A NOVEL HUMAN DNA SEQUENCE WITH TUMOR METASTASIS SUPPRESSIVE ACTIVITY

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ABSTRACT

Objective: To isolate human tumor metastasis suppressive DNA sequence and to study the molecular mechanisms regulating tumor metastasis. Methods: A mouse lung adenocarcinoma cell clone 12 derived from its parent cell line LM2, which had been transduced with normal human genomic DNA, was previously reported. Compared with LM2, the metastatic potential of clone 12 was very much decreased. Clone 12 was used in this study to amplify the human DNA fragments by Inter Alu PCR technique. The human DNA fragments obtained were then transfected into LM2 cells and their malignant phenotype was tested in vitro and in vivo, and compared with that of the untransfected LM2 cells. *Results* Three human DNA fragments of 700, 500 and 300 bp were isolated. DNA sequencing revealed that the 700bp fragment does not show homology with hitherto reported genes and was accepted by the Genbank (pt712 U67835). In vitro proliferation and colony formation in soft agar of the 700 bp fragment-transfected LM2 cells were significantly inhibited as compared to the untransfected LM2 cells. Upon subcutaneous inoculation to syngeneic T739 mice, the 700bp–transfected LM2 cells grew more slowly and smaller tumors developed compared to the untransfected ones. Moreover, lung metastasis was not found in 6 of 10 mice inoculated with the 700bp-transfected LM2 cells, while it was found in 9 of 10 mice inoculated with the untransfected LM2 cells. The difference was statistically significant (P<0.001). The frequency of lymph node metastasis was also statistically different between the 2 groups of mice. *Conclusion* The newly isolated 700bp human DNA fragment may be a metastasis suppressor gene of malignant tumor.

Key words: Neoplasm metastatic suppressor gene, Human genomic DNA, Gene transfection, Sequence analysis, Inter Alu PCR

In recent years, many tumor metastasis researchers have devoted themselves to identify tumor metastasis suppressor genes in order to study the molecular mechanisms of tumor invasion and metastasis. In general, a gene that in some way inhibits the formation of metastasis may be defined as a metastasis suppressor gene. New concept "phenotype cloning" [1,2] and other new approaches [3] has been applied to isolate novel human tumor metastasis suppressor genes in our recent research. It was found several morphologically flat revertants of mouse lung adenocarcinoma cells transfected with human genomic DNA [4]. Some transfected clones showed significent suppression of metastatic phenotype. By a sensitive Inter Alu PCR method human DNA fragments were amplified from these transfected tumor cells of which the metastatic phenotype had been suppressed. One DNA fragment was sequenced and its similarity was compared with the Genbank sequence Databases. This fragment of human genomic DNA was transfected into the highly metastatic mouse lung adenocarcinoma cells LM2, and a series of in vitro and in vivo experiments were carried out in order to examine the metastatic suppressing effects of that human DNA fragment.

MATERIALS AND METHODS

Plasmid DNA, Cell Line and Animals

PSV2neo, eukaryotic expression vector, preserved in our laboratory. LM2 cell line, mouse lung adenocarcinoma cells with high metastatic potential, was established in our laboratory [4]. It is presented

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round or fusiform appearance loosely attached to the cultured bottle bottom in vitro. Clone cell 12, the metastatic revertant of mouse lung adenocarcinoma cells LM2 transfected with human genomic DNA in medium containing G418 and Ouabain, the rate of cell growth as well as colony formation in agar were lower than the maternal untransfected cells [4]. T739 mice, 5~7 weeks old, 20~25 g weight, female, were purchased from the Animal Center of Cancer Institute, Chinese Academy of Medical Sciences.

Amplification the human DNA fragments by Inter Alu PCR [5,6]

The Alu primer was a human-specific primer designed by us and the primer does not show any homology with mouse DNAs detected by "Goldkey" software. The Alu PCR primer sequence is as follows: 5'CCGAATTCAGAGCGAGACTCCGTCTC3', amplification with this Alu primer generated human DNA fragments from hybrid DNA, [7] and no mouse DNA amplification. PCR reaction was performed for 35 cycles of 94°C denaturation (1 min), 55°C annealing (30 sec), and 72°C extension (100 sec) in an automated thermal cycler (PTC-51B DNA amplificator). Initial denaturation was 5 min at 95°C. PCR's productions were separated by 1.5% agarose electorphoresis and purified by Wizard™ PCR purification kit (Promega).

Constructing recombinant human DNA, DNA sequencing and Homology searching

One human DNA fragment was ligated to pGEM-T vector (Promega), the recombinant DNA sequence was determined by automated DNA sequencing machine (Applied Biosystems 373, USA), the similarity of the newly obtained sequence was compared with the Genbank Sequence Database over the Internet.

Cell transfection

Cell transfection was performed by the method described by Lipofectamine transfection kit (Promega).

