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World J Gastroentero, 2001;7(3):335 - 339
World Journal of Gastroenterology
 ISSN 1007 - 9327

Alterations in metastatic properties of hepatocellular carcinoma cell following H-ras oncogene transfection

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Subject headings liver neoplasms/pathology; carcinoma, hepatocellular/pathology; genes, ras; neoplasm metastasis

Wang Q, Lin ZY, Feng XL. Alterations in metastatic properties of hepatocellular carcinoma cell following H-ras oncogene transfection. ***World J Gastroenterol***, 2001;7(3):335-339

Abstract

AIM To demonstrate the relationship between H-ras oncogene and hepatocellular carcinoma (HCC) metastasis.

METHODS Activated H-ras oncogene was transfected into SMMC 7721, a cell line derived from human HCC, by calcium phosphate transfection method. Some metastasis related parameters were detected in vitro, including adhesion assay, migration assay, expression of collagenase IV (c IV ase) and epidermal growth factor receptor (EGFR).

RESULTS The abilities of H-ras-transfected cell clones in adhesion to laminin (LN) or fibronectin (FN), migration, c IV ase secretion increased markedly, and the expression of EGFR elevated moderately. More importantly, these alterations were consistent positively with the expression of p21, the protein product of H-ras oncogene.

CONCLUSION H-ras oncogene could induce the metastatic phenotype of HCC cell in vitro to raise its metastatic potential.

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Received 2001-02-06 **Accepted** 2001-02-12

INTRODUCTION

Hepatocellular carcinoma (HCC) is common in China[1-6], and metastasis occurs early with poor prognosis[7-13]. Numerous studies on various human solid tumors have shown that H-ras oncogene is associated with tumor metastasis[14-21]. However, therelationship between H-ras and HCC metastasis remains an open question[22-25]. In the present study, we transferred activated H-ras genes into SMMC 7721, a cell line derived from human HCC, by the method of calcium phosphate transfection. The metastatic properties of ras-transfected clones were detected *in vitro*. This research was conducted to investigate the influence of H-ras oncogene on the metastatic characteristics of this liver cancer cell line from each link in the chain of tumor metastasis: adhesion-degration-migration, in order to reveal the relationship between H-ras oncogene and metastatic behavior of HCC cell.

METERIALS AND METHODS

Materials

Carrier plasmid pSV_2-neo and recombinant plasmid pSV_2-neo-*ras* (with activated H-*ras* DNA inserted at BamH I site) were gifts from Professor Luo, Director of Department of Biophysics, Fudan University. Human HCC cell line SMMC 7721 was provided by the Liver Cancer Institute, Zhongshan Hospital. Calcium phosphate transfection kit was purchased from Promega Company. DACO $p21^{ras}$ antibody, purchased from Sigma Company, could recognize specifically the 126-140 amino-acids of C-terminal. Antibodies of c IV ase and EFGR were products of Oncogene Company.

Methods

Transfer of recombinant plasmid into SMMC 7721 The method of calcium transfection, was used according to the protocol in the kit. Transfected clones were selected by G418.

Southern blotting The presence of the transfected ras oncogene in the DNA of the clones was assessed by Southern blot. Briefly, total DNA was digested with *Bam* H I, separated by electrophoresis in a 8 $g \cdot L^{-1}$ agarose gel, and transferred to nitrocellulose. The filter was then probed with H-*ras*-T_{24} DNA (6.6kb *Bam* H I fragment of plasmid pT_{24}) which had been radiolabeled with [^{32}P]dCTP.

Following hybridization, the filter was washed and X-rayed.

Immunochemistry The avidin-biotin-peroxidase complex (ABC) method was employed to detect the expression of c IV ase, EFGR and p21. The results were graded according to the percentage of positively stained cells: - less than 5%; + 5%-25%; ++ 25%-50%; +++ 50%-75%; and ++++ above 75%.

