The microcell mediated transfer of human chromosome 8 into highly metastatic rat liver cancer cell line C5F

Hu Liu, Sheng-Long Ye, Jiong Yang, Zhao-You Tang, Yin-Kun Liu, Lun-Xiu Qin, Shuang-Jian Qiu, Rui-Xia Sun

AIM: Our previous research on the surgical samples of primary liver cancer with CGH showed that the loss of human chromosome 8p had correlation with the metastatic phenotype of liver cancer. In order to seek the functional evidence that there may harbor metastasis suppressor gene(s) for liver cancer on human chromosome 8, we tried to transfer normal human chromosome 8 into rat liver cancer cell line C5F, which had high metastatic potential to lung.

METHODS: Human chromosome 8 randomly marked with neo gene was introduced into C5F cell line by MMCT and positive microcell hybrids were screened by double selections of G418 and HAT. Single cell isolation cloning was applied to clone microcell hybrids. Finally, STS-PCR and WCP-FISH were used to confirm the introduction.

RESULTS: Microcell hybrids resistant to HAT and G418 were obtained and 15 clones were obtained by single-cell isolation cloning. STS-PCR and WCP-FISH proved that human chromosome 8 had been successfully introduced into rat liver cancer cell line C5F. STS-PCR detected a random loss in the chromosome introduced and WCP-FISH found a consistent recombination of the introduced human chromosome with the rat chromosome.

CONCLUSION: The successful introduction of human chromosome 8 into highly metastatic rat liver cancer cell line builds the basis for seeking functional evidence of a metastasis suppressor gene for liver cancer harboring on human chromosome 8 and its subsequent cloning.

INTRODUCTION

With the practice of diagnosis of primary liver cancer at early stage, surgical resection of small hepatocellular carcinoma (HCC) and other improvements in medical diagnosis, imaging, nonsurgical therapies, etc, important progresses have been made toward the control of liver cancer. For example, surgical resection of small HCC has resulted in a marked increase in 5-year survival rate from 20-30 % to 40-60 %. In our institute, the 5-year survival rate of 963 patients with small HCC (≤5 cm) resection was 65.1 %. However, recurrence and metastasis still remain to be major challenges for clinical workers[1-3]. The main obstacle to control of metastasis for liver cancer is the lack of sensitive predictive biomarkers and novel molecular targets for biotherapies[4-5]. The discovery of metastasis suppressor genes for liver cancer will undoubtedly be of vital importance to the prognostic diagnosis and intervention therapy for overcoming the metastasis of liver cancer.

Our previous study suggested that there may harbor metastasis suppressor genes on human chromosome 8p[6]. To seek functional evidence for this possibility, we tried to introduce the normal human chromosome 8 into the highly metastatic rat liver cancer cell line C5F[7] through microcell mediated chromosome transfer (MMCT). The positive microcell hybrids were screened through drug selection and confirmed by sequence tagged sites-polymerase chain reaction (STS-PCR) and whole chromosome painting-fluorescence in situ hybridization (WCP-FISH).

MATERIALS AND METHODS

Cell lines

The highly metastatic rat liver cancer cell line C5F[7] was generously provided by Dr. Kumiko Ogawa (the First Department of Pathology, Nagoya City University, Nagoya, Japan). The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % fetal bovine serum (FBS). The human chromosome 8 donor cell line A9 (neo8) was purchased from Japanese Collection of Research Bioresources (JCRB) Cell Bank, Osaka, Japan. A9 (neo8) is a mouse fibroblast cell line that contains a human chromosome 8. The human chromosome 8 was randomly marked with neo resistant gene. The A9 (neo8) cells were cultured in DMEM containing 10 % FBS and 800 µg mL⁻¹ geneticin (G418).

