Study on the Metastatic Mechanisms of Human Giant-Cell Lung Carcinoma Comparison Between the Strains C and D

Liu Mei, Chen Lezhen, Zhao Po, Gu Zheng, Lu Yali and Li Nan

The process of metastasis consists of a series of linked sequential steps. In order for cells to carry out a successful metastasis, a group of cells with the primary tumor must invade through the host tissue cells and extracellular matrix (ECM). For this to occur, the release of certain degradative enzymes including plasmin (activated by plasminogen activator), cathepsins and type IV collagenase appears to be necessary. The structures and components of the tumor cell surface are also associated with reduced cellular adhesion to the extracellular matrix and tumor-host-cell interactions involved in tissue arrest and metastatic colony formation. Tumor cells then enter the lymph or blood vessels. During circulation, tumors cells aggregate with platelets, lymphocytes and neutrophils, leading to the formation of emboli that can become lodged in the capillary web of a distant organ. Once tumor cells migrate through the vascular wall, new growth begins and this is fostered by the release of angiogenesis factors. Activation of a number of oncogenes has been associated with the invasive, metastatic phenotype in different tumor type. Overexpression of c-myc, erb, c-Ki-ras and hst oncogenes has been observed in metastatic gastric cancer and mdm 2 gene amplification has been seen in metastatic osteosarcomas. It is of the utmost importance to know which cellular genes are involved in the expression of the metastatic phenotype and to learn how they are regulated.

SUMMARY The biologic characteristics of the two human giant-cell lung carcinoma strains with high (strain D) and low metastatic potential (strain C) were studied, including karyotype of chromosome, intracellular free calcium, morphologic changes of cell surface and the expression of nm23-H1, p53, ras, c-myc, c-erbB2, bcl-2 genes and PCNA. The correlation between different biologic features and the metastatic potential of the two strains was analyzed. We found: 1) Both strains had the karyotypic abnormality of -13, -14, -15, +20, +21 with seven same marker chromosomes. Only strain D had the karyotypic abnormality of +7, -17, -18, +X, 7p+; 2) [Ca\(^{2+}\)] of the strain C (984.7 ± 573.8) and D (517.6 ± 216.6) was significantly different (p < 0.05). The amplitude of intracellular calcium oscillations of strain C was lower than the one of strain D; 3) strain C had more villous-like protrusions on the cell surface, whereas strain D had more bubble-like protrusions; 4) The expression of nm23-H1 and p53 protein of strain C was all higher than that of strain D. The expression of PCNA of strain C was lower than strain D; 5) nm23-H1 mRNA levels of strain C was lower than that of strain D. We consider that the karyotype of chromosomes, intracellular free calcium, the structure of cell membrane and the expression of nm23-H1 gene, p53 gene, PCNA could be closely related to the metastatic potential of human giant-cell lung carcinoma. They could be used as the sign for judging whether the tumor will metastasize in clinical practice as well as in judging the prognoses of patients.
nm23-H1 mRNA levels were associated with metastatic potential in breast cancers and colorectal cancers.11,12 Antonio13 has reported that nuclear accumulation of p53 protein was significantly linked with the metastasis of the non-small cell lung carcinoma (NSCLC). The expression of bcl-2 gene in NSCLC was correlated with the metastatic involvement of lymph nodes.14

Giant-cell lung carcinoma is uncommon, invades blood vessels, is more widely metastatizing, and is the most rapidly fatal of lung carcinomas. Increased expression of p53 protein and mutation of p53 gene in PG cell line derived from a lung giant cell carcinoma of the Pathology Department of Beijing Medical University was found.15 There is still little known about the metastatic mechanism of giant-cell lung carcinoma. Human giant-cell lung carcinoma cell line (PLA-801) was established in our department in 1981.16 The subline with high metastatic potential (strain D) and the subline with low metastatic potential (strain C) were ideal models for the study of tumor metastasis of lung carcinomas. Both sublines were isolated from PLA-801 by single cell cloning technique in 1989.17 In this study we hoped to find the factors influencing the metastatic potential of human giant-cell lung carcinoma through comparing biologic phenotypes of strains C and D. Invasion and metastasis can be facilitated and blocked by some factors. Here we could only select four aspects on metastatic generation and modulation to study for limited efforts, namely karyotype of chromosome, [Ca²⁺], morphology of cell surface, the expression of nm23-H1, p43, ras, myc, c-erbB-2, bcl-2 gene and proliferation cell nuclear antigen (PCNA) in tumor cells. Then the correlation between the different biologic features and different metastatic potential was analyzed.