Cell adhesion assay The cell adhesion assay was performed as previously described by Busk *et al*. In brief, some wells of polystyrene 96-well flat-bottom microtiter plates were coated with increasing concentrations of laminin (LN) or fibronectin (FN), and additional wells with poly-lysine (positive control) or 10 g·L^{-1} bovin-serum albumin (BSA) (negative control) respectively. Cells were added after all the plates were blocked with 10 g·L^{-1} BSA. The plates were then incubated for 2 h at 37 ℃ in humidified CO_2. Non-adherent cells were removed and the attached cells were fixed and stained. The relative number of cells in each well was evaluated by measuring the absorbance (A) at 595 nm with a Microplate Reader. The percentage of cells attached to the experimental wells was calculated according to the formula as follows:

$$\frac{A\text{ (experimental well) - A(mean of BSA-coated wells)}}{A\text{ (mean of poly-lysine well) -A (mean of BSA-coated wells)}} \times 100\%$$

The data were expressed as $x \pm s_x$ of triplicate wells.

Cell migration assay The wound assay described by Birch *et al* was used to determine the random migration capacity of various clones. Cells were plated into the wells of 24-well plates and incubated until the cultures were subconfluent. A wound track (approximately 4 mm in size) was scored in each well. Replicate wells were terminated at 8, 16 and 24 h after wounding by fixing and staining the cell cultures with 10 g·L^{-1} crystal violet in methanol. The stained cells were then examined under an inverted microscope.

RESULTS

Identification of transfected cell clones

The four clones transfected with recombinant plasmid pSV_2-neo ras (named RC_1, RC_2, RC_3 and RC_4) and the two clones transfected with carrier plasmid pSV_2-neo (named NC_1 and NC_2), along with SMMC 7721, were tested for both Southern blot analysis and p21 expression. The presence of the transfected ras oncogene in RC_1-RC_4 was confirmed by Southern blotting, while it was absent in NC_1, NC_2 and SMMC 7721. The immunochemistry staining showed that the percentage of positive stained cells of RC_1-RC_4 was 71%, 76%, 55% and 49%, respectively. However, it was less than 5% in SMMC 7721, NC_1 and NC_2. The staining grade of these cell clones is presented in Table 1. The results showed that H-*ras* DNA had been transferred into SMMC 7721 successfully and it could express its protein product normally.

Table 1 Expression of p21, c IV ase and EGFR in different cell clones

	Staining grade						
	SMMC 7721	NC_1	NC_2	RC_1	RC_2	RC_3	RC_4
p21	-	-	-	+++	++++	+++	++
c IV ase	+	+	+	+++	++++	+++	+++
EGFR	+	+	+	+++	+++	++	++

Detection of metastasis-related parameters

Adhesive ability When ras transfected clones were assessed for their ability to bind LN or FN, it was found that there was a substantial difference in the adhesive capabilities of these variants (Figure 1). The attachment percentage of RC_1-RC_4 to LN increased by different degree as compared with SMMC 7721, the maximal was up to 69.7%, 74.4%, 38.5% and 55% respectively. Similar results were observed in adhesion assay to FN. The adhesive capabilities of NC_1 and NC_2 had no significant difference from that of SMMC 7721, suggesting that the carrier plasmid itself had no effect on cell metastatic properties.