Reagents and instruments

Colcemid, cytochalasin B, phytohemagglutinin A (PHA), polyethylene glycol (PEG, MW 300-1 600, Hybrid-Max®) and Dimethylsulfoxide (DMSO, Hybrid-Max®) were purchased from Sigma, USA. DMEM high glucose, 100x HAT (10 mM hypoxanthin, 40 µM aminopterin, 1.6 mM thymidine) and G418 were ordered from Life Technologies, GIBCO BRL, USA. Isoproyl™ polycarbonate membranes were purchased from Millipore, USA. WCP paint DNA probes for chromosome 8 (SpectrumGreen™) were purchased from Vysis Inc, IL, USA. Primers were ordered from Sangon Biotechnology Company, Shanghai, China. Taq polymerase, dNTP and 100bp DNA ladder marker were the products of Sino-America Biotechnology Company, China. Fetal bovine serum (FBS, define) and Bovine Calf Serum (BCS) were purchased from Hyclone, USA. PE-2400 thermocycler was the product of Perkin-Elmar Company, USA. The high speed Hitachi himac CR21G centrifuge was the product of Hitachi, Japan.

Cyto
Vision™ Chromosome Analysis System (Cytovision™ Image Analysis Workstations, USA) was the product of Applied Imaging, USA.

MMCT [8-10]

Micronucleation of A9(neo8) A9(neo8) cells were seeded in six straight-neck T25 flasks respectively. Colcemid was added to the culture to the final concentration of 0.05 µg·mL⁻¹ when the cells reached confluence of 80 %. The cells were subsequently incubated at 37 °C in 50 mL·L⁻¹ CO₂ incubator for 40-48 h.

Enucleation and filtration Media was removed from culture and cells were washed twice with PBS. The flasks were filled to the neck with DMEM containing 20 µg·mL⁻¹ cytchalasin B which had been prewarmed at 37 °C. Flasks were sealed with parafilm, and then put into a R12A3 rotor (fixed-angle 28°) to centrifuge at 7 500×g at 36 °C for 75 min. Pellets were resuspended with DMEM. Suspension was serially filtered through 8 µm, 5 µm and 5 µm polycarbonate membranes. Microcells were observed and counted on hemacytometer under microscope. Microcells were collected again by centrifuging at 2 000×g at 4 °C for 20 min and thereafter resuspended with 2 mL of DMEM with 100 µg·mL⁻¹ PHA-P-A.

Microcell fusion & drug selection of microcell hybrids The recipient cell line CSF was trypsinized to make single cell suspension and counted on a hematocytometer for its cell density. CSF cells equivalent to 1/10 of microcells were taken and washed twice with PBS to get rid of remnant serum and thereby collected by centrifugation and resuspended with the PHA-P solution containing the microcells. The mixture was incubated at 37 °C for 15 min and subsequently centrifuged at 2 000×g for 15 min. Supernatant was removed as much as possible. Microcells were fused with the recipient cells for 30-60 s with 1 mL of 50 % PEG, 10 % DMSO in DMEM. Fusion reaction was terminated immediately by adding 10 mL of DMEM containing 5 % DMSO to remove the PEG medium. Pellets were resuspended with DMEM supplemented with 10 % FBS. The culture was recovered at 37 °C for 48 h and replaced with selective media of 1×HAT and 800 µg·mL⁻¹ G418. The selective medium was refreshed twice per week.

Single cell Isolation cloning [31] Viable cells in selective media of G418 and HAT were trypsinized to make single cell suspension and counted on a hematocytometer. Fifty to one hundred cells were added into a P100 culture plate with serum free DMEM. Single cells were picked up with a P20 pipette under microscope in laminar flow. It was assured that there was only one cell in view under a low-power objective to exclude the possibility of aspirating more than one cell each time. The opening of tip was pointed by the side of the selected cell and 5 µL media was aspirated each time. The single cell could be seen disappear into the tip under the low magnification microscope. Single cells were added to 96-well cell culture plate containing 0.1 mL of selective media (DMEM supplemented with 20 %FBS containing 1×HAT and 800 µg·mL⁻¹ G418) in each well. After about 10 days, clones that were actively proliferating were picked up and transferred to a 24-well plate. After about 7 days, cells were transferred again to T25 flask. When cells reached large quantity, they were frozen down in liquid nitrogen to keep in stock.

