In addition, understanding the nature of tumor multifocality can serve to further our understanding of the genetic basis of tumor progression.

Based on previous findings, we hypothesize that some molecules produced by the cancer cells can exist in the blood and/or tissues and accumulate in local organs, lymph nodes or distant organs rich in blood supply, which may alter the normal micro-environment and subsequently change the gene expression profile resulting in malignant transformation of cells and occurrence of new metastatic tumor. This hypothesis can explain some problems encountered in clinical practice including that radiotherapy and chemotherapy cannot eliminate these molecules. If the non-cell transfer hypothesis is confirmed, the treatment strategy for cancer will undergo major change from the "target cells" to the "target molecules", and maybe there is great change in cancer treatment.

In the present study, the Lewis lung cancer (LLC) cells carrying sex-determining region of the Y (SRY) gene were inoculated to the female C57BL/6 mice to establish the model of spontaneous metastasis. The SRY gene was employed as a marker of tumor cell clones in primary tumor to detect the metastatic tumor (Gubbay et al., 1992). Polymerase chain reaction (PCR) and *in situ* hybridization (ISH) were done to detect the SRY gene in the macroscopic metastatic tumor and the micrometastatic tumor to determine the origin of cancer cells in the spontaneous metastatic tumor. When the SRY gene was absent in the metastatic tumor, the cancer cells were not related to the primary tumor, but are the *in situ* cancer cells, which confirmed our hypothesis.

MATERIALS AND METHODS

Main reagents

Taq deoxyribonucleic acid (DNA) Polymerase, proteinase K (Merk), dNTPs (Research Genetics), Marker DL2000 (Sigma, USA), TIANamp Genomic DNA kit (TIANGEN) and Mouse SRY BIO ISH kit (Tianjin Haoyang) were used in the present study.

Animals and cell line

A total of 32 female C57BL/6 mice aged 4 ~ 6 weeks and one male C57BL/6 mouse were purchased from the Animal Center of Southern Medical University. The LLC cells were from the Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences, CAS. SRY gene was identified in these cancer cells. Our study has been approved by the Ethics Committee of Nanfang Hospital of Southern Medical University.

Design of primers

The sequences of SRY and β-actin were obtained from GenBank. β-actin served as an internal reference of SRY gene. The primers were designed using the PRIMER3.0 program online and synthesized by Guangzhou Invitrogen. Primers are displayed in Table 1.

Detection of sex determining region of the Y (SRY) gene in the Lewis lung cancer (LLC) cells by polymerase chain reaction (PCR)

(1) Extraction of DNA of Lewis lung cancer cells: The cells were collected and centrifuged at 12000 g for 15 min at 4℃. The supernatant was removed and 1 ml of water phenol, 1 ml of chloroform and 40 µl of isoamyl alcohol were added followed by centrifugation at 12000 g for 15 min at 4℃. The 4/5 of upper solution was collected and mixed with 0.5 ml of isopropanol. The mixture was kept at 4℃ for 20 min and then centrifugation was performed at 12000 g for 10 min at 4℃. The supernatant was removed and 1 ml of 75% alcohol was added followed by centrifugation at 12000 g for 10 min at 4℃. The supernatant was removed and the sediments were air-dried and dissolved in 35 µl of ultra-pure water. The mixture was stored at 4℃ for use.

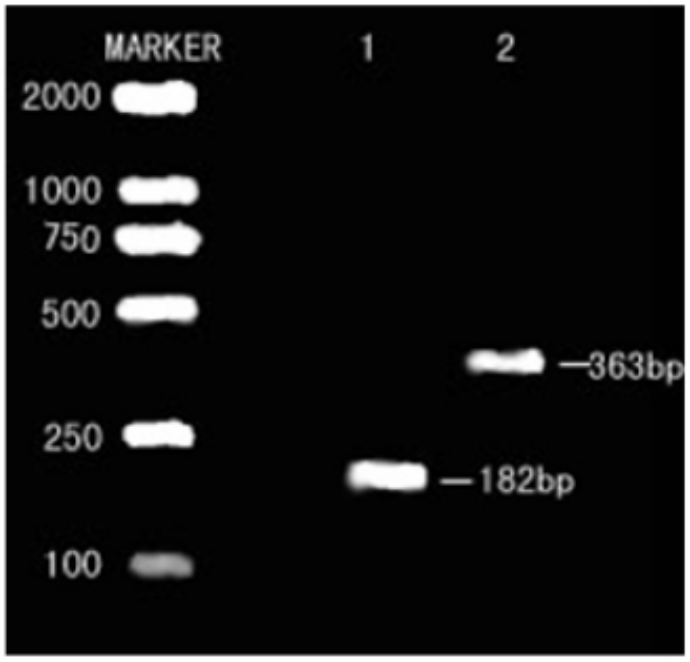

Figure 1. SRY gene in LLC cells by PCR 1: SRY-182; 2: β-actin.

