Establishment and Characterization of a Human Lung Cancer Cell Line NCI-H460-LNM35 with Consistent Lymphogenous Metastasis via Both Subcutaneous and Orthotopic Propagation

Ken-ichi Kozaki, Osamu Miyaishi, Tetsuya Tsukamoto, Yoshio Tatematsu, Toyoaki Hida, Toshitada Takahashi, and Takashi Takahashi

ABSTRACT

Lymphogenous metastasis is a common feature of human lung cancers, but very little is known about the underlying mechanism. In the present study, in vivo selection was carried out to obtain a highly lymphogenous metastatic subline of a human large cell carcinoma of the lung, NCI-H460. The resulting subline, termed NCI-H460-LNM35 (LNM35), was shown to metastasize to regional lymph nodes with a 100% incidence not only as a result of orthotopic intrabronchial (i.b.) implantation, but also as a result of conventional s.c. implantation. LNM35 has a short latency period, allowing for the collection of experimental data within 28 days after i.b. inoculation and 45 days after s.c. inoculation. It was noted that orthotopically i.b.-propagated LNM35 closely mimicked the clinical manifestations of human lung cancer patients by infiltrating into lymphatic vessels and metastasizing to the mediastinal lymph nodes. The LNM35 cell line is, to the best of our knowledge, the first human lung cancer cell line to be reported as having lymphogenous metastatic properties, and the observed 100% incidence by s.c. inoculation gives LNM35 a significant advantage even over previously reported human cancer cell lines of other origins. Comparisons between LNM35 and its parental NCI-H460 cell lines were also made with regard to expression levels and/or activities of various molecules that are thought to play a part in the metastatic process. We show here that the expression of cyclooxygenase 2 is increased in LNM35 and that a specific cyclooxygenase 2 inhibitor, nimesulide, can inhibit the invasion of LNM35 in vitro through Matrigel containing basement membrane components.

INTRODUCTION

It has now been clearly established that lung cancer is a disease caused by the accumulation of multiple genetic alterations in both oncogenes and tumor suppressor genes (1). Despite considerable advances in the understanding of the molecular pathogenesis of lung cancer, only one in eight patients diagnosed as having lung cancer can be cured at present, while the rest of the cases eventually fail because of widespread metastases (2). The degree of lymphogenous spread is known to be an important parameter for the staging and assignment of treatment and useful for the assessment of patients' prognoses (3). An inverse correlation between the extent of lymph node metastasis and postoperative survival of lung cancer patients (4) suggests that lymphogenous metastasis reflects the malignant potential of tumor cells and contributes to fatality.

The expression of certain molecules, such as adhesion receptors and ligands (5–17) as well as metalloproteinases (18–22), has been suggested to play a role in the development of metastatic lesions. Metastasis occurs via two distinct pathways, and tumor cells spread through blood and LVs (4). Although a large number of studies have been conducted, yielding considerable information about the metastatic processes, very little is known about how cancer cells propagate lymphogenous metastasis. Identification of molecules with a crucial role in the lymphogenous spread of cancer cells has been hampered by lack of an appropriate experimental model system. To date, a few cell lines derived from several types of human malignancies have been reported to have a high potential to metastasize regional lymph nodes when the tumor cells were injected at orthotopic sites (23–29), although they were found to metastasize to a much lesser extent when propagated s.c. However, no cell lines have been reported thus far as being useful for studies of the lymphogenous metastasis of human lung cancers.

In the present study, we describe an in vivo selection resulting in the establishment of a human lung cancer cell line, NCI-H460-LNM35 (LNM35), which consistently and spontaneously metastasizes to lymph nodes when injected either s.c. or orthotopically. Comparisons between LNM35 and its parental H460 cell lines are also made with regard to expression levels and/or activities of various molecules that are thought to play a part in the metastatic processes. We show that expression of COX-2 is increased in LNM35 and that a specific COX-2 inhibitor, nimesulide, can inhibit the invasion of LNM35 in vitro through Matrigel containing basement membrane components.