Genomic DNA extraction from cell lines [32] About 1×10⁶ cultured cells were harvested by trypsinization and centrifugation, into which 0.5 mL of cell lysis buffer (100 mmol·L⁻¹ NaCl, 1 mmol·L⁻¹ EDTA, 0.5 %SDS, 50 mmol·L⁻¹ Tris-HCl, pH 8.0) and 10 µL of Proteinase K (2 g·L⁻¹) were added. The mixture was incubated at 37 °C in water bath for 6 h. Genomic DNA was extracted with phenol and chloroform, precipitated with ethanol, dissolved with 20 µL of sterile dd-H₂O, quantitated by spectrophotometer, diluted to 10 µg·µL⁻¹ with sterile dd-H₂O, and finally aliquoted and stored at -20 °C.

STS-PCR [31,32] A human STS, D8S277, which located at 8p23.3-p22, was randomly chosen to confirm the result of MMCT. Primers were designed according to the published sequences on NCBI UniSTS database (http://www.ncbi.nlm.nih.gov/genome/sts/). Forward primer: CCAGGGTAGTATATCAATCTTGAG; reverse primer: TGAGAGGGCAGATGACCTCC. PCR product size: 148-180 (bp). PCR reaction solution (10 mmol·L⁻¹ Tris, 50 mmol·L⁻¹ KCl, 2 mmol·L⁻¹ MgCl₂, 0.001 % Gelatin, 200 mmol·L⁻¹ dNTPs, 0.5 mmol·L⁻¹ primers and 2 U of Taq polymerase) was added into 50 ng of DNA template for each sample respectively. PCR program was run as: 94 °C 2 min, 1 cycle; 95 °C 40 s 60 °C 40 s 72 °C 40 s, 35 cycles in all; 72 °C 4 min, 1 cycle; keep at 4 °C. Results were checked on 2 % agarose gel electrophoresis stained with ethidium bromide.

Chromosome slide preparation [35] Microcell hybrids at logarithmic phase were treated with colcemid at a final concentration of 0.02 µg·mL⁻¹ for 45 to 60 min at 37 °C and trypsinized subsequently to make single cell suspension. Cells were pelleted and then exposed to 5 mL of hypotonic solution (0.075 M KCl) by incubating for 10 to 15 min at 37 °C in a water bath. Chromosomes were repetitively fixed with methanol: glacial acetic acid (3:1, volume ratio) for 3 times, and finally resuspended with 0.5-1 mL of fixative. Three to four drops were dropped evenly on a cold wet slide, which was allowed to dry later. It was examined under low magnification phase objective (10× or 16×) to check the cell density and spread of metaphase chromosomes.

WCP-FISH [41] WCP-FISH was performed according to the protocol provided by Vysis Inc (IL, USA). Briefly chromosome slides were immersed in denaturation solution (700 mL·L⁻¹ formamide/2×SSC) at 73 °C±1 °C in water bath for 5 min and subsequently dehydrated serially in 700 mL·L⁻¹, 850 mL·L⁻¹ and 1 000 mL·L⁻¹ ethanol and dried at 45 °C-50 °C. The WCP probe mix (7 µL of WCP hybridization buffer, 2 µL of purified H₂O and 1 µL of WCP paint DNA probe for human chromosome 8 Spectrum Green™) was incubated at 73 °C±1 °C in water bath for 5 min and placed at 45 °C-50 °C ready for use. The probe mix was applied to the target area and coverslip was immediately applied which was sealed with rubber cement. Slides were placed in a prewarmed humidified box, which were put into a 37 °C incubator for 16 h. Coverslips were removed from slides, which were immediately immersed in 0.4×SSC/ 3 mL·L⁻¹ NP-40 prewarmed to 73 °C±1 °C agitating for 1-3 s. Slides were removed after 2 min and immersed into 2×SSC/ 1 mL·L⁻¹ NP-40 at ambient temperature agitating for 1-3 s and removed after 5 s to 1 min. Slides were air dried in darkness. 10 µL of DAPI counterstain was applied to the target area of slide and coverslip was applied. Slides were viewed using DAPI/Green Vysis filter set on an optimally performing fluorescence microscope and images were captured using CytoVision™ Chromosome Analysis System (Applied Imaging).

