

Correlation between metastatic potential and variants from colorectal tumor cell line HT-29

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Abstract

AIM: To evaluate the relationship between uPA, PAI-1, CEA, PI3K and metastatic potential in three colorectal tumor cell lines.

METHODS: Metastatic model in nude rats was established by variants HT-29c and HT-29d cell lines and the metastatic potential of two tumor cell variants was compared. Urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor type 1 (PAI-1) were determined using ELISA in colorectal carcinoma WiDr, HT-29 and HT-29d cell lines with different metastatic potentials. Expression of carcinoembryonic antigen (CEA) and phosphoinositide 3-kinase (PI3-Kinase) was analyzed using immunohistochemistry (IHC) in these cell lines *in vitro* and *in vivo*. CEA expression was compared using fluorescence activated cell sorter (FACS) *in vitro*.

RESULTS: The number of HT-29d cells arrested in liver dramatically decreased within the initial 24 hours after injection. The taking rate of liver metastases in the variant HT-29d increased as compared with parental HT-29 cells (70 % versus 50 %) and a variant HT-29b cells (70 % versus 60 %), and extensive organs were synchronously involved in metastases. The uPA concentration of variant HT-29d cell line was significantly higher than that of the non-metastatic WiDr and the low metastatic HT-29 cell lines. The variant HT-29d cells produced stronger PI3-kinase expression as compared with the non-metastatic WiDr cells and the low metastatic HT-29 cells *in vivo*.

CONCLUSION: The selected variant HT-29d cell exhibited an enhanced metastatic potential. The level of uPA and PAI-1 is positively correlated with the metastatic capacity of tumor cells. The expression of PI3-kinase correlates with tumor development and metastasis.

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INTRODUCTION

The malignancy of a solid tumor is due to its ability to invade and metastasize. Metastasis is a major cause of cancer death. Cancer metastasis consists of multiple sequential and selective steps. The aggressiveness of a tumor is primarily dependent on its ability to invade adjacent tissues and to metastasize to distant sites. In recent years special attention has been paid to tumor-associated protease systems, such as urokinase-type plasminogen activator (uPA)^[1]. A role of uPA in regulating tumor cell invasiveness has been proposed on the basis of generally increased uPA activity in several metastatic tumors^[2,3]. For many malignant tumors, there is a significant correlation between the production of uPA and tumor invasion^[4,5]. The activation of uPA is controlled by well-characterized plasminogen activator inhibitors: type 1 (PAI-1). Numerous studies have shown that PAI-1 antigen levels in primary tumor with lymph node involvement were significantly higher than those in cancers without lymph node involvement^[6,7], and in metastatic lymph nodes their levels were also increased compared to primary tumors^[8].

Carcinoembryonic antigen (CEA) is one of the most common tumor-associated substances produced by colorectal carcinoma, and it has been used in numerous studies as a tumor marker. Although the functional role of CEA in liver metastasis from colorectal carcinomas has been assumed to be an adhesion factor or receptor binding to Kupffer cells^[9,10], a conclusive evidence *in vivo* has not been clarified yet.

Phosphoinositide 3-kinase (PI3-kinase) is a key signaling enzyme implicated in a variety of receptor-stimulated cell responses^[11]. This lipid product is believed to act as the second messenger in a variety of signaling processes including cell survival and migration^[12,13]. Numerous studies have implicated PI3K in signal transduction pathways correlated with cell proliferation, cell cycle progression, cell apoptosis, tumorigenesis, tumor angiogenesis and tumor invasiveness^[14,15].

In this study, uPA, PAI-I, CEA and PI3K were determined in three colorectal cancer cell lines with different metastatic capacities *in vitro* and *in vivo*, and the relationship between metastatic potential and these biological factors in colorectal tumor was investigated.

MATERIALS AND METHODS

Experimental animals

Three-week old male athymic Rowett nude rats (Hsd:RH-nu/nu) were obtained from Harlan/Winkelmann (Borchen, Germany). All rats were housed under special pathogen-free conditions in a laminar flow cabinet (EHRET, DIPL.-ING. W. EHRET GmbH, Germany) with constant temperature (24-26 °C), humidity (40-50 %) and 12-hour light/12-hour dark cycle. The rats were fed with standard rat food and water. All cages, bedding and operative equipments were autoclaved at 121 °C for 30 minutes. The nude rats were stabilized for one week in the laboratory before the experiments were started.

Cell lines and cell culture

Human colonic adenocarcinoma cell lines WiDr and HT-29

as well as their variants HT-29c and HT-29d were used in the present study. WiDr cell line was established from a moderately differentiated human primary rectosigmoid adenocarcinoma with non-distant metastatic capability and obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). HT-29 cell line was established from a moderately differentiated human colon adenocarcinoma and was a gift of Dr. Dippold (Mainz, Germany). HT-29c and HT-29d cell lines were two variant cell lines after three and four cycles of selection of liver metastases from parental HT-29 cells^[16].