MATERIALS AND METHODS

Animals and Cell Lines. Five-week-old female athymic nude mice and SCID mice were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan) and maintained under specific pathogen-free conditions. The NCI-H460 (H460) cell line at passage 136 (ATCC HTB 177), which was originally established by Carney et al. (30), was obtained from the American Type Culture Collection (Rockville, MD). NCI-H460 is a human large-cell lung carcinoma line with mutant K-ras and wild-type p53 (31, 32). Derivation by in vivo selection of the high-lung-metastatic LuM1 and low-lung-metastatic NM11 sublines derived from a murine colon adenocarcinoma 26 tumor cell line was described previously (22, 33, 34). All cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum.

In Vivo Selection. In vivo selection was carried out to establish a high-lung-metastatic subline of H460, using the procedures described by Fidler (35) and also in a previous study of ours (22). In brief, 1.0 × 10^7 of the parental H460 cells in 100 μl of serum-free RPMI 1640 medium were injected in the s.c. tissue of the left abdominal wall of 7-week-old female SCID mice. Lung tissues containing the metastatic tumor cells were excised, minced, and reimplanted in the abdominal wall of new recipient mice for the selection of high-metastatic tumor cells. After two rounds of in vivo selection by means of sequential implantations, metastatic nodules in the lung tissues were harvested.
to initiate in vitro culture of the metastatic tumor cells. Further selection was then carried out by injecting the resulting cell line into s.c. tissue, followed by in vitro propagation of tumor cells obtained from spontaneous ALN metastasis. A clonal cell line was established from the cultured cell mixture with the limiting dilution method and use of 96-well culture plates. These cell lines were then maintained in RPMI 1640 medium with 10% fetal bovine serum.

**Spontaneous Metastasis Assay.** Cells (1.0 × 10^5) in 100 μl of serum-free RPMI 1640 medium were implanted in the s.c. tissues of the left abdominal wall of 7-week-old female SCID mice or nude mice. At 45 days (SCID mice) or 55 days (nude mice) after s.c. implantation, mice were sacrificed by cervical dislocation under deep anesthesia, and internal organs, including lung, liver, kidney, and spleen as well as lymph nodes and s.c. T.s, were resected. The resected specimens were weighed, fixed with 4% paraformaldehyde, and processed for light microscopic examination of the paraffin-embedded sections stained with H&E. The lung-metastatic nodules were examined and counted under a dissecting microscope.

**Orthotopic Implantation.** Mice were anesthetized by i.p. injection with 0.28 mg/kg body weight of 2,2,2-tribromoethanol (Aldrich Chemical Company, Milwaukee, WI). A 1-cm long ventral midline incision was made in the neck to expose the trachea for direct inspection of the orotracheal intubation of a 20-gauge catheter/needle unit (Terumo, Tokyo, Japan), which was advanced through the oral cavity to a depth of 2.1 cm from the incisor teeth under visual inspection through the exposed trachea. The needle was then pulled out, leaving only the outer 20-gauge catheter, through which a blunt-ended 25-gauge needle (Top, Tokyo, Japan) was inserted a depth of 4.4 cm. Next, 1.0 × 10^5 of the cultured LNM35 cells in 50 μl of serum-free RPMI 1640 medium were directly inoculated through the inserted needle into the bronchial tree, and the skin incision was closed with two stitches. The mice were sacrificed as described above 28 days after orthotopic implantation, and lung and mediastinum were removed en bloc and fixed with 4% paraformaldehyde. Metastasis to the MLNs was examined under a dissecting microscope and confirmed by histological examination of paraffin-embedded sections stained with H&E.

**Growth Curves.** Cells (1.0 × 10^5) were inoculated onto 3.5-cm dishes. At daily intervals, triplicate samples were harvested and counted with a Coulter counter (Coulter Electronics, Luton, United Kingdom), and cell numbers were averaged for each time interval.

