ASIAN PACIFIC JOURNAL OF ALLERGY AND IMMUNOLOGY (1998) 16: 167-176

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

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 ($[Ca^{2+}]_i$), 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) $[Ca^{2+}]_i$ 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 matastasize in clinical practice as well as in judging the prognoses of patients.

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),[1] cathepsins[2] and type IV collagenase[3] 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.[4,5] 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.[6] Once tumor cells migrate through the vascular wall, new growth begins and this is fostered by the release of angiogenesis factors.[7] Activation of a number of oncogenes has been associated with the invasive, metastatic phenotype in different tumor type. Overexpression of *c-myc, c-erb, c-Ki-ras* and *hst* oncogenes has been observed in metastatic gastric cancer[8] and *mdm 2* gene amplification has been seen in metastatic osteosarcomas.[9] 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. Steeg *et al.*[10] has demonstrated that *nm23* was a new suppressor gene.

From the Department of Pathology, Chinese PLA General Hospital, Fuxing Road No.28, Beijing 100853, China.
Correspondence: Liu Mei

nm23-H1 mRNA levels were associated with metastatic potential in breast cancers and colorectal cancers.[11,12] Antonio[13] 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, $[Ca^{2+}]_i$, 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 metatatic 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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[22] and three equal total RNA were used for reverse transcription. A pair of primers were *nm23H-1* (366 base pairs): 5'- TTGAGCGTACCTCCATTGCGATC-3'and 3'-TTTGCACTCTCCACAGAATCACT-5'. RT mixtures contained 1.316 µg RNA, AMV reverse transcriptase 3 U, 25 pM each primers, RNasin 20 U, 200 µM each deoxynucleotide, 50 µM 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.[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