(2) Preparation of SRY mixture for PCR: the mixture (50 µl) for PCR included 10 µl of 5×PCR buffer, 1 µl of forward primer, 1 µl of reverse primer, 1 µl of dNTPs (10 mM), 1 µl of Taq DNA Polymerase, 5 µl of DNA plate and ultra-pure water. (3) Conditions for PCR: pre-denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 15 s, annealing at 65°C for 30 s and extension at 72°C for 30 s, and a final extension at 72°C for 10 min.

The products were then subjected to 2% agarose gel electrophoresis (90 V, 30 min) and the bands were visualized and photographed.

Preparation of animal model and sample collection

Preparation of animal model and grouping: LLC group: 30 female C57BL/6 mice received the injection of 100 µl of LLC cells (2×10^6 cells) carrying SRY gene at hind footpad. In negative control group, 2 female C57BL/6 mice received injection of 100 µl of Dulbecco's modified eagle medium (DMEM) without cells. One male C57BL/6 mouse served as a positive control. (2) Sample collection: LLC group: Mice were sacrificed at 4 weeks after injection. Those died within 4 weeks were subjected to same procession. The bilateral lungs and liver were collected, and the subcutaneous primary tumors and macroscopic metastatic tumors in the lungs and liver were obtained with the help of microscope. The primary and metastatic tumors in the lungs and liver were divided into two: one was fixed in 40% formaldehyde at 4°C followed by H and E staining, the other was stored in the liquid nitrogen followed by extraction of DNA. The lungs and liver without macroscopic metastatic tumors were fixed in 4% formaldehyde at 4°C for ISH. In negative control, part of liver was stored in liquid nitrogen followed by extraction of DNA, and the remaining liver and lungs were fixed in 4% formaldehyde at 4°C for ISH. One male C57BL/6 mouse served as a positive control in which part of liver was stored in liquid nitrogen for DNA extraction and the remaining were fixed in 4% formaldehyde at 4°C for ISH.

Detection of sex determining region of the Y (SRY) gene in the metastatic tumor by polymerase chain reaction (PCR)

(1) Extraction of DNA: the extraction of DNA was carried out according to the manufacturer's instructions. (2) Detection of SRY gene in the macroscopic metastatic tumor by PCR: The mixture and conditions for amplification of SRY gene by PCR were identical to those described above. The DNA in negative and positive controls was used as controls.

Detection of sex determining region of the Y (SRY) gene in the metastatic tumor by *In situ* hybridization (ISH)

(1) Sample processing: The lungs and liver without macroscopic metastatic tumor in three groups were collected and fixed in 4% paraformaldehyde, embedded in paraffin and cut into sections (5 µm). The two consecutive sections were subjected to H and E staining and ISH, respectively. (2) Procedures for ISH: The de-paraffinized section was treated with pore solution at 37°C for 8 min and then with 0.1 M tablespoons (TBS) (pH7.8) three times (3×5 min). The section was incubated in complex digestion solution at 37°C for 20 min, which was then discontinued by addition of glycine followed by washing with 0.1 M TBS (pH7.8) three times (3×5 min). After incubation in 0.1 M TBS (pH8.9) 95°C for 15 min, the section was rinsed with 0.1 M TBS (0°C) three times (3×5 min) and then incubated at 0°C for 20 min. After incubation in pre-hybridization working solution at 37°C for 1 h, sections were washed 0.2×SSC thrice (3×5 min) and then treated with hybridization working solution at 37°C for 4 h. After washing with 2×SSC thrice (3×5 min) at 37°C and with 0.1 M TBS (pH7.8) thrice (3×5 min), sections were treated with high-sensitivity streptavidin peroxidase complex solution at 37°C for 40 min and then washed with 0.01 M phosphate buffered saline (PBS) thrice (3×5 min). Color development, dehydration and transparentization were performed followed by mounting. The sections were observed under a light microscope and photographed. The cells with brown nucleus were positive for SRY.