**MATERIALS AND METHODS**

**Karyotypical analysis of chromosome**

Strains C and D at passage 19 were provided by cell culture laboratory of Pathology Department of Chinese PLA General Hospital. G-banding and cytogenetic analysis followed routine methods. The lymphocytic chromosomal karyotype of age-matched health persons were referred as the normal control. Chromosome counts were obtained from at least 20 metaphase cells, and karyotypes were prepared from at least 5 of these cells, whenever possible. Chromosome identification and designation were in accordance with the ISCN (1991).

**Intracellular free calcium**

Strains C, D and human lung fibroblast cell strain (as normal control) (strain F) cells were passed on to 96 well plates. The cells were incubated at 37°C for 30 minutes in RPMI 1640 medium containing 20 μmol/ml Fluo-3/AM (Meridine, USA) and 6% F-127 (Meridine, USA). Following two washes in PBS, the cells were incubated in RPMI1640 at 37°C. Then [Ca²⁺], transients were measured by laser confocal scanning microscope (Insight, USA).18 For Fluo-3/AM imaging, the cells were excited at 488nm and the fluorescence was measured through a 520 nm filter. The gray value of cellular fluorescence represented [Ca²⁺], and the fluorescence signals were recorded by computer. The gray values of strains C, D and F were compared by analysis of variance and Student-Newman-Keul (SNK) test. For each oscillating cell studied, the amplitude of oscillation was expressed as percentage above the base level.

**Morphology**

Scanning electron microscopic examination of the morphological differences between strains C, D and F cells was performed as previously described.19

**Immunohistochemical staining**

Strains C, D and F cells were cultured on coverslips. The cells were stained for the presence of nm23-H1 protein using a monoclonal antibody (Santa Cruz Biotechnology, California, USA), c-erbB-2 protein using a monoclonal antibody (Santa Cruz Biotechnology, California, USA), p53 protein using a monoclonal antibody (mutant and wild forms; Zymed, South San Francisco, USA), bcl-2 protein using a polyclonal antibody (Zymed, South San Francisco, USA), c-myc and ras using a monoclonal antibody (Bangding, Beijing, China). The alkaline phosphatase anti-alkaline phosphatase (APAAP) method was performed on the immunostaining of nm23-H1 and bcl-2 protein.20 The avidin-biotin-peroxidase (ABC) method was performed on the staining of p53, PCNA, c-erbB-2, c-myc and ras.21 The strain F cells provided a normal control. Immunoreactivity including staining intensity (value of gray) and positivity (percentage) was recorded by imaging analysis system and evaluated by t-test.
Reverse transcription-PCR amplification of nm23H1 mRNA

Total RNA of strains D, C and F was isolated by the guanidinium thiocyanate-CsCl method\(^\text{22}\) and three equal total RNA were used for reverse transcription. A pair of primers were \(nm23H-1\) (366 base pairs): 5'- TTGAGCGTACC-TCCATTGCGATC-3' and 3'-TTTGCACTCTCCACAGAATC-25'. RT mixtures contained 1.316 \(\mu\)g RNA, AMV reverse transcriptase 3 U, 25 pM each primers, RNasin 20 U, 200 pM each deoxynucleotide, 50 \(\mu\)M Tris HCl, 25 mM KCl, 10 mM MgCl\(_2\), 10 mM DTT and 0.5 mM spermidine. After reverse transcription, first-strand cDNA was directly used and 6 U \(Taq\) DNA polymerase, 25 pM of each primer was added to the RT mixtures for the later PCR. PCR cycles included denaturation for 45 seconds at 94°C, annealing for 30 seconds at 55°C and polymerization for 90 seconds at 72°C with 35 cycles. In the RT-PCR all the reagents of the three cell strains were equal for better comparison. The PCR products were separated on agarose gel in TAE buffer, stained by ethidium bromide and checked by dideoxysequencing.\(^\text{23,24}\)

RESULTS

Karyotypical analysis of chromosomes

Near-triploid was the most common chromosome number in each of the strains with 63-64 chromosomes in strain D and 62-65 chromosomes in strain C (Fig. 1). Most cells of strain D showed numerical changes of monosomies 13, 14, 15, 17, 18, trisomies 7, 20, 21, double X chromosome and the