RESULTS

Selective screening of microcell hybrids and single cell isolation cloning Three to four weeks after MMCT, viable floating cells were
observed among dead cells in selective media containing G418 and HAT. Figure 1. The viable cells had good refraction and round shape while the dead cells had poor refraction and morphologic shrinkage.

Figure 1 Double selection of microcell hybrids in HAT and G418 (10×25 magnification).

Fifteen microcell hybrid clones were obtained by single cell isolation cloning, which were named neo8/C5F-1~15 respectively. It took about 4 weeks for the progress of enlargement culture serially from 96- to 24-well cell culture plate and finally to T25 flask. Feeder cells are proven unnecessary for the single cell isolation cloning of C5F microcell hybrids. Figure 2 showed the microcell hyrid clone obtained in 96-well plate containing selective media of HAT (1×) and G418 (800 µg·mL⁻¹) one week after single cell isolation.

Figure 2 Single-cell isolation cloning in 96-well cell culture plate (10×25 magnification).

STS-PCR
The chromosome donor cell line A9 (neo8) was taken as positive control and the recipient cell line C5F as negative control. DNA extracted from A9 (neo8), C5F and microcell hybrids was used as template. PCR was performed with the primers for D8S277, which is a unique STS located on human chromosome 8p23.3-p22. PCR products, when checked on 2 % agarose gel electrophoresis, were found of the same length with those reported in UniSTS database of NCBI. Figure 3 showed that neo8/C5F-3 had the deletion of D8S277.

Figure 3 STS-PCR Amplification of D8S277. 1: chromosome donor cell line A9 (neo8); 2: recipient cell line C5F; 3-7: microcell hybrids neo8/C5F-1-5; M: 100bp ladders.

WCP-FISH
WCP-FISH was performed using whole chromosome painting DNA probe for human chromosome 8 (WCP 8 probe SpectrumGreen™, Vysis) to confirm its presence. Figure 4 (a and b) showed that the human chromosome 8 had been successfully introduced into rat liver cancer cell line. Meanwhile, the recombination of human chromosome 8 with rat chromosome could be observed clearly by comparing the probe with its DAPI counterstain image. This recombination was found to be consistent in different metaphase cells.

Figure 4a WCP-FISH analysis of neo8/ C5F microcell hybrids.

Figure 4b WCP-FISH of neo8/ C5F microcell hybrids DAPI counterstain.

DISCUSSION
Metastasis is a major problem puzzling both specialists of cancer biology and of clinical oncology. Thousands of cancer patients die of it each year while clinicians can do little to deal with it. The main reason is that the molecular mechanisms of metastasis have not been totally clarified yet. Researchers fascinated by metastasis hope that the discovery of metastasis suppressor genes could shed light on the solution of this problem, while hitherto few of them have been discovered[17,18].

Metastasis suppressor genes suppress the formation of spontaneous, macroscopic metastases without affecting the growth rate of the primary cancer. Until presently, only five genes, nm23, KAI1, KISS1, MKK4 and BrMS1, have been proved to meet this criteria[17,18]. Despite the first metastasis suppressor gene nm23 was discovered by subtractive hybridization, most of encoding regions of metastasis suppressor genes have been discovered with the methodology of MMCT[18], which is thought to be the methodology particularly suitable for the functional localization of metastasis suppressor genes[19]. MMCT is established on somatic cell
All in all, the human chromosome 8 has been successfully transferred into the highly metastatic rat liver cancer cell line C5F, which builds solid basis for future researches on the discovery of metastasis suppressor genes for liver cancer.