RESULTS

Sex determining region of the Y (SRY) gene in Lewis lung cancer cells

As shown in Figure 1, the bands were clear and identified at 182 bp and 363 bp respectively, which were consistent with the anticipated size. This finding confirmed the presence of SRY gene in Lewis lung cancer cells.

Sex determining region of the Y (SRY) gene in the metastatic tumor

The female C57BL/6 mice were inoculated with Lewis lung cancer cells carrying SRY gene. H and E staining was performed in the subcutaneous primary tumor, and macroscopic metastatic tumor in the lungs and liver (Figure 2). The SRY gene was detected by PCR (Figure 3). In negative control (lanes 1 and 2), no band was observed. In positive control (lane 3) and the subcutaneous primary tumor, and metastatic tumors in the lungs and liver, similar bands were identified (lanes 4-10). Our results showed, after inoculation of Lewis lung

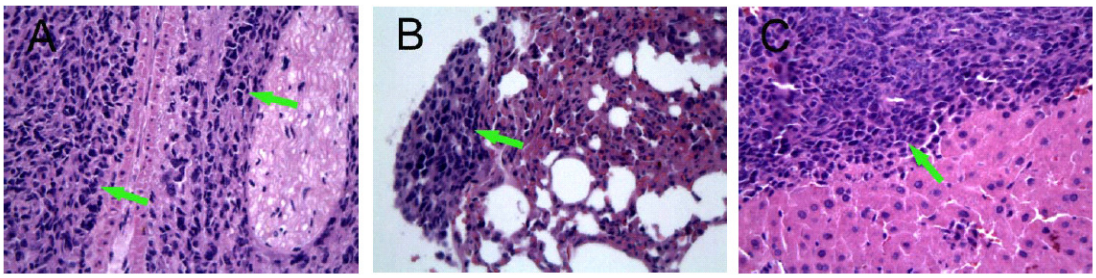

Figure 2. Subcutaneous primary tumor and macroscopic metastatic tumor. Arrows indicated tumors (×400).

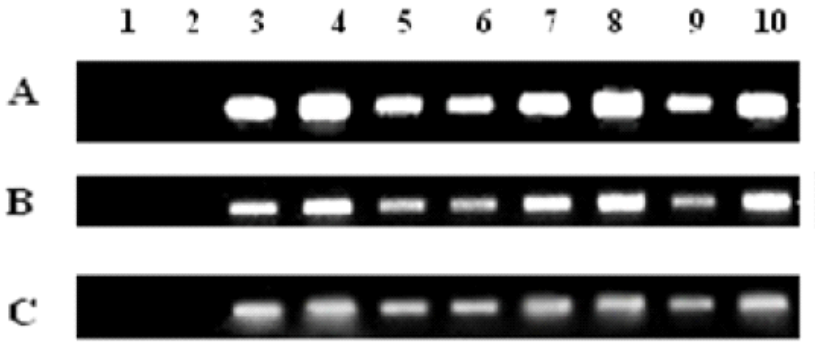

Figure 3. SRY gene in the macroscopic metastatic tumor. 1 and 2, Negative control; 3, positive control; A, 4-10: subcutaneous primary tumor; B, 4-10: metastatic tumor in the lungs; C, 4-10: metastatic tumor in the liver.

cancer cells carrying SRY gene into female C57BL/6 mice, the SYR gene was detectable in the subcutaneous primary tumor as well as macroscopic metastatic tumors in the lungs and liver.

Sex determining region of the Y (SRY) gene in the metastatic tumor by *In situ* hybridization (ISH)

As shown in Figure 4, the SRY gene was detectable in the lung and liver of male mouse demonstrated by ISH. As shown in Figure 5, in negative controls, the ISH did not show the SRY gene in the lung and liver. As shown in Figure 6, after inoculation of Lewis lung cancer cells into female C57BL/6 mice, the metastatic tumors were found in the lungs and liver by H and E staining. ISH also demonstrated the presence of SRY gene.

DISCUSSION

Currently, two theories have been proposed to explain the frequent occurrence of tumor multifocality. One theory suggests that, the multiple tumors are of monoclonal origin, arising from a single malignant transformed cell which proliferates and spreads through infiltration and migration of cancer cells. The second theory explains tumor multifocality as developing secondary to a field-cancerization effect precipitated by carcinogens causing independent genetic alterations at different sites and leading to the development of multiple, genetically unrelated tumors (Jones et al., 2005). The issue of monoclonal versus oligoclonal origin of multifocal carcinomas is clinically important because an understanding of patterns of early tumor development must be considered in the development of appropriate treatment and surgical strategies and in the genetic detection of recurrent or residual tumor cells. There continues to be no consensus on which of the theories is most important in the development of multifocal urothelial carcinoma.