**Antibodies.** SNH-3 (specific to sialyl Lewis X), 2F3–6 (specific to sialyl Lewis X-variant), and 2D-3 (specific to sialyl Lewis A) MoAbs (36, 37) were generous gifts of Dr. R. Kannagi (Aichi Cancer Center Research Institute). Anti-E-cadherin and anti-CD44 MoAbs as well as anti-integrin MoAbs used in this study were obtained from the generous gifts of Dr. R. Kannagi (Aichi Cancer Center Research Institute). Anti-E-cadherin and anti-CD44 MoAbs as well as anti-integrin MoAbs used in this study were obtained from Medical and Biological Laboratories, Inc. (Nagoya, Japan) except for TS2/7 and J143, which were the generous gifts of Dr. R. Kannagi (Aichi Cancer Center Research Institute). The following anti-integrin MoAbs were used (17): β1, K2, β2, BLS, β3, S2Z1, α1, TS2/7, α2, G9h, α3, J143, e4, HP2/1, e5, SAM1, α6, GoH3; and αv, AM7F. Rabbit anti-TIMP-2 polyclonal antibody was obtained from Chemicon International Inc. (Temecula, CA), affinity-purified FITC-conjugated goat antirabbit and antirat IgG were obtained from Protos Immunoresearch (San Francisco, CA), and FITC-conjugated rabbit antirabbit IgG was obtained from Cappel Inc. (Malvern, CA). Biotin-conjugated affinity-purified goat antirabbit IgG and horseradish peroxidase-conjugated streptavidin were obtained from Vector Laboratories (Burlingame, CA).

**Fluorescence-activated Flow Cytometry.** Aliquots (100 μl) containing 1.0 × 10^5 cells were subjected to indirect immunofluorescence staining for the detection by FACScan (Becton Dickinson Immunocytometry Systems, Mountain View, CA) of the expression of various adhesion molecules and carbohydrate determinants. MoAbs were used at a concentration of 10 μg/ml and incubated for 30 min at room temperature.

**Detection of TIMP-2 and Gelatinases A and B.** Cells (1 × 10^5) were cultured for 2 days in 1 ml of serum-free RPMI 1640 culture medium in culture dishes 3.5 cm in diameter followed by harvesting of the culture supernatant. Each medium sample was concentrated 10-fold with the aid of Centricron-10 (Amicon, Beverly, MA). TIMP-2, which had been secreted into the serum-free conditioned medium, was detected by means of Western blot analysis using anti-TIMP-2 polyclonal antibody and a POD immunostaining kit (Wako Pharmaceutical Industries, Ltd., Osaka, Japan), as previously described (38). Gelatinases A (MMP-2, M, 72,000 type IV collagenase) and B (MMP-9, M, 92,000 type IV collagenase), which had been secreted into the serum-free-conditioned medium, were detected by means of zymography with gelatin (2 mg/ml) as the substrate, as described previously (38).

**Northern Blot Analysis of COX-2.** Extraction of RNA from cell lines and Northern blot analysis were conducted according to the standard procedures. A human COX-2 cDNA probe was generated by PCR with the aid of a sense primer, 5′-TTCATTGAGATTGTGCTATGCTGC-3′, and an antisense primer, 5′-AGATCTCTGCTGAGTATCTTCT-3′.

**In Vitro Motility and Invasion Assay.** To quantify the in vitro motility and invasion assay, transwell-chamber culture systems were used. The upper surface of filters 6.4 mm in diameter with 8-μm pores (Becton Dickinson Labware, Franklin Lakes, NJ) were coated with 100 μl of 0.1 mg/ml Matrigel (Collaborative Research Inc., Bedford, MA) in the case of the invasion assay. Filters were filled with 0.5 ml of serum-free RPMI 1640 medium and placed on culture plates with 24 wells filled with 1 ml of the medium. LNM35 cells (1 × 10^5 cells in motility assay; 1 × 10^6 cells in invasion assay) were then added to the upper chambers and cultured. After 24 h of incubation, the filters were fixed with 70% ethanol and stained with Giemsa, and the cells on the lower surface of the filters were counted in triplicate. Nimesulide was provided by Hisamitsu Pharmaceutical Co. (Tsou, Japan).