Total RNA isolation and Northern blot Analysis

Total RNA was isolated from cells either control or cells transfected by human 700bp DNA fragment. Human 700bp DNA fragment as probe was labelled by Biotin. Slot hybridization were done according to the method in "Molecular Cloning" [10].

In Vitro Cell proliferation assay and Growth in soft agarose

According to the methods as described [8,9]

In Vivo Gene Administration

T739 mice were randomly divided into two groups. Each group has 10 mice. 1×10^6 / 0.2ml cells were inoculated subcutaneously into the back region of the mice. The growth of tumor was checked once each two days, and tumor volume was determined by the formula $V\ (mm^3) = L\times W^2/2$, where "L" was the longest diameter and "W" was the shortest diameter of the tumor nodule. The mice were killed when the tumor >2 cm in diameter. Tissues from the lung, lymph nodes and tumor were also taken for pathohistological analysis.

RESULTS

Sequence analysis of the human DNA fragment in metastatic revertant Clone 12

Inter Alu PCR was performed on genomic DNA extracted from the metastatic revertant clone 12. A continuum of human genomic DNA fragments was obtained, which was about 700bp, 500bp and 300bp long. One DNA fragment that is about 700bp long was sequenced and its similarity was compared with the Genebank Sequence Databases. The Genebank accepted the 700bp DNA nucleotide sequences, as a new human genomic DNA sequence. The registration code is pt712 U67835. The part of the human DNA sequence is as follows:

ATCTGCACTCCAGCCTGGGGTATATGAGATCAAGCTGGGACTTAGTCTTCGCATAACTG
GATGCTCATGACTATACATTATGTATAGGACGTGATATGGATATGGCCTTAATATTAATGA
TTTTCCCCCTAGAGAAGAGAAATAAAATTTTTGAAAGTTTAAGTGATAGAGCAATATTT
GTAGTCATGAATTCTTACTTCTTTCCTTCTG CTTGTCTTGTGGTTGGATATTGCATGGGT
GGTCCTGATGTTGGCTTCTAATTTGAGTTTATGCTTAAGAGTTCCAGAAGATCAATGTCT
GGAAAATTGGTATCTACCTATCTGTTTGTCTACTGGTGAAAGCAAAGGGTATTTTAGCTG
TATCTGTCTCACTGCTCGTTTTTCCTGTATAGTGTGGTGGTGTGTGTGGTGTGTGTGT
GTGTGTGTGTAACCATTAATGCAGTAATCCNCCAATTGGACCTCATGCCCAGGCTGGGA
AGTGCAGAATCCACNAAGGTGCGGGCCGCCTGCAN

Expression of human DNA in h700 DNA-transfected clone cell

Total RNA (10μg each sample) was isolated from h700 DNA-transfected clone (LM2-h700) cells and control (LM2) cells, the LM2-h700 clone cell was the highly metastatic mouse lung adenocarcinoma LM2 cells transfected by 700bp fragment of human genomic DNA. Northern slot hybridization to human 700bp DNA probe revealed the presence of an intensive hybridization signal barely in RNA sample of the h700 DNA-transfected clone cells. The results indicated that human DNA fragment achieved efficient expression in h700 DNA-transfected clone (LM2-h700) cells.

Effect on cell morphology changes after transfection by h700 DNA fragment

After transfection by h700 fragment, mouse LM2 cell showed significant morphological changes into flat and mosaic appearance with decreased in refractivity and steroscopy as compared to the short spindle-like or round control untransfected LM2 cell with highly refractivity and steroscopy.

Effect on the proliferation ability

The proliferation ability of control LM2 cells and h700 DNA-transfected clone cells was examined by the cell count method, the results indicated that the proliferation of h700 DNA-transfected clone cells was greatly reduced as compared to that of control LM2 cells. (P<0.001, Figure 1)

Effect on growth in soft agarose

Compared with control LM2 cells, the colony formation rate of the human 700bp DNA transfected cells in soft agarose was greatly decreased. (Table 1)

Effect on tumor growth and metastasis

Tumor growth was observed post inoculation and tumor nodule volume was determined by the formula $V(mm^3)=L\times W^2/2$. The tumor growth curve were drawn in Figure 2. It shows that the tumor volume increased in the group of 700bp fragments-transfected cells was obviously slowly, therefore smaller tumors developed as compared to the control ones (P<0.001). Pathohistological analysis of the tumor tissues revealed that lung metastasis was not found in 6 of 10 mice inoculated with the 700 bp-transfected LM2 cells, while it was found in 9 of 10 mice inoculated with the untransfected LM2 cells. The difference was statistically significant (P<0.001). The frequency of lymph node metastasis was also statistically different between the 2 groups of mice (Table 2).