All the cell lines were grown in RPMI-1640 medium (Life Technologies, Eggenstein, Germany) plus 10 % fetal bovine serum (FCS, Life Technologies) supplemented with 2 mM L-glutamine and 1 mM sodium pyruvate (Life Technologies) and maintained in a 37 °C incubator (Heraeus, Germany) with 100 % relative humidity and 5 % CO₂. Cells were seeded and grown as monolayers in 75 cm² culture flasks (Falcon, England).

Establishment of metastatic model

Cells were harvested from exponential growth phase by brief trypsinization. The number of cell concentration was determined using a hemocytometer (Marienfeld, Germany). The cell suspensions were resuspended in HBSS at a final concentration of 4×10⁷ cells/ml. A total of 12 rats were anesthetized, an abdominal incision about 2 cm long was made in midline and the portal vein and mesenteric vein were isolated and exposed. Single-cell suspension of 0.5 ml containing 2×10⁷ cells of HT-29c or HT-29d was injected into the mesenteric vein of 2 or 10 rats, respectively. The rats were explored for the first time about 4 weeks after injection and killed and autopsied at 6-10 weeks or when moribund after injection. The liver, lung and other organs were removed and frozen in liquid nitrogen, then stored at -80 °C for IHC.

To investigate the distribution and fate of tumor cells in the liver at the initial 48 hours after injection, 3-4 rats were killed at 1, 24, 48 hours after injection, respectively. The livers were removed, frozen in liquid nitrogen immediately, then the liver tissues were sliced at 5 µm cryosections and analysed for tumor cells by IHC with anti-CK mAb KL-1.

Quantitative detection of uPA and PAI-1

Six-well tissue culture plates were used. The single cell suspension of WiDr cells, HT-29 cells and HT-29d cells containing 3×10⁵ tumor cells in supplemented RPMI-1640 was seeded into each well, respectively, and incubated at 37 °C in a humidified 5 % CO₂ incubator. After incubation for 24 hours, the medium of each well was sucked away and freshly supplemented RPMI-1640 medium (1.5 ml/per well) was added. The cells were incubated under the same conditions as above for 24 and 48 hours, respectively. Then the medium of each well was removed into Eppendorf tubes and centrifuged at 10 000 rpm for 15 minutes at 4 °C. The supernatant was for detection of uPA and PAI-1. To measure the concentration of uPA and PAI-1 antigen in tumor cell culture supernatants, the uPA and PAI-1 ELISA kits (Imubind, American Diagnostica, Greenwich, CT, USA) were used. The sample value was multiplied by the dilution factor to determine the uPA and PAI-1 concentration of the cell culture supernatant. Meanwhile, the cells in six-well tissue culture plates were harvested by brief trypsinization and cell number was determined using a hemocytometer.

Determination of PI3-kinase expression

Tumor cells were seeded on 10-well mask slides at a density of 2×10⁵ cells/ml supplemented medium and incubated at 37 °C in an incubator with 100 % relative humidity and 5 %

CO₂ as described above. 48 hours later, the slides were washed twice with PBS and fixed in cold acetone for 5 minutes. Tumor tissue samples were cut into 5 µm cryosections and placed on microscope slides. The 10-well mask cell slides or tissue cryosections were fixed in cold acetone (4 °C) for 5 minutes. The rabbit polyclonal antibody against PI3-kinase p85α and P110α (Santa Cruz Biotechnology) was used for immunohistochemical evaluation. The standard ABC immunostaining method was employed as immunohistochemical staining with ABC kit (Vector Laboratories, CA, USA). A semiquantitated score of 5 categories based on color intensity was assigned as -: no reaction, +: weak positive reaction, ++: moderate positive reaction, +++: strong positive reaction. The percentage of positive tumor cells was determined by calculating 1 000 tumor cells in 5 random areas of one section.