### RESULTS

#### In Vivo Selection.** In vivo selection was carried out by direct s.c. implantation of lung-metastatic nodules, which were barely obtainable by s.c. injection of H460 cells into the left abdominal wall of SCID mice. After two rounds of in vivo selection, lung-metastatic nodules were minced and cultured in vitro to yield a continuously growing cell line termed H460-Lu. Then, 1.0 × 10^5 cells of H460-Lu were injected in the s.c. tissue of the left abdominal wall of two SCID mice. Although both mice developed s.c. T.s, one of them also showed metastasis in the left ALN, from which a metastatic nodule was harvested at day 50 to establish a tumor cell line growing in vitro. Limiting dilution using 96-well culture plates was then carried out to isolate a clonal cell line, which yielded NCI-H460-LNM35 (LNM35).

**Morphological and Growth Characteristics of LNM35 in Vitro.** Under a phase-contrast microscope, both parental H460 and in vivo-selected LNM35 cells demonstrated the polygonal shape typical of epithelial cells, although adhesion of H460 to the culture dish and intercellular junction tended to be tighter than that of LNM35. No significant differences were observed in in vitro cell growth rates between parental H460 and LNM35 cell lines. Furthermore, microsatellite analysis using D17S250 and D17S513 to confirm derivation of LNM35 from H460 showed identical patterns for H460 and LNM35 (data not shown).

**Spontaneous Metastatic Properties of LNM35.** Spontaneous metastatic properties were examined by means of s.c. injection of 1 × 10^5 cells of LNM35. Three independent experiments were carried out, and the injected mice were sacrificed after 45 or 55 days. At autopsy, s.c. injection of parental H460 cells as well as of in vivo-selected LNM35 cells consistently yielded a similar sized tumor mass with similar microscopic features of large cell undifferentiated carcinoma (Fig. 1, s.c.; Table 1). Markedly enlarged LVs (Fig. 2, arrowheads) draining to the ALNs (Fig. 2, ALN) were observed in all mice injected with LNM35 cells, implying the occurrence of carcinomatous lymphangitis. Upon histological examination, the enlarged LVs were shown to be filled with the tumor cells (data not shown), while lymph node metastasis was also confirmed (Fig. 1, ALN).

Furthermore, LNM35 cells characteristically caused carcinomatous lymphangitis on the visceral pleura and in intrapulmonary LVs surrounding lung-metastatic nodules (Fig. 1, lung, arrow). Metastasis to the inguinal lymph nodes was detected in a few cases, and tumor infiltration of LNM35 cells into blood vessels was detected in some cases (data not shown). In contrast, no lymphatic involvement was detected in any mice injected with parental H460 cells. It was also
noted that the number of lung-metastatic nodules in mice inoculated with LNM35 cells was markedly larger than that seen in mice injected with parental NCI-H460 or LNM35 cells. Both LNM35 and NCI-H460 show the typical histological features of large cell undifferentiated carcinoma of the lung. Note that infiltration of LNM35 cells is evident in a peribronchial LV (arrow). Bars, 50 μm.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Subcutaneous tumor weight (g)</th>
<th>ALN metastasis</th>
<th>Lung metastasis</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>No. of nodules</td>
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<tr>
<td>Experiment 1 (SCID mice, 1.0 × 10^7/100 μl, examined after 45 days)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>H460</td>
<td>5.1 ± 1.0</td>
<td>0/6 (0%)</td>
<td>6.8 ± 4.6</td>
</tr>
<tr>
<td>LNM35</td>
<td>5.3 ± 0.6</td>
<td>6/6 (100%)</td>
<td>456.2 ± 252.3</td>
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<tr>
<td>Experiment 2 (KSN nude mice, 1.0 × 10^7/100 μl, examined after 55 days)</td>
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<tr>
<td>H460</td>
<td>8.5 ± 2.9</td>
<td>0/8 (0%)</td>
<td>8.9 ± 7.7</td>
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<tr>
<td>LNM35</td>
<td>10.1 ± 0.7</td>
<td>5/5 (100%)</td>
<td>131.4 ± 95.2</td>
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<td>Experiment 3 (KSN nude mice, 1.0 × 10^7/100 μl, examined after 55 days)</td>
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</tr>
<tr>
<td>H460</td>
<td>9.1 ± 0.9</td>
<td>0/9 (0%)</td>
<td>20.4 ± 17.8</td>
</tr>
<tr>
<td>LNM35</td>
<td>11.0 ± 0.6</td>
<td>5/5 (100%)</td>
<td>184.4 ± 108.0</td>
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</table>