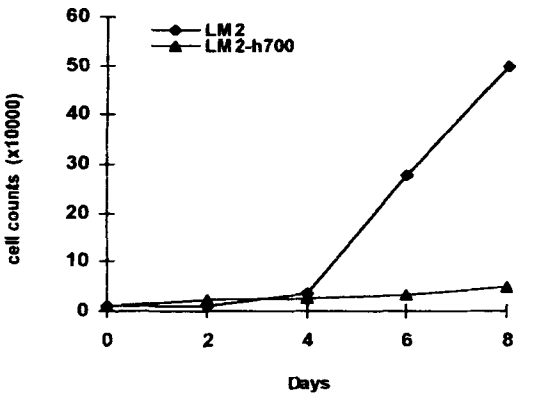

Fig 1. Proliferation curves of LM2 and transfected cell LM2-h700.

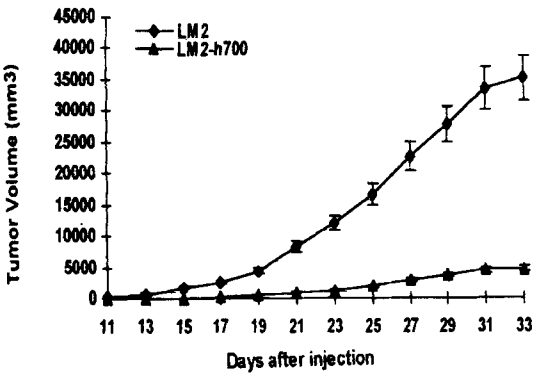

Fig. 2. Growth curve of the tumors.

Table 1. Compared with LM2 and LM2-h700 on growth in soft agarose

Cells	Cells/plate	Number of colonies				$\bar{x} \pm s^D$
		1	2	3	4	
Contols (LM2)	5×10^3	106	91	101	110	102±8.2*
Transfectants (LM2-h700)	5×10^3	38	25	30	29	30.5±5.5*

*Compared each other, P<0.001

Table 2: Metastasis to lung and lymph nodes

Groups	Lung metastasis /five pieces of lung	Lymph node metastasis /numbers of lymph node	Metastasis ratio(%)*
Controls (LM2)	31/50	7/35	100(10/10)
Transfectants (LM2-h700)	11/50	2/38	50 (5/10)

*Inside the ( ) are the numbers of mice which had lung or lymph node metastasis/the numbers of mice which had tumor nodules

DISCUSSION

"Phenotype cloning" [1] is a current concept by which novel genes are functionally identified. In contrast to positional cloning, phenotype cloning has the potential to save a great deal of repetitive work, so it has greater resolution and sensitivity. We could describe the isolation of genes by virtue of their effect alone and without requiring prior knowledge of their biochemical function or map position in the genome. The prospects for direct isolation new oncogenes or metastasis relating genes but of unknown genome position were revived by several recent papers.

Recently we use this reverse mode of thinking to isolate unknown human metastasis suppressor genes or relating human DNA sequences. Normal human genomic DNA fragments were transfected into highly metastatic mouse lung adenocarcinoma cloned cells, the existence human DNA fragments were amplifical by Inter Alu PCR from the transfected tumor cell clones of which the metastatic phenotype had been suppressed. One DNA fragment that is about 700bp long was sequenced and accepted as a new human genomic DNA sequence by the Genbank (the registration number is pt712 U67835). Using PCGENE software for the preliminary analysis of this sequence, it was found that the sequence may contain a single open reading frame of 228bp, the predicted protein consists of 76 amino acids, but limited with its length, the structure analysis is open for further clarify.

We should pay attention to the fact that in vitro and in vivo studies the metastatic phenotype of the mouse lung adenocarcinoma cells transfected with the 700bp fragment of human genomic DNA were moderately suppressed. Several possible mechanisms may lead to the results above-mentioned: 1) the expression of the normal human DNA fragment 700bp directly suppressed the metastasis activity in transfected cells; 2) the human DNA fragment maybe a regulation sequence or a control unit which could inhibit the expression of other metastasis genes in the transfected cells; 3) the transfection of human DNA fragment may be affected the function of some other genes in the highly metastasis tumor cells and gave the inhibition of metastatic phenotype of transfected cells.

Seeing that the invasion and metastasis of tumor cells is a complex process influenced by many genes and factors, the definite mechanism of regulation tumor growth and metastasis of the mouse lung adenocarcinoma cells transfected by the 700bp fragment of human genomic DNA should be further studied. The aforesaid experiment facts might be enough to encourage us to continue our research work, include: obtaining the integrated gene by screening genomic DNA libraries using this DNA fragment as a probe, analyzing and forecasting the structure and function of the integrated gene; introducing this integrated gene into human highly metastasis tumor cells, observing the effects of integrated gene transfected on tumor metastasis in vitro and in vivo, and determining the interrelation between this gene and tumor metastasis; testing and verifying the common effect of metastasis inhibition in different types of tumor cells by transfection of the goal gene; using antisense RNA of the goal gene to downregulate the human low metastasis tumor cells, observing the metastasis phenotype changes in vivo.

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