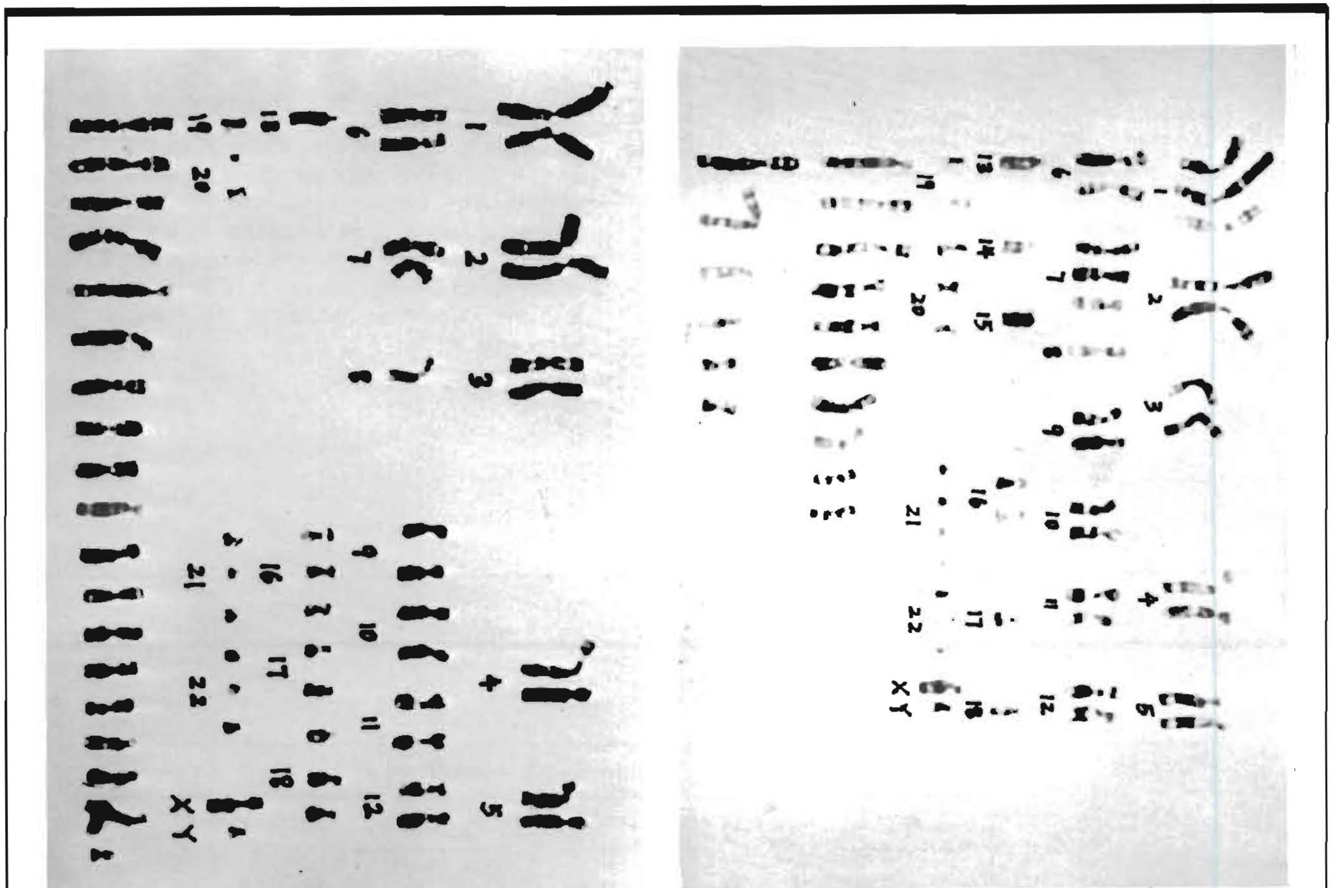


Fig. 1 The chromosome karyotype of strains C and D. The karyotype of strain C showed numerical changes of monosomies 8, 13, 19, trisomies 16, 18, 21, 22 and the loss of double chromosome 14, 15 (A). The karyotype of strain D showed numerical changes of monosomies 8, 13, 14, 15, 17, 18, trisomies 1, 7, 22, trisomies 20, 21 and structural changes of partial gain of 7p (B). Both strains had some marker chromosomes, seven of which were the same.