To confirm the hypothesis of non-cell metastasis, the SRY gene was used as a marker to detect the SRY gene in the primary tumor and metastatic tumor *in vivo*. Our results showed the metastatic cancer cells originated from primary tumor, which deny our hypothesis.

We speculate that animal study has its own limitation. The progression of cancers of humans cannot be

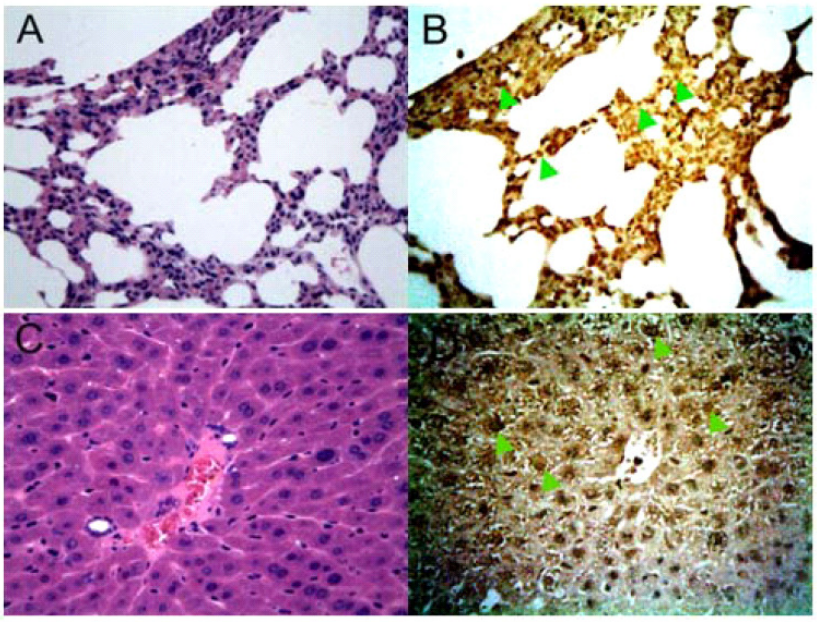

Figure 4. H and E staining and ISH of the lung and liver of male mouse. Green triangles indicated SRY gene in the nucleus (×400). DAB staining: the brown nucleus represents positive staining; A, H and E staining of the lung; B, ISH of the lung; C, H and E staining of the liver; D, ISH of the liver.

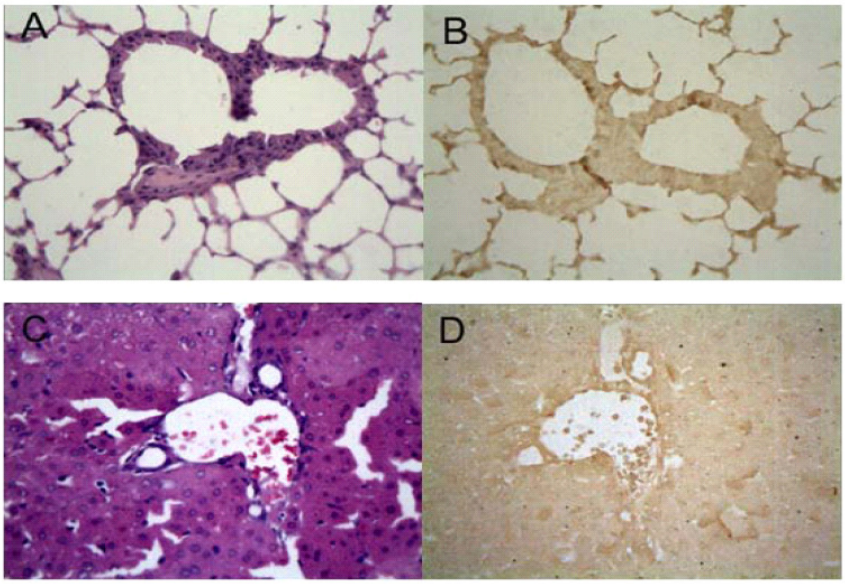

Figure 5. H and E staining and ISH of the lung and liver of female mice. DAB staining: the brown nucleus represents positive staining (×400); A, H and E staining of the lung; B, ISH of the lung; C, H and E staining of the liver; D, ISH of the liver.