![Fig. 1](image) The chromosome karyotype of strains C and D. The karyotype of strain C showed numerical changes of monosomies 8, 13, 19, trisomies 16, 18, 21, 22 and the loss of double chromosome 14, 15 (A). The karyotype of strain D showed numerical changes of monosomies 8, 13, 14, 15, 17, 18, trisomies 1, 7, 22, trisomies 20, 21 and structural changes of partial gain of 7p (B). Both strains had seven marker chromosomes, which were the same.
Fig. 2 Intracellular calcium oscillations in strains C, D, and F. The frequencies of oscillations were similar. The amplitude of the oscillation appeared different. The amplitude of the strain F cells (C) was 1.07 at the top level, the amplitude of the strain C cells (A) was 1.12 at the top level and the highest amplitude of the strain D (B) reached 1.35.

Table 1 Chromosomal aberration of strains D and C

<table>
<thead>
<tr>
<th>Strain</th>
<th>7</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>17</th>
<th>18</th>
<th>20</th>
<th>21</th>
<th>X</th>
</tr>
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<tbody>
<tr>
<td>Aberration*</td>
<td>T</td>
<td>PG</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>LD</td>
<td>M</td>
<td>M</td>
<td>T</td>
</tr>
<tr>
<td>frequency</td>
<td>D</td>
<td>2/5</td>
<td>4/5</td>
<td>2/5</td>
<td>2/5</td>
<td>5/5</td>
<td>0/5</td>
<td>5/5</td>
<td>3/5</td>
</tr>
<tr>
<td>C</td>
<td>0/5</td>
<td>0/5</td>
<td>3/5</td>
<td>3/5</td>
<td>1/5</td>
<td>2/5</td>
<td>0/5</td>
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<td>2/5</td>
</tr>
</tbody>
</table>

*L.D: loss of double chromosome, M: monosomy, PG: partial gain, T: trisomy and/or tetrasomy.
structural change of partial gain of 7p. Some cells of strain D had tetrasomies 20, 21. Most cells of strain C had monosomies 13, 14, 15 and trisomies 18, 20, 21. Some cells of strain C had terasomies 20, 21 and the loss of double chromosome 15. Both strains had eight marker chromosomes, seven of which were the same (Table 1).

Intracellular free calcium

The differences in \([\text{Ca}^{2+}]_j\) among strains C, D and F were significant (p < 0.01). The difference in \([\text{Ca}^{2+}]_j\) between any two strains was also significant (p < 0.05). \([\text{Ca}^{2+}]_j\) of D cells was the lowest, \([\text{Ca}^{2+}]_j\) of F cells was the highest and \([\text{Ca}^{2+}]_j\) of C cells was in the middle (Table 2). Intracellular calcium oscillations occurred in the three strain cells and the frequencies of oscillation were similar. The amplitude of the oscillation appeared different (Fig. 2). The amplitude of most cells of F was lower than the one of C cells and the amplitude of most C cells was lower than the one of most D cells.

Cell morphology

Strains D and C cells had round shape, numerous membrane protrusions and membrane ruffle. The cells were connected to each other (by long protrusions). The strain D cells had more bubble-like protrusions and ruffle on the cell surface, whereas the strain C cells had more villous-like protrusions (Fig. 3). There were few protrusions on the cell surface of strain F.

Immunostaining

The immunostaining intensity of nm23-H1 protein of strain C

**Table 2** \([\text{Ca}^{2+}]_j\) of the strains C, D and F

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Number of cells</th>
<th>Gray of fluorescence (U) X ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>30</td>
<td>517.6 ± 216.6</td>
</tr>
<tr>
<td>C</td>
<td>30</td>
<td>984.7 ± 573.8</td>
</tr>
<tr>
<td>F</td>
<td>30</td>
<td>1,727.7 ± 203.2</td>
</tr>
</tbody>
</table>

Fig. 3 The membrane structure of strains C and D. The strain D cells had more bubble-like protrusions (A). The strain C cells had more villous-like protrusions (B).
(122.3 ± 37.6 U) was higher than that of strain D (48.4 ±37.9 U), p < 0.05 (Fig. 4). The positivity for p53 protein of strain D (34%) was lower than that of strain C (100%), p < 0.05 (Fig. 5). The positivity for PCNA of strain D (39%) was stronger than that of strain C (5%), p < 0.05. There was no differences between the expression of bcl-2 gene of the two strains (Table 3). The expression of c-erbB-2, c-myc and ras gene of the two strains was negative. The immunostaining for nm23-H1 and bcl-2 gene in the strain F cells was positive and for other protein the staining was negative.