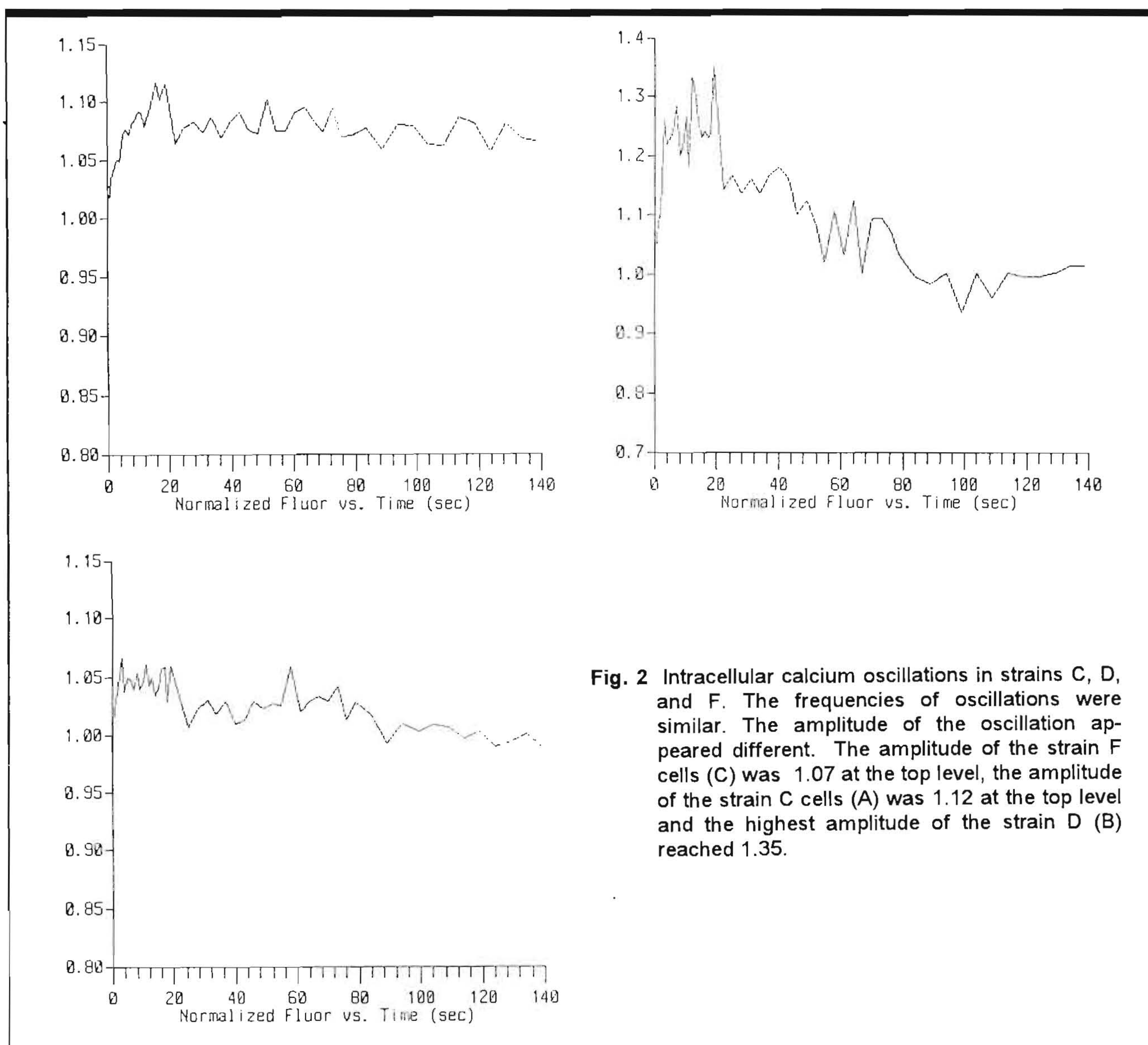


Fig. 2 Intracellular calcium oscillations in strains C, D, and F. The frequencies of oscillations were similar. The amplitude of the oscillation appeared different. The amplitude of the strain F cells (C) was 1.07 at the top level, the amplitude of the strain C cells (A) was 1.12 at the top level and the highest amplitude of the strain D (B) reached 1.35.

Table 1 Chromosomal aberration of strains D and C

	Strain	Chromosome number											
		7		13	14	15		17	18		20	21	X
Aberration*		T	PG	M	M	M	LD	M	M	T	T	T	T
frequency	D	2/5	4/5	2/5	2/5	5/5	0/5	5/5	3/5	0/5	5/5	5/5	3/5
	C	0/5	0/5	3/5	3/5	1/5	2/5	0/5	0/5	2/5	4/5	5/5	0/5

*LD: loss of double chromosome, M: monosomy, PG: partial gain, T: trisomy and /or terasomy

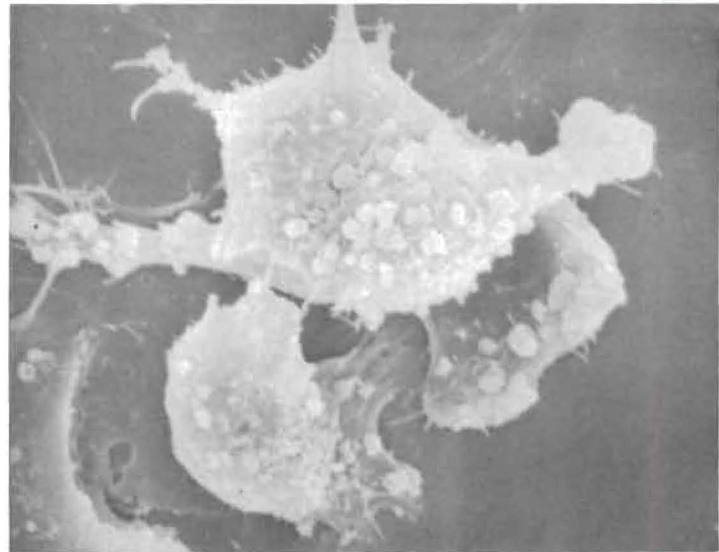
structural change of partial gain of 7p. Some cells of strain D had tetrasomies 20, 21. Most cells of strain C had monosomies 13, 14, 15 and trisomies 18, 20, 21. Some cells of strain C had terasomies 20, 21 and the loss of double chromosome 15. Both strains had eight marker chromosomes, seven of which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

Table 2 $[Ca^{2+}]_i$ of the strains C, D and F

Cell line	Number of cells	Gray of fluorescence (U) X ± SD
D	30	517.6 ± 216.6
C	30	984.7 ± 573.8
F	30	1,727.7 ± 203.2

Intracellular free calcium

The differences in $[Ca^{2+}]_i$ among strains C, D and F were significant ($p < 0.01$). The difference in $[Ca^{2+}]_i$ between any two strains was also significant ($p < 0.05$). $[Ca^{2+}]_i$ of D cells was the lowest, $[Ca^{2+}]_i$ of F cells was the highest and $[Ca^{2+}]_i$ 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.