CEA determination

FACS analysis of cell lines WiDr, HT-29 and HT-29d cells were harvested from exponential growth phase with 0.002 % EDTA in PBS and inactivated with supplemented medium, then centrifuged at 1 410 rpm for 5 minutes. The pellet was washed in Na-azide-PBS (7.5 mM) at 4 °C, and cells were counted with 5×10⁵ cells removed to each FACS tube. The cells were washed in 1 ml Na-azide-PBS and centrifuged again. The supernatant was sucked away, primary mAb to human CD66e (clone 85A12, 100 µg/ml, Cymbus Biotechnology, Chandlers Ford, Hants, UK) was diluted (2:3) with 3 % BSA in azide-PBS and added to each tube. The tube was vortexed and incubated on ice at 4 °C for 1 hour. One ml azide-PBS was added for inactivation and the cells were centrifuged. The supernatant was sucked away, the second antibody of dichlorotriazinyl amino fluorescein (DTAF)-conjugated affiniPure goat anti-mouse IgG+IgM (1.3 mg/ml, Dianova, Hamburg, Germany) diluted (1:100) with 3 % BSA in azide-PBS was added to each tube. The tube was vortexed and incubated on ice in the dark at 4 °C for 30 minutes. One ml azide-PBS was added for inactivation and the cells were centrifuged. The supernatant was sucked away, 300 µl of azide-PBS was added to each tube and vortexed, then 100 µl of 4 % paraformaldehyde was added. The samples were stored at 4 °C in the dark for FACS analysis. The primary antibody and secondary antibody were replaced by 3 % BSA in azide-PBS as negative controls, the primary antibody was replaced by 3 % BSA in azide-PBS as the second antibody control.

Immunohistochemistry analysis To investigate the CEA expression of different cell lines, s.c tumor and liver metastasis tumor, mouse monoclonal antibody to human CD66e were used for immunohistochemical evaluation. The standard ABC immunostaining method and a semiquantitated score were performed as described above.

Statistical analysis

The data were presented as mean ± standard deviation. Statistical analyses were performed using the *F* test and χ^2 test. Differences were considered significant if the *P*-values were <0.05.

RESULTS

Phenotype of HT-29 variants with enhanced tumorigenicity in vivo

The distribution and fate of tumor cells HT-29d in the liver at the initial phase after inoculation were observed in the rats of each group at different time intervals, respectively. The number of HT-29d cells arrested in the liver was dramatically decreased within the initial 48 hours after injection. By 1 hour after mesenteric vein injection, many tumor cells could be found in

Table 1 Macroscopical pattern of HT-29c/d cell metastases

Cell	Hepatic metastasis				Lung metastasis			Other organs
	No of metast ^a	I	II	III	No of metast ^a	I	II	
HT-29c	1/2	0/2	0/2	1/2	1/2	1/2	0/2	2/2
HT-29d	7/10	2/10	3/10	2/10	3/10	1/10	2/10	9/10

a: metastasis.

the livers, with a mean of 14.7 tumor cells/mm², and the single and clustered cells in the liver were located surrounding portal vein and in hepatic sinusoids. By 24 hours, however, the number of tumor cells surviving in the liver was significantly decreased. Only a few cells were found, with a mean of 2.3 tumor cells/mm² (range, 0-9 tumor cells/mm²), and the cells were located mostly in parenchyma of the liver. At 48 hours after injection, a similar tumor cell distribution was found.

Metastatic potential of HT-29c and HT-29d cells was studied by injecting tumor cells into the mesenteric vein of nude rats. All rats survived the surgery and tumor-cell injection. Exploration was performed at the fourth week after injection. The rats were killed 8 to 10 weeks after injection or when moribund. The metastases were determined either by macroscopy or by microscopy. Liver tumor burden was evaluated based on the number of lesions as Class I: histological evidence of tumor growth, Class II: <10 tumor foci (macroscopically), Class III: >10 tumor foci (macroscopically). Lung metastasis was evaluated as Class I: histological evidence of tumor growth, Class II: macroscopically positive. The results are shown in Table 1. One of the two rats injected HT-29c cells developed extensive hepatic metastases, in which 60 % of the right liver was occupied by tumor tissue. The other developed micrometastases in the lung, diaphragm, left kidney with hemorrhagic ascites. The two rats developed mesenteric metastases. Seven of the ten rats (70 %) injected HT-29d cells had either liver macrometastases or micrometastases. Still three of the ten rats (30 %) had lung metastasis, of which two developed extensively dispersed metastatic nodules in their lungs. Three of the ten rats (30 %) developed metastatic tumors in their thoracic cavity. Five developed metastatic tumors in the diaphragm. Two produced hemorrhagic ascites. Nine developed extensive mesenteric metastases within 8 to 10 weeks after injection of HT-29d. One suffered from hindlegs paralysis at day 30 after injection.

Level of uPA and PAI-1

The concentrations of uPA and PAI-I in cell supernatants were statistically significantly different among WiDr, HT-29 and HT-29d cells. The uPA and PAI-I concentrations of variant HT-29d cell line were significantly higher than those of HT-29 and WiDr cell lines at 24 hours (F value was 17.4, $P=0.03$) and at 48 hours (F value was 98.3, $P=0.001$). The uPA and PAI-I concentration of variant HT-29 cell line was also higher than that of WiDr cell line (Table 2).