ACKNOWLEDGEMENTS

We are much grateful to Dr. Kumiko Ogawa (First Department of Pathology, Nagoaya City University, Japan) for his generous offer of C5F cell line, Prof. Hong-Xuan Lin (Plant Physiology and Ecology Institute, Chinese Academy of Science, Shanghai, China) for his warmhearted provision and assistance in the usage of Hitachi high speed centrifuge and Dr. M.Z.Zdzienicka and Wouter Wiegant (Leiden University Medical College, Netherlands) for kindly providing us the detailed protocol of MMCT.

REFERENCES

1. Tang ZY. Hepatocellular carcinoma- cause, treatment and metastasis. World J Gastroenterol 2001; 7: 445-454
2. Tang ZY. Hepatocellular carcinoma, J Gastroenterol Hepatol 2000; 15 (Suppl): G1-G7
3. Tang ZY, Yu YQ, Zhou XD, Ma ZC, Wu ZQ. Progress and prospects in hepatocellular carcinoma surgery. Ann Chir 1998; 52: 558-563
4. Qin LX, Tang ZY. The prognostic significance of clinical and pathological features in hepatocellular carcinoma. World J Gastroenterol 2002; 8: 193-199
5. Qin LX, Tang ZY. The prognostic molecular markers in hepatocellular carcinoma. World J Gastroenterol 2002; 8: 385-392
6. Qin LX, Tang ZY, Sham JS, Ma ZC, Ye SL, Zhou XD, Wu ZQ, Trent JM, Guan XY. The association of chromosome 8p deletion and tumor metastasis in human hepatocellular carcinoma. Cancer Res 1999; 59: 5662-5665
7. Ogawa K, Nakashima H, Takeshita F, Futakuchi M, Asamoto M, Imaida K, Tatematsu M, Shirai T. Establishment of rat hepatocellular carcinoma cell lines with differing metastatic potential in nude mice. Int J Cancer 2001; 91: 797-802
8. Fournier RE. A general high-efficiency procedure for production of microcell hybrids. Proc Natl Acad Sci U S A 1981; 78: 6349-6353
9. McNeill CA, Brown RL. Genetic manipulation by means of microcell-mediated transfer of normal human chromosomes into recipient mouse cells. Proc Natl Acad Sci U S A 1980; 77: 5394-5398
10. Kraakman-van der Zweit M, Overkamp WJ, Jaspers NG, Natarajan AT, Lohman PH, Zdzienicka MZ. Complementation of chromosomal aberrations in AT/NBS hybrids: inadequacy of RDS as an endpoint in complementation studies with immortal NBS cells. Mutat Res 2001; 485: 177-185
11. Cowell JK. Single-cell Isolation Cloning. Human Chromosome Principles and techniques, Verma RS and Babu A, eds. 2nd ed. Mc Graw-Hill Inc USA 1995: 37-38
12. Sambrook J, Russell DW. Molecular cloning: A laboratory manual. 3rd ed. New York: Cold Spring Harbor Laborator Press 2001: 542-545
13. Schuler GD. Electronic PCR: bridging the gap between genome mapping and genome sequencing. Trends Biotechnol 1998; 16: 456-459
14. Nelson DL. Applications of polymerase chain reaction methods in genome mapping. Curr Opin Genet Dev 1991; 1: 62-68
15. Verma RS, Babu A. Human Chromosome Principles and techniques. 2nd ed. Mc Graw-Hill Inc USA 1995: 12-13
16. Jenkins RB, Le Beau MM, Kraker WJ, Borell TJ, Stalboerger PG, Davis EM, Penland L, Fernald A, Espinosa R 3rd, Schaid DJ. Fluorescence in situ hybridization: a sensitive method for trisomy 8 detection in bone marrow specimens. Blood 1999; 73: 3307-3315
17. Welch DR, Rinker-Schaeffer CW. What defines a useful marker of metastasis in human cancer? Natl Cancer Inst 1990; 91: 1330-1353
18. Yoshida BA, Sokoloff MM, Welch DR, Rinker-Schaeffer CW. Metastasis-suppressor genes: a review and perspective on an emerging field. J Natl Cancer Inst 2000; 92: 1717-1730
19 Mashimo T, Watabe M, Cuthbert AP, Newbold RF, Rinker-Schaeffer CW, Helfer E, Watabe K. Human chromosome 16 suppresses metastasis but not tumorigenesis in rat prostatic tumor cells. Cancer Res 1998; 58: 4572-4576