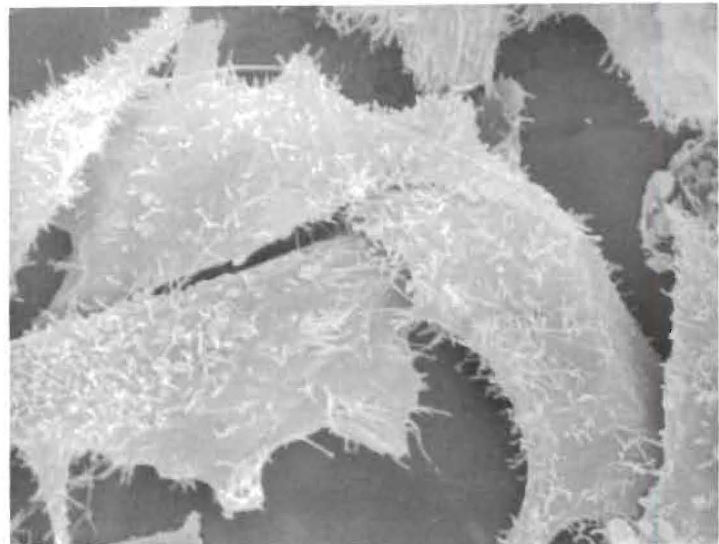


Fig. 3 The membrane structure of strains C and D. The strain D cells had more buble-like protrusions (A). The strain C cells had more villous-like protrusions (B).

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

(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.