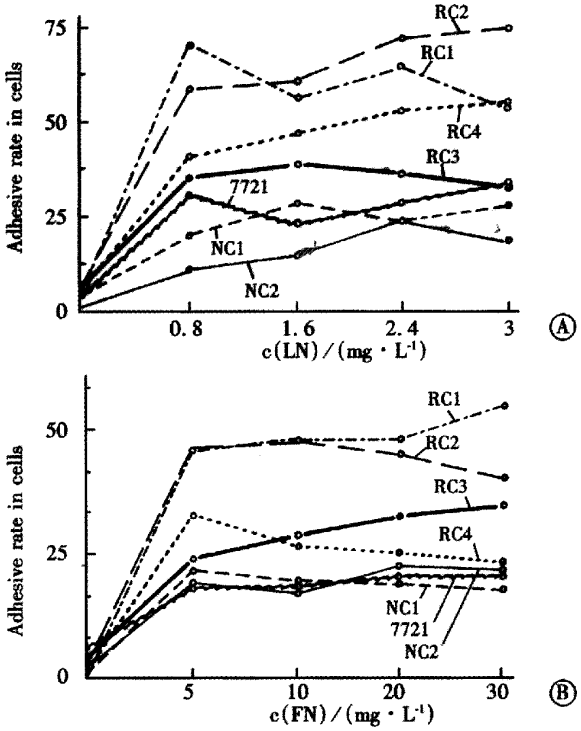

Figure 1 Attachment of different cell clones to LN or FN ($x \pm s_x$).
A: To increasing concentrations (0, 0.8, 1.6, 2.4 and 3mg·L^{-1}) of LN
B: To increasing concentrations (0, 5, 10, 20 and 30 mg·L^{-1}) of FN