20 Ramshaw IA, Carlsson S, Wang HC, Badenoch-Jones P. The use of cell fusion to analyze factors involved in tumor cell metastasis. Int J Cancer 1983; 32: 471-478

21 Overhauser J. Somatic Cell Hybrid Mapping Panels: Resources for Mapping Disease Genes. In: Human Genome Methods, Adolph KW ed. CRC Press LLC USA 1998: 258-264

22 Dong JT, Lamb PW, Rinker-Schaeffer CW, Vukanovicj, Ichikawa T, Issacs JT, Barrett JC. KAI1, a metastasis suppressor gene for prostate cancer on human chromosome 11p11.2. Science 1995; 268: 884-886

23 Chekmareva MA, Hollowell CM, Smith RC, Davis EM, LeBeau MM, Rinker-Schaeffer CW. Localization of prostate cancer metastasis suppressor activity on human chromosome 17. Prostate 1997; 33: 271-280

24 Nihei N, Kouprina N, Larionov V, Oshima J, Martin GM, Ichikawa T, Barrett JC. Functional evidence for a metastasis suppressor gene for rat prostate cancer within a 60-kilobase region on human chromosome 8p12. Cancer Res 2002; 62: 367-370

25 Ichikawa T, Ichikawa Y, Dong J, Hawkins AL, Griffin CA, Issacs WB, Oshimura M, Barrett JC, Isaacs JT. Localization of metastasis suppressor gene(s) for prostate cancer to the short arm of human chromosome 11. Cancer Res 1992; 52: 3486-3490

26 Yoshida BA, Dubauskas Z, Chekmareva MA, Christiano TR, Stadler WM, Rinker-Schaeffer CW. Mitogen-activated protein kinase kinase 4/ stress-activated protein/ Erk kinase 1 (MKK4/ SEK1), a prostate cancer metastasis suppressor gene encoded by human chromosome 17. Cancer Res 1999; 59: 5483-5487

27 Miele ME, Jewett MD, Goldberg SF, Hyatt DL, Morelli C, Gualandi F, Rimessi P, Hicks DJ, Weissman BE, Barbanti-Brodano G, Welch DR. A human melanoma metastasis-suppressor locus maps to 6q163-q23. Int J Cancer 2000; 86: 524-528

28 Lee JH, Miele ME, Hicks DJ, Phillips KK, Trent JM, Weissman BE, Welch DR. KiSS-1, a novel human malignant melanoma metastasis-suppressor gene. J Natl Cancer Inst 1996; 88: 1731-1737

29 Mashimo T, Goodarzi G, Watabe M, Cuthbert AP, Newbold RF, Pai SK, Hirota S, Hosobe S, Miura K, Bandyopadhyay S, Gross SC, Watabe K. Localization of a novel tumor metastasis suppressor region on the short arm of human chromosome 2. Genes Chromosomes Cancer 2000; 28: 285-293

30 Goodarzi G, Mashimo T, Watabe M, Cuthbert AP, Newbold RF, Pai SK, Hirota S, Hosobe S, Miura K, Bandyopadhyay S, Gross SC, Balaji KC, Watabe K. Identification of tumor metastasis suppressor region on the short arm of human chromosome 20. Genes Chromosomes Cancer 2001; 32: 33-42

31 Seraj MJ, Samant RS, Verderame MF, Welch DR. Functional evidence for a novel human breast carcinoma metastasis suppressor, BRMS1, encoded at chromosome 11q13. Cancer Res 2000; 60: 2764-2769

Edited by Zhang J