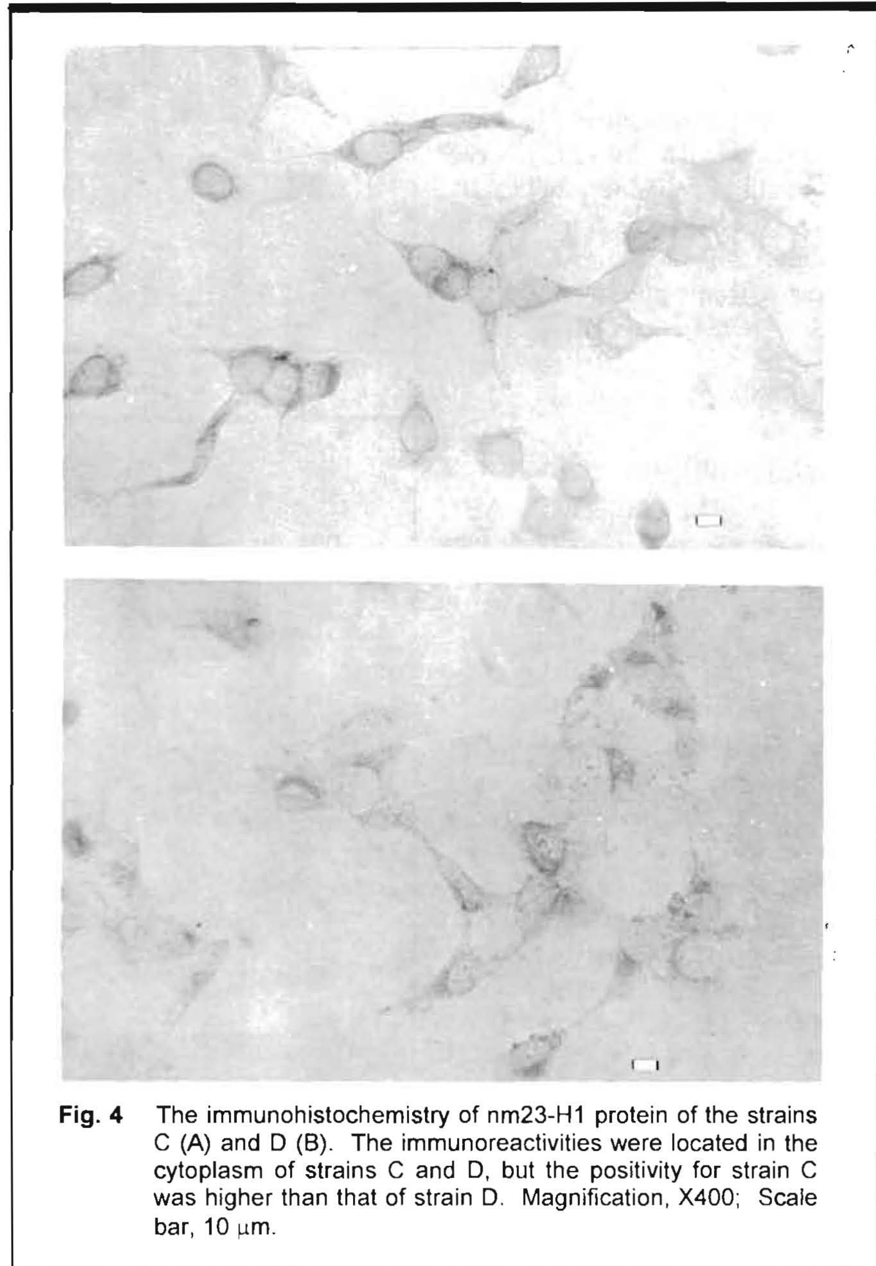


Fig. 4 The immunohistochemistry of nm23-H1 protein of the strains C (A) and D (B). The immunoreactivities were located in the cytoplasm of strains C and D, but the positivity for strain C was higher than that of strain D. Magnification, X400; Scale bar, 10 μm.

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 3 Comparison between the results of immunostaining in strains C and D

Protein	Gray (Units)			Positivity (%)		
	C (X ± SD)	D (X ± SD)	Comparison	C	D	Comparison
nm23-H1	122.3 ± 37.6	48.4 ± 37.9	p < 0.001	100	100	-
p53	73.3 ± 35.8	79.7 ± 6.7	p > 0.05	100	34	p < 0.001
PCNA	91.0 ± 28.9	100.1 ± 7.6	p > 0.05	5	39	p < 0.001
bcl-2	134.6 ± 24.6	141.9 ± 12.9	p > 0.05	100	100	-