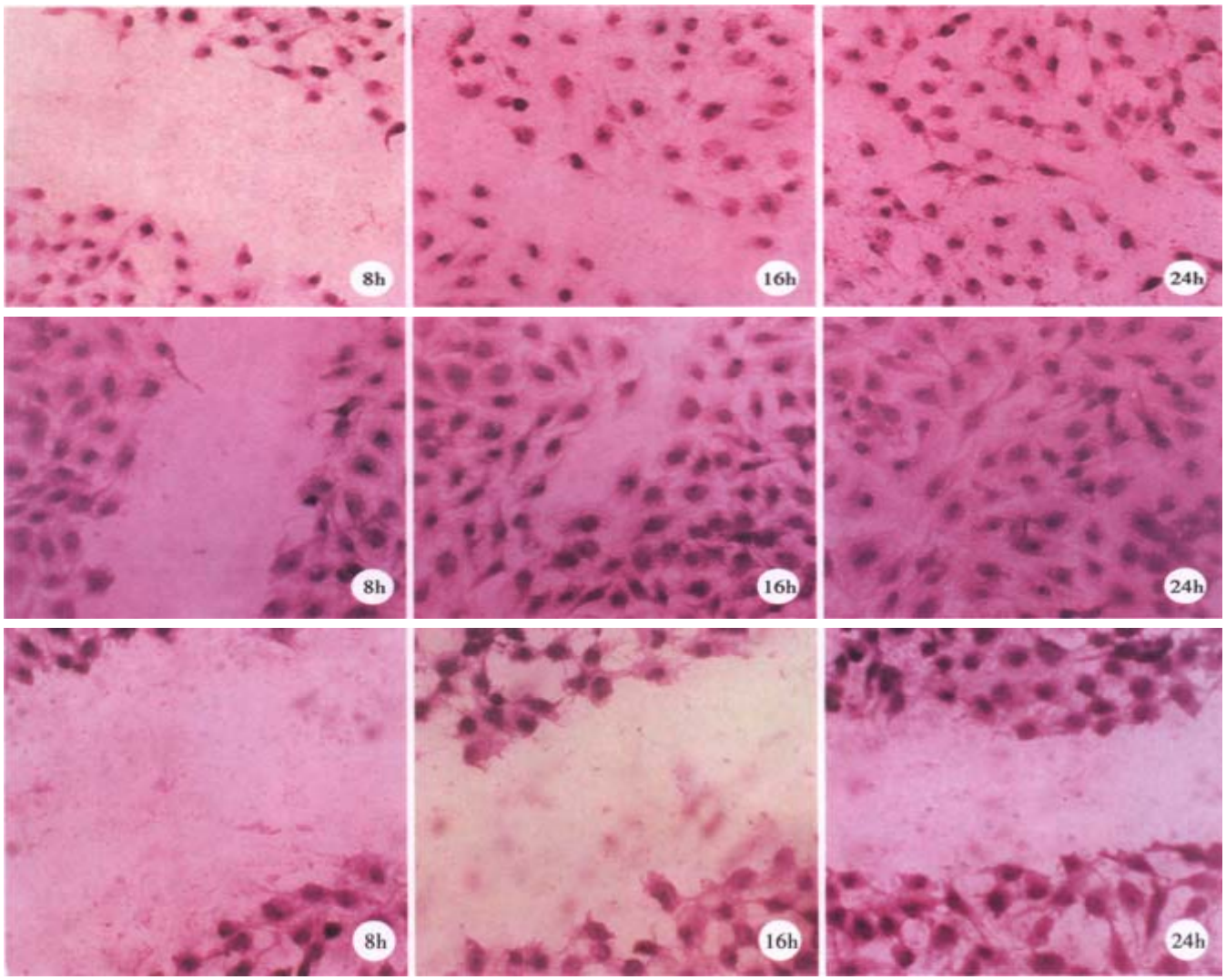

Figure 2 Migration ability of representative clones. Subconfluent monolayers of the clones were "wounded" at time 0. The cells were allowed to migration into the cell-free area for 24 h then fixed and stained with crystal violet.
A: RC_1; B: RC_2; C: SMMC 7721

Migration assay The migration of the different clonal lines was analyzed by using the "wound" system *in vitro*. Wounds of approximately4mm were made in subconfluent monolayers of the different clones and cells were allowed to migrate into the cell-free area over a 24 h period. Representative experiments using three clones are illustrated in Figure 2. The cell-free areas were filled up with cells within 24 h in the tests of RC_1-RC_4, but they still remained empty even after 24 h in the tests of SMMC 7721, NC_1 and NC_2.

Expression of c IV aes and EGFR The expressions of c IV aes and EGFR were significantly different before and after ras transfection (Table 1) and these alterations were consistent positively with the expression of p21.

DISCUSSION

The process of tumor invasion and metastasis can be divided into three steps at molecular level: adhesion, degration and migration. This consecutively complex process involves many kinds of cytokines, enzymes and cell surface receptors [26-29]. Ras gene has been implicated in these processes through the signal transduction pathway [30-42]. In malignant tumors, cell-matrix interactions are very important for tumor invasion and metastasis. LN and FN, major components of the basement membrane, are involved in several biologic activities. We investigated the adhesive abilities of H-*ras* transfected SMMC 7721 cells to LN and FN. The results showed that the adhesive abilities of different cell clones raised in different

degree. The reason that the adhesive ability of RC_3 to LN had no significant increase as against SMMC 7721, may be contributed to the heterogeneity of transfected clones. Some data have shown that the property of transfected clones is not expressed stably and that heterogeneity may develope during the growth of the clones. Experimental studies with several different tumors have suggested that the instability causing the heterogeneity of metastatic properties is due to a variety of genetic and epigenetic processes.

c IV ase is also associated with tumor metastasis[43]. It is considered that activated or overexpressed H-*ras* gene can induce the secretion and synthesis of c IV ase directly. Ura *et al* revealed that the transcription level of c IV ase gene was obviously higher in BEAS-2B cells transformed by H-*ras* gene than in their parent cells. The c IV ase secretion ability of these cells increased, and metastatic behavior *in vitro* was positively related to c IV ase secretion *in vivo*. Our results showed that the c IV ase expression level increased markedly following *ras*-transfection, the percentage of positively stained cells increased 2 to 5 times after transfection. It was indicated that oncogenic p21 ras may upregulate translational efficiency by activating the eukaryotic translation initiation factor 4E (eIF-4E), thereby enhancing the protein expression of ras-induciable genes.

EGFR is known to be interrelated with and interact on ras gene in cell signal transduction pathway[44-47]. It has some effects on tumor cell attachment, secretion of proteolytic enzymes, cytoskeleton structure and cell migration[48-52]. We found that the expression of EGFR in ras-transfected clones increased moderately, and the expressions of EGFR and c IV ase had definite relevance to p21 expression.

In summary, we have demonstrated that H-*ras* oncogene can induce the metastatic phenotype of human HCC cell *in vitro*, to raise its metastatic potential. The detections of some metastasis-related parameters, such as cell adhesion ability, migration ability, expressions of c IV ase and EGFR may have predictive value in the metastatic potential of HCC clinically.

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Edited by Ma JY