replicated in animal model. For example, the lifespan of human is significantly different from that of mouse. Thus, some pathological features the progression of which requires a long time cannot be observed in short time. First, the duration of animal study is relatively short, and the accumulation of molecules or the time of action cannot reach the threshold. Thus, the changes in the molecules are not obvious. Secondly, the molecules induced metastasis may occur later than infiltration of cancer cells does, which was demonstrated by the fact

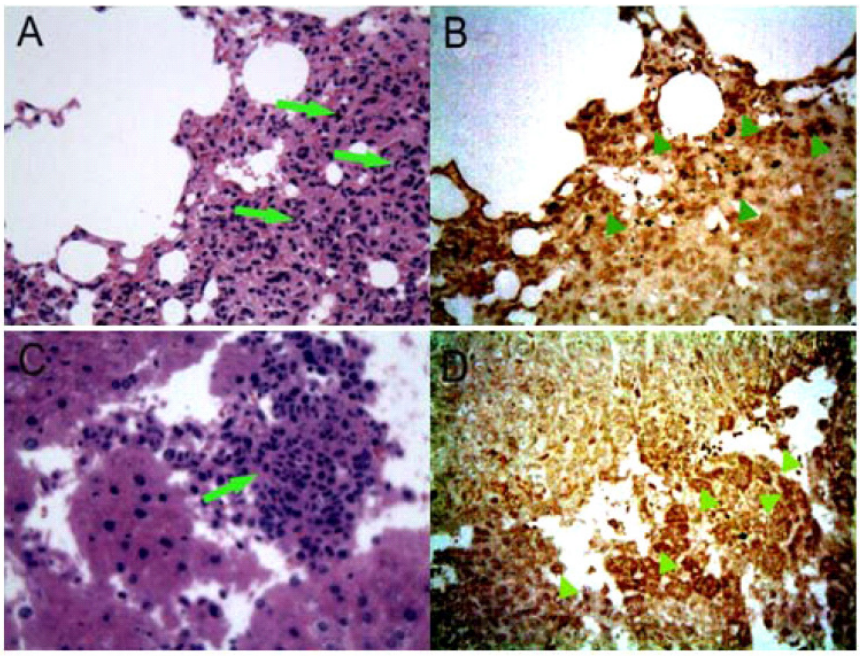

Figure 6. Microscopic metastatic in lung and liver of LLC. Green arrow indicated tumors. Green triangles indicated SRY positive nucleus (×400). DAB staining: the brown nucleus represents positive staining; A, H and E staining of the lung; B, ISH of the lung; C, H and E staining of the liver; D, ISH of the liver.

that our results showed infiltration induced metastasis but not molecule induced metastasis in the short time. Thirdly, although Lewis lung cancer cells are derived from C57BL/6 mouse (Bertram and Janik, 1980), that the genetic condition of C57BL/6 mice in the present study was identical to that of Lewis lung cancer cells cannot be assured. The C57BL/6 mice in our study had no congenital genetic defects (such as chromosome fragile sites) that the host of Lewis lung cancer cells has. In addition, the mice in the present study did not undergo the influence of carcinogens for a long time. Therefore, the mice in our study had no acquired genetic impairment that the host of Lewis lung cancer cells has due to the presence of carcinogens, which may affect the molecule induced gene expressions. Finally, the Lewis lung cancer cells used in our study were screened and susceptible to cell metastasis. Other types of cancer cells may have the possibility to induce the non-cell metastasis. Taken together, the animal study may have difficulties to demonstrate our hypothesis.

Thus, other strategies are undertaking to further elucidate our hypothesis. Based on the primary findings in the present study, we postulate the selection of experimental conditions and cell line is the key component for the confirmation of our hypothesis. These conditions require the molecules with enough amounts and the action of sufficient duration, which is critical to induce the genetic alteration and visible morphological changes.

In summary, we proposed a hypothesis of non-cell metastasis of cancer cells. It is generally recognized that tumor metastasis is caused by the spread of tumor cell infiltration, while the effect of radiotherapy and chemotherapy to kill tumor cells is poor. If the non-cell transfer hypothesis is confirmed, the treatment strategy for cancer will undergo major change from the "target cells" to the "target molecules", and maybe there is great change in cancer treatment. Further studies with other strategies are required to elucidate our hypothesis due to the presence of limitations of the present study.

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