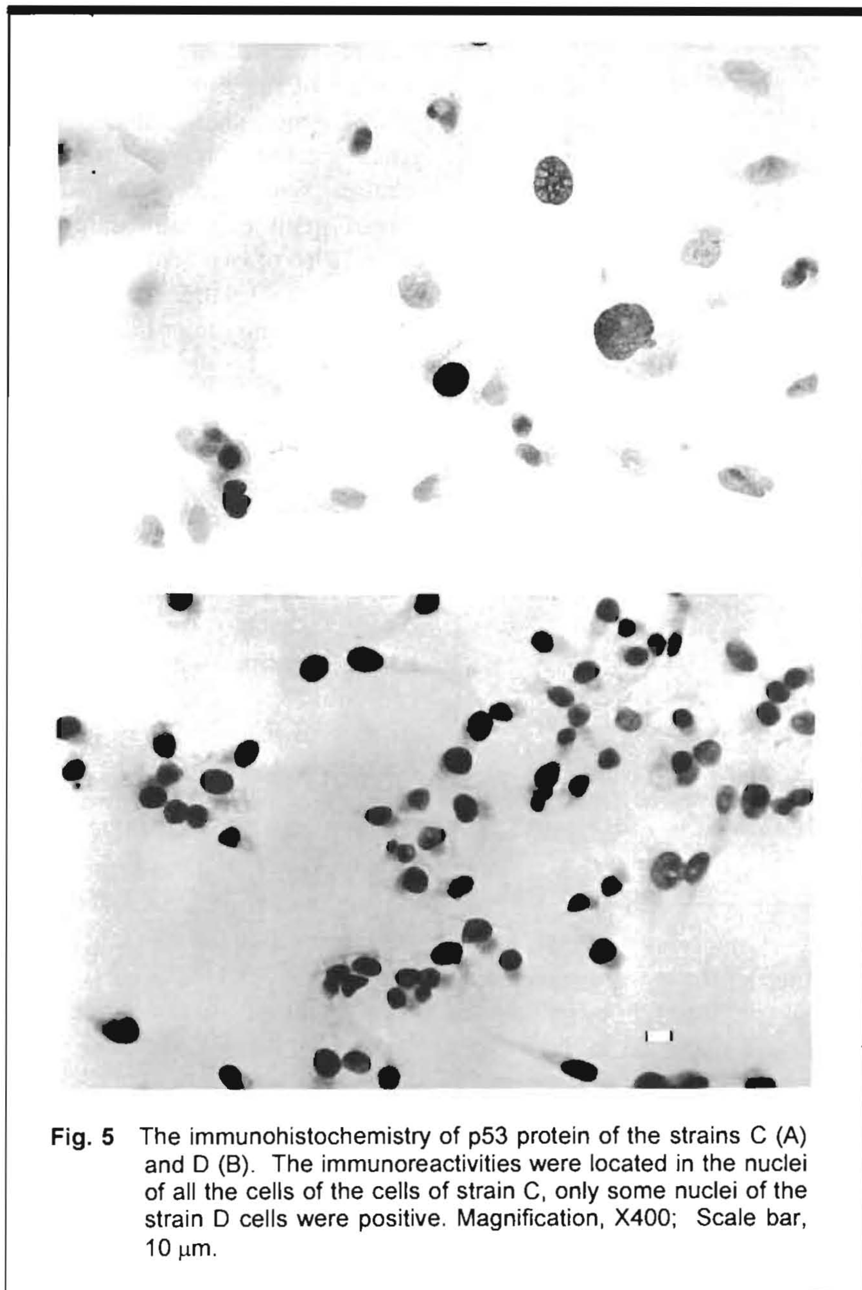


Fig. 5 The immunohistochemistry of p53 protein of the strains C (A) and D (B). The immunoreactivities were located in the nuclei of all the cells of the cells of strain C, only some nuclei of the strain D cells were positive. Magnification, X400; Scale bar, 10 μm.

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.[25] Similar findings have been reported for breast cancer,[26] bladder cancer[27] and pancreatic cancer.[28] John *et al.*[29] 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 chromotin functions.[30] Several cellular proto-oncogenes including *erB*,[31] *met*[32] were also located in chromosome 7. In the present study not strain C but stain 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.[10] Bevilacqua *et al.*[11] 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.[33] Its expression increases in the late G1 phase, reaches its maximum in the S phase,[34] declines during the G2

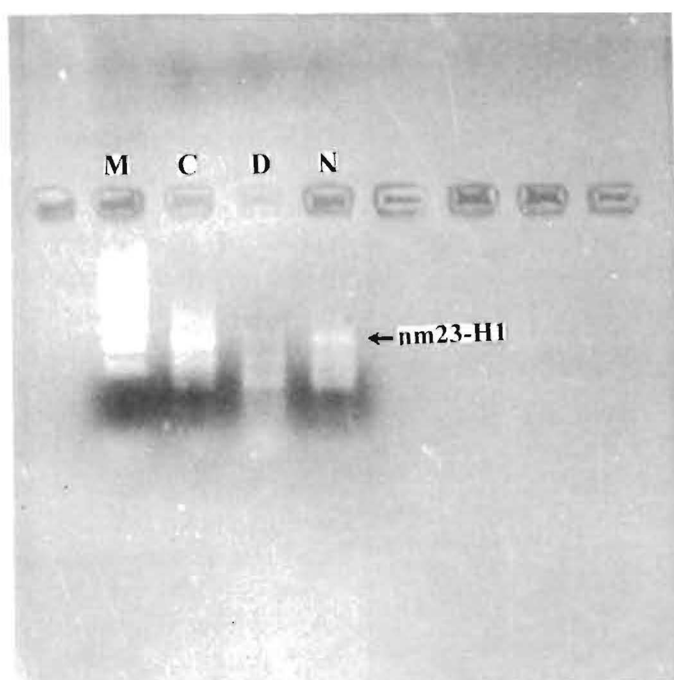


Fig. 6 RT-PCR showing nm23-H1 mRNA (size 366 bp) in strains C, D and F. nm-23H1 mRNA level in strain C was lower than that in normal control (strain F). There was no product in strain D. C, strain C; D, strain D; N, strain F; M, PCR markers (1543, 994, 695, 515, 377 and 237 bp).

phase, and is absent during the mitotic phase of the cell cycle.[35] Enhua *et al.*[36] 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.*[13] found a significant association between the overexpression of p53 protein and metastasis in NSCLC. Haruhisa *et al.*[37] 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 Ca^{2+} ($[Ca^{2+}]_i$).[38] Weber pointed out that a high concentration of inorganic phosphate would evidently be incompatible with a high concentration of $[Ca^{2+}]_i$; the maintenance of $[Ca^{2+}]_i$ at very low concentration levels allowed the phosphate-oriented metabolism essential for life. In this study, lower $[Ca^{2+}]_i$ 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. $[Ca^{2+}]_i$; 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.*[39] 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^{185new} 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 $[Ca^{2+}]_i$, 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 $[Ca^{2+}]_i$ 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.