RT-PCR

A 366-base pair PCR product was detected in normal control (strain F) and strain C (Fig. 6). The amplified product was not found in strain D.

DISCUSSION

The cellular and genetic control of malignant tumor metastasis is still poorly understood. Cytogenetic studies can help to identify very precisely the chromosomal sub-location of metastasis-related genes. Strains D and C were isolated from human giant cell lung

<table>
<thead>
<tr>
<th>Protein</th>
<th>Gray (Units)</th>
<th>Positivity (%)</th>
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<tr>
<td></td>
<td>C (X ± SD)</td>
<td>D (X ± SD)</td>
</tr>
<tr>
<td>nm23-H1</td>
<td>122.3 ± 37.6</td>
<td>48.4 ± 37.9</td>
</tr>
<tr>
<td>p53</td>
<td>73.3 ± 35.8</td>
<td>79.7 ± 6.7</td>
</tr>
<tr>
<td>PCNA</td>
<td>91.0 ± 28.9</td>
<td>100.1 ± 7.6</td>
</tr>
<tr>
<td>bcl-2</td>
<td>134.6 ± 24.6</td>
<td>141.9 ± 12.9</td>
</tr>
</tbody>
</table>

Fig. 4 The immunohistochemistry of nm23-H1 protein of the strains C (A) and D (B). The immunoreactivities were located in the cytoplasm of strains C and D, but the positivity for strain C was higher than that of strain D. Magnification, X400; Scale bar, 10 μm.
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Fig. 5 The immunohistochemistry of p53 protein of the strains C (A) and D (B). The immunoreactivities were located in the nuclei of all the cells of the cells of strain C, only some nuclei of the strain D cells were positive. Magnification, X400; Scale bar, 10 μm.

carcinoma cell line PLA-801. The same chromosome abnormalities found in strains D and C, such as -13, -14, -15, and +20, +21, perhaps correspond to the generation and development of human giant cell lung carcinoma. The same chromosome abnormalities indicate that the two strains may be similar in some biologic behaviors. An increased number of chromosome 7 has been detected in advanced metastatic melanomas. Similar findings have been reported for breast cancer, bladder cancer, and pancreatic cancer. John et al. have obtained evidence for the existence of genes residing on chromosome 7 controlling invasion and metastasis by somatic cell fusion studies. Located in chromosome 7 were several genes critical to cell proliferation and chromatin functions. Several cellular proto-oncogenes including erbB, met were also located in chromosome 7. In the present study not strain C but strain D had trisomy 7. The observation suggests that more copies of chromosome 7 help to increase metastatic potential of human giant cell lung carcinoma. More work needs to be done to make sure whether there are metastasis-associated genes on chromosome 7. The metastasis suppressor gene nm23, located on the short arm of chromosome 17 at 17q22, was significantly higher on melanoma cell line with low metastatic activity in comparison to that with high metastatic activity. Bevilacqua et al. reported that low nm23 RNA levels were associated with high metastatic potential in breast carcinomas. Therefore, we deduce that a loss of chromosome 17 in strain D might decrease the expression of nm23-H1 protein and mRNA level of strain D, which results in a higher metastatic potential of strain D. The RT-PCR result also supports our view that the mRNA level of nm23-H1 gene in strain C was higher than that in strain D and nm23-H1 protein expressed in strain C was also higher than that in strain D. There are no reports about the relationship between chromosomes 18 and X and metastasis regulation of tumors. We think the different changes of chromosomes 18 and X in strain C and D may supply a clue to study the metastasis-related genes on both chromosomes.

PCNA is an acid nuclear protein that is directly involved in DNA synthesis. Its expression increases in the late G1 phase, reaches its maximum in the S phase, declines during the G2.
Fig. 6 RT-PCR showing nm23-H1 mRNA (size 366 bp) in strains C, D and F. nm-23H1 mRNA level in strain C was lower than that in normal control (strain F). There was no product in strain D. C, strain C; D, strain D; N, strain F; M, PCR markers (1543, 994, 695, 515, 377 and 237 bp).