Table 1 Metastatic properties of s.c.-propagated LNM35 in comparison with its parental line, H460

* Lung metastatic nodules were counted by using a dissecting microscope. Number of lung nodules is expressed as mean ± SD.

is relatively easy and requires an average of 10 min for completion. Table 2 summarizes the observed frequencies of successful tumor propagation in the lung and those of histologically confirmed metastases to the MLNs in cases with i.b.-implanted tumors (Table 2 and Fig. 3). Histological examination also showed intravasation of LNM35 cells into the LVs (Fig. 3, i.b.T., arrows) and blood vessels (data not shown) of the lung as well as into the lymphatic afferent vessels of MLNs (LV in Fig. 3, MLN). These macroscopic and microscopic features of this orthotopic propagation model are similar to those seen in lung cancer cases, which suggests that the lymphogenous metastatic processes of LNM35 mimic those occurring in patients.

Expression of Gelatinases and Their Specific Inhibitor and of Adhesion Molecules in LNM35 and Parental H460. Zymographic examinations of the concentrated conditioned medium revealed that...
Lymphogenous metastatic potential of LNM35 is suggested by its ability to form spontaneous lymphogenous metastases to MLNs at a 100% incidence after orthotopic implantation. LNM35 is the first human lung cancer cell line capable of spontaneous metastasis (Table 3). The LNM35 cell line is, to the best of our knowledge, the first human lung cancer cell line to be reported as having such a biological property. Moreover, LNM35 has significant advantages over previously reported cell lines. LNM35 spontaneously metastasizes at a 100% incidence not only as a result of orthotopic i.b. propagation but also as a result of conventional s.c. injection. This proven highly reproducible nature of lymphogenous metastases makes LNM35 very different from others because s.c. inoculation of other human cancer cell lines have been described in the literature thus far as being useful for studies of lymphogenous metastasis (Refs. 23–29; Table 3). The LNM35 cell line is, to the best of our knowledge, the first human lung cancer cell line to be reported as having such a biological property. Moreover, LNM35 has significant advantages over previously reported cell lines. LNM35 spontaneously metastasizes at a 100% incidence not only as a result of orthotopic i.b. propagation but also as a result of conventional s.c. injection. This proven highly reproducible nature of lymphogenous metastases makes LNM35 very different from others because s.c. inoculation of other cell lines has been reported as being useful for studies of lymphogenous metastasis (Refs. 23–29; Table 3).

**COX-2 Expression and Effect of Nimesulide on In Vitro Motility and Invasion.** Because our previous immunohistological studies of COX-2 expression in human lung cancer patients suggested a possible association of the increase in COX-2 expression with invasion and metastasis as well as with poor prognosis, expression of COX-2 was also examined in LNM35 and H460. Northern blot analysis showed that COX-2 expression was significantly increased in LNM35 when compared with that in H460 (Fig. 4A). As an initial step toward elucidation of the potential relationship of the increased expression of COX-2 with the highly metastatic phenotype of LNM35, we examined the effects of a specific COX-2 inhibitor, nimesulide, in LNM35 in vitro. Nimesulide was shown to be potent in the inhibition of invasion through Matrigel as well as of cell motility in a dose-dependent manner at concentrations significantly lower than that required for the inhibition of cell growth (Fig. 4B).