REFERENCES

1. Grondahl-Hansen J, Christensen IJ, Rosenquist C *et al.* High levels of urokinase-type plasminogen activator and its inhibitor PAI-1 in cytosolic extracts of breast carcinomas are associated with poor prognosis. Cancer Res 1993; 53: 2513-6.
2. Murnane MJ, Sheahan K, Ozdemirli M, Shujia SD. Stage-specific increase in cathepsin B messenger RNA content in human colorectal carcinoma. Cancer Res 1991; 51: 1137-9.
3. Sefter REB, Sefter EA, Stetler-Stevensen WG, Hendrix MJC. The 72 kDa type IV collagenase is modulated via differential expression of $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins during human melanoma cell invasion. Cancer Res 1993; 53: 3433.
4. Easton EW, Bolscher JGM, Eijnden DH. Enzymatic amplification invading glycosyltransferases forms the basis for the increased size of asparagine-linked glycans at the surface of NIH 3T3 cells expressing the N-*ras*. J Biol Chem 1991; 266: 21674-8.
5. Willingham MC, Yamada KM, Yamada SS, Poussegur J, Pastan I. Microfilament bundles and cell shape are related to adhesiveness to substratum and are dissociable from growth control in cultured fibroblasts. Cell 1977; 10: 375-7.
6. Wood S, Robinson JRR, Marzocch B. Factors influencing the spread of cancer: locomotion of normal and malignant cells *in vivo*. In: Wissler RW, Dao TL and Wood S, eds, Endogenous factors influencing host-tumor balance. University of Chicago Press, Chicago, 1967; pp. 223-37.
7. Forkman J, Shing Y. Angiogenesis. J Biol Chem 1992; 267: 10931-8.
8. Ranzani GN, Pellegata NS, Previdere C *et al.* Heterogeneous protooncogene amplification correlates with tumor progression and presence of metastasis in gastric cancer patients. Cancer Res 1990; 50: 7811-20.
9. Ladanyi M, Cha C, Lewis R, Jhanwar SSC, Huvos AG, Healey JH. MDM2 gene amplification in metastatic osteosarcoma. *Cancer Res* 1993; 53: 16-8.
10. Steeg PS, Bevilacqua G, Kopper L *et al.* Evidence for a novel gene associated with low tumor metastatic potential. J Natl Cancer Inst 1998; 80: 200-4.
11. Bevilacqua G, Sobel ME, Liotta LA, Steeg PS. Association of low nm23 RNA levels in human primary infiltrating ductal breast carcinomas with lymph node involvement and other histopathological indicators of high metastatic potential. Cancer Res 1989; 49: 5158-90.
12. Alvaro L, Ursula FC, Richter K *et al.* Reduced tumor incidence, metastatic potential and cytokine responsiveness of nm23-transfected melanoma cells. Cell 1991; 65: 25-35.
13. Antonio M, Fiamma B, Giorgio M *et al.* p53 alterations in non-small cell lung cancers correlate with metastatic involvement of hilar and mediastial lymph nodes. Cancer Res 1993; 53: 2846-51.
14. Fontanini G, Vignati S, Bigini D. Bcl-2 protein: a prognostic factor inversely correlated to p53 in non-small cell lung cancer. B J Cancer 1995; 71: 1003-7.
15. Bo Z, Wen C, Yingtang Z. Mutation of *p53* gene in a highly metastatic human lung cancer cell line. Chinese J Oncol 1995; 17: 279-82.
16. Lezhen C, Jingxiang H, Lianbi Y, Weihua L. Establishment of human pulmonary giant cell carcinoma cell line (PLA-801) and observations of its

biologic properties. Chinese J Oncol 1983; 5: 409-13.