Enhua et al. reported that the expression of PCNA in lung carcinomas was significantly linked with metastatic involvement of lymph nodes and distant metastasis \( (p < 0.05) \). In our study, the expression of PCNA in strain D was significantly higher than that in strain C \( (p < 0.05) \). Therefore, the nuclear accumulation of PCNA in human lung giant cell carcinomas might be correlated with the metastatic capacity of tumor cells. Antonio et al. found a significant association between the overexpression of p53 protein and metastasis in NSCLC. Haruhisa et al. also reported that the incidence of nuclear p53 staining of adenocarcinoma of the lung was significantly higher in cases with distant metastasis than that in those without \( (p < 0.05) \). In our study, the result was opposite to theirs, but our conclusion does not contradict those reports. If only \( p53 \) gene had an influence on the metastatic potential of human large cell lung carcinoma, the metastatic capacity of the strain D cells would be higher than that of the strain C cells. In fact \( nm23-H1, p53 \) gene and PCNA, perhaps other effectors, might take part in regulating the metastatic process of strains C and D, coordinately and differently in degree. The positivity for p53 protein of strain D (34%) was significantly lower than that of strain C (100%). Therefore, we deduce that not single p53 protein but \( nm23-H1 \) gene, PCNA, p53 protein, etc. altogether can have an influence on the metastatic potential of human giant cell lung carcinoma. In the present study there was no expression of \( ras, c-myc, bcl-2 \) and \( c-erbB-2 \) genes, that indicates these genes perhaps have little correlation with the metastasis of human giant cell lung carcinoma although many publications reported the relationship between these genes and tumor metastasis.

A primary intracellular regulator of cellular function such as movement, secretion, metabolization and differentiation was the intracellular concentration of free \( \text{Ca}^{2+} \). Weber pointed out that a high concentration of inorganic phosphate would evidently be incompatible with a high concentration of \( [\text{Ca}^{2+}] \); the maintenance of \( [\text{Ca}^{2+}] \) at very low concentration levels allowed the phosphate-oriented metabolism essential for life. In this study, lower \( [\text{Ca}^{2+}] \) perhaps makes the widespread use of more phosphate-containing compound as metabolic fuel for strain D cells, thus may be beneficial to the invasion and metastasis of strain D cells. \( [\text{Ca}^{2+}] \) was found to oscillate in strains D and C. The oscillatory frequencies in strains D and C were in common, but the oscillatory amplitude of most strain D cells was higher than that of most strain C cells. Koopman et al. proposed that the information present in the oscillatory component (the oscillation frequency and possibly the amplitude) might regulate processes which require prolonged activation, such as gene transcription and the intracellular transport of secretory vesicles. We further supposed the difference between the oscillatory amplitude of strains C and the one of strain D might influence some metastasis-associated gene transcription and expression of the two
strain cells differently, thus resulting in different metastatic potential of the strain D and C cells.

The expression of certain membrane components at the cell surface may affect the physiological state of cells and its interaction with the environment. Changes of physiological state can be reflected by changes of morphology.\(^{40}\) Yu et al.\(^{41}\) also reported that the NCL-H460 transfectants overexpressed transmembrane glycoprotein p\(^{18}\)new with increased metastatic potential after they introduced the human c-erbB-2/neu gene into the parental NCCl-H460 cells. We propose that the structure and function of the strain C and D cell surfaces might be modulated by the expression of some metastasis-associated gene despite the fact that we have not found such genes in our study. Thus the different membrane structures of the strain C and D cells, the strain C with a more villous-like membrane process and D with a more bubble-like membrane process, may not only reflect different metastatic potential but also make a difference to the metastatic activity of the two strains.

In conclusion, according to the above-mentioned, we consider that in our study the karyotype of +7, -17, -18, +X, 7p+, the lower [Ca\(^{2+}\)], the bubble-like protrusions on the cell surface and the expression of p53 protein, PCNA could benefit the metastasis of human giant-cell lung carcinomas. The expression of nm23-H1 protein and the higher nm23-H1 mRNA level might suppress the metastatic capacity of human giant-cell lung carcinomas. The structure and numerical changes of some chromosomes as well as the [Ca\(^{2+}\)], of the strains C and D cells could influence the expression of some metastasis-related genes. The cellular surface structure may be modulated by some metastasis-associated gene. Thus all these metastatic effectors perhaps take part in controlling metastasis of strains C and D and result in their different metastatic potential. Because a group of coordinated cellular processes are responsible for tumor metastasis, it is hard to say which is the most important and determinative among the four biologic features.

**ACKNOWLEDGEMENTS**

We thank Dr Jin Cuizhen for providing technological support and indication on karyotypic analysis. We also thank Dr Li Xianghong for his critical reading of the manuscript.

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