**DISCUSSION**

In the present study, we successfully established a human lung cancer cell line, LNM35, which is capable of spontaneous metastasis to lymph nodes with a 100% incidence. A number of tumor cell lines have been shown to possess hematogenous metastatic potential and proved to be useful for studies of underlying mechanisms and in the search for new therapeutics (22, 35, 40–43). However, only a few human cancer cell lines have been described in the literature thus far as being useful for studies of lymphogenous metastasis (Refs. 23–29; Table 3). The LNM35 cell line is, to the best of our knowledge, the first human lung cancer cell line to be reported as having such a biological property. Moreover, LNM35 has significant advantages over previously reported cell lines. LNM35 spontaneously metastasizes at a 100% incidence not only as a result of orthotopic i.b. propagation but also as a result of conventional s.c. injection. This proven highly reproducible nature of lymphogenous metastases makes LNM35 very different from others because s.c. inoculation of other cell lines has been reported as being useful for studies of lymphogenous metastasis (Refs. 23–29; Table 3).
Lymphogenous metastatic lung cancer line LNM35

Table 3 Comparison of LNM35 with other human cancer cell lines previously reported as having lymphogenous metastatic potentials

<table>
<thead>
<tr>
<th>Human cell line</th>
<th>Tumor type</th>
<th>Implantationa</th>
<th>Lymph node metastasis incidence (%)</th>
<th>Days to autopsy</th>
<th>References</th>
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<td>Lung cancer</td>
<td>Ectopic (s.c.)</td>
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<td>PC-3</td>
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<td>112</td>
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<td>M4Be</td>
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<td>57</td>
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<td>Pancreatic cancer</td>
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<td>MeWo</td>
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<td>20</td>
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* a pro., intraprostatic injection; m.f.p., injection into the mammary fat pad.
* b Incidence in cases with orthotopically propagated lung tumors of LNM35.

Table 3. Comparison of LNM35 with other human cancer cell lines previously reported as having lymphogenous metastatic potentials.

Comparison of LNM35 with other human cancer cell lines previously reported as having lymphogenous metastatic potentials

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* a pro., intraprostatic injection; m.f.p., injection into the mammary fat pad.
* b Incidence in cases with orthotopically propagated lung tumors of LNM35.

The present extensive search for differentially expressed molecules, however, allowed us to identify that COX-2 expression was significantly increased in LNM35. We could also show that motility and invasion of LNM35 could be inhibited significantly at least in vitro by treatment with nimesulide. These findings are of considerable interest because we previously found that COX-2 is expressed intensely in lung cancer cells infiltrating into the surrounding stromal tissues and in the corresponding lymph node metastases (45) and that an increase in COX-2 expression may be associated with a poor prognosis in patients undergoing surgical resection of early stage lung adenocarcinomas (46). Although an increase in expression of COX-2 has been suggested to play a significant role in the carcinogenesis of colorectal and lung adenocarcinomas (45, 47, 48), it has also been reported that introduction of the COX-2 gene may intensify invasiveness of colon cancer cells but that this invasiveness could be reduced by treatment with sulindac sulfide, a known COX inhibitor (49). Although further in vivo studies are necessary, the present study together with previous observations suggest the potential involvement of COX-2 in the metastatic processes of lung cancers. Studies such as expression profiling using LNM35 and its parental H460 are also warranted to search for additional differentially expressed molecules to elucidate the complex mechanisms of tumor metastasis.

In conclusion, we successfully established for the first time a human lung cancer cell line, LNM35, with high capability for developing spontaneous lymphogenous metastasis when inoculated in mice. The highly reproducible metastasis of LNM35, even as a result of conventional s.c. inoculation, as well as its short latency period should prove to be suitable for the screening of agents active in the suppression of metastasis in human lung cancer, which may ultimately lead to the development of novel means of diagnosis and nonsurgical therapy of this fatal disease.

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LYMPHOCENIC METASTATIC LUNG CANCER LINE LNM3


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