17. Yinglin L, Jingxiang H, Xianghong L, Hongfen L, Lezhen C. Spontaneous metastasis of clonal cell subpopulations of human lung giant cell carcinoma after subcutaneous inoculation in nude mice. Chinese J Oncol 1989; 11: 1-7.
18. Stenphen WP. To boldly glow: applications of laser scanning confocal microscopy in development biology. BioEssays 1994; 16: 357-65.
19. Nicodon GL, Custead SE. Effects of chemotherapeutic drugs on platelet and metastatic tumor cell-endothelial cell interaction as a model for assessing vascular endothelial integrity. Cancer Res 1985; 45: 331-6.
20. Elliot WM, Hayashi S, Hogg JC. Immunodetection of adenoviral EIA proteins in human lung tissue. Am J Respir Cell Mol Biol 1995; 12: 642-8.
21. Hsu SM, Raire L, Fanger H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (APA) procedures. J Histochem Cytochem 1981; 29: 577-80.
22. Ullrich A, Shire J, Chingwin J *et al.* Rat insulin genes: construction of plasmids containing the coding sequences. Science 1977; 196: 1313-6.
23. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 1977; 74: 5463-6.
24. Maniatis T, Fritsch, Sambrook J. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, New York, 1982.
25. Koprowski H, Herlyn M, Balaban G *et al.* Expression of the receptor for epidermal growth factor correlates with increased dosage of chromosome 7 on malignant melanoma. Somatic Cell Mol Genet 1985; 11: 297-301.
26. Sainsburg JRC, Sherbet GV, Furndon JR, Harris AL. Epidermal-growth-factor receptors and oestrogen receptors in human breast cancer. Lancet 1995; 1: 364-6.
27. Neal DE, Bennet MK, Hall RR *et al.* Epidermal-growth-factor receptors in human bladder cancer. J Natl Cancer Inst 1985; 1: 366-8.
28. Korc M, Meltzer P, Trent J. Enhanced expression of epidermal growth factor correlates with alterations of chromosome 7 in human pancreatic cancer. Proc Natl Acad Sci USA 1986; 83: 5141-44.
29. John GC, Monique VD, Annehieke S. Location of genes involved in invasion and metastasis on human chromosome 7. Cancer Res 1987; 47: 6666-70.
30. Smith M, Spence MA. Report of the committee on the genetic constitution of chromosome 7, 8 and 9. Cytogenetic Cell Genet 1985; 40: 156-78.
31. Spurr NK, Solomon E, Jansson M *et al.* Chromosomal localization of the human homologous to the oncogenes *erbA* and *B*. EMBOJ 1984; 3: 159-64.
32. Cooper CS, Park M, Blair DG *et al.* Molecular cloning of a new transforming gene from a chemically transformed human cell line. Nature 1984; 311: 29-33.
33. Mathewa MB, Bernstein RM, Franza BR, Garrels JI. Identity of the proliferating cell nuclear antigen and cyclin. Nature 1984; 303: 374-6.
34. Kurki P, Ogata K, Tan EM. Monoclonal antibodies to proliferating cell nuclear antigen (PCNA/cyclin) as probes for proliferating cells by immunofluorescence microscopy and flow cytometry. J Immunol Methods 1988; 109: 49-59
35. Hall PA, Levison DA, Woods AL *et al.* Proliferating cell nuclear antigen (PCNA) immunolocation in paraffin sections: an index of cell proliferation with evidence of deregulated expression in some neoplasms. J Pathol 1990; 162: 285-94.
36. Enhua W, Guinan L, Mingchang H. PCNA expression and AgNORs image analysis as factors in judging the prognosis of lung cancer. Chung Hua Ping Li Hsueh Tsa Chih 1995; 24: 143-5.
37. Haruhisa H, Yoshihiro M, Harubumi K. Clinical pathological significance of nuclear accumulation of tumor suppressor gene *p53* product in primary lung cancer. Jpn J Cancer Res 1992; 83: 101-6.
38. Ernesto C. Intracellular calcium homeostasis. Ann Rev Biochem 1987; 56: 395-433.
39. Koopman WJ, Scheen WJ, Rowbos EW, Jenks BG. Kinetics of calcium steps underlying calcium oscillation in melanotrope cells of *Xenipus laevis*. Cell Calcium 1997; 22: 167-8.
40. Valerie W, Livio M. Cell surface components in cell morphology and growth. In: Letnansky K, ed. Biology of the cancer cell. H Charlesworth, Co Ltd. Huddersfield, 1980; pp. 227-34.
41. Dihua Y, Shunshue W, Kim MD, Chunming Y, Garth LN, Mieachie H. C-erbB-2/neu overexpression enhances metastatic potential of human lung cancer cells by induction of metastasis-associated properties. Cancer Res 1994; 54: 3260-6.