Expression of CEA in cell lines and tumor tissues

The results by FACS showed that mAb CD66e could react with CEA on the surface of cells *in vitro*, but no differences were observed between WiDr, HT-29 and HT-29d cell lines. Their fluorescence intensities were 212, 215 and 207, respectively, (F value was 0.07, $P=0.93$).

CEA expressions among WiDr, HT-29 and HT-29d cell lines *in vitro* by IHC were not significantly different (χ^2 value was 2.311, $P=0.315$). However, HT-29d showed a slightly higher ratio of positive cells and stronger staining compared with WiDr and HT-29 in s.c. tumor, without significant difference (χ^2 value was 4.397, $P=0.111$). The ratio of positive

cells and staining intensity showed no significant difference in liver metastases between HT-29 and HT-29d (χ^2 value was 0.823, $P=0.364$) (Table 3).

Expression of PI3-kinase

For detection of expression of PI3-kinase, we stained PI3-kinase with Abs against p85 α and p110 α subunits of PI3-kinase in WiDr, HT-29 and HT-29d cell lines. Immunochemical reactivity with polyclonal antibodies p85 α and p110 α subunits of PI3-kinase was present among all tumor cell lines *in vitro*. No significant difference was observed in positive cell ratio and staining intensity among WiDr, HT-29 and HT-29d cell lines (χ^2 value was 2.041, $P=0.360$).

PI3-kinase expression of HT-29d showed stronger staining intensity compared to WiDr and HT-29 in s.c. tumor, and the ratio of positive cells of HT-29 and HT-29d was significantly higher than that of WiDr (χ^2 value was 6.161, $P=0.046$). HT-29d cells exhibited stronger staining and a slightly higher ratio than that of the parental HT-29 in liver metastatic tumor using mAb p85 α and p110 α (Table 3).

Table 2 uPA and PAI-1 level of different cell lines at 24 h

Cell	Level(pg/10 ⁵ cell)	
	uPA($\bar{x}\pm s$)	PAI-1($\bar{x}\pm s$)
WiDr	16.7 \pm 5.8	1 613 \pm 90
HT-29	80.01 \pm 8.1 ^a	2 650 \pm 1154 ^a
HT-29d	87.0 \pm 20.8 ^b	9 707 \pm 2450 ^b

^a $P<0.05$ vs WiDr, ^b $P<0.05$ vs HT-29.

Table 3 Expression of CEA and PI3-Kinase *in vitro* and *in vivo*

Cell lines and tumor	CEA		PI3-Kinase	
	Positive cell(%)	Intensity	Positive cell (%)	Intensity
WiDr	93 \pm 4.4	+++	99 \pm 0.8	++
HT-29	90 \pm 2.1	+++	98 \pm 1.6	++
HT-29d	94 \pm 3.0	+++	97 \pm 3.6	++
WiDr s.c. ^a	29 \pm 2.6	++	14 \pm 0.8	+
HT-29 s.c.	31 \pm 4.6	++	22 \pm 0.8	+
HT-29d s.c.	40 \pm 6.0	+++	23 \pm 2.2	++
HT-29 Lm ^b	71 \pm 7.9	+++	28 \pm 0.8	+
HT-29d Lm	66 \pm 7.0	+++	34 \pm 0.8	++

a: s.c.: s.c. tumor, b: Lm: liver metastases.

DISCUSSION

Tumor metastasis in the liver is a complex process of tumor-cell invasion into normal tissue on the one hand and the anti-invasive mechanisms of host defense system on the other hand. Lodgment of tumor cells in a blood vessel is an important step of tumor cell invasion. The survival of cancer cells after they enter the circulation depends upon their abilities to successfully reach the microcirculation, invade the capillary endothelium, establish a microenvironment for subsequent vascularization and growth. In the present study the capacity of arrested tumor

cells in the liver was observed in HT-29d cell line. One hour after tumor cell injection via the mesenteric vein, the number of tumor cells arrested in the liver was very high. Most tumor cells were located in the portal vein and its surrounding hepatic sinusoids. Furthermore, at 24 hours, the number of tumor cells surviving in the liver was significantly decreased, most tumor cells had been removed from the liver, the survived cells were located mostly in parenchyma of the liver. The remaining tumor cells were likely to be able to proliferate and to develop metastatic colonies. The results suggested that not all cells that entered the liver via hematogenous dissemination produced metastasis, the majority of circulating tumor cells died and only a minority of the cells escaped from host defense systems (e.g. NK cell activity in nude rats), arrested and survived in the liver could develop metastasis.

Neoplasms are heterogeneous with regard to invasion and metastasis, they contain a variety of subpopulations of cells with different metastatic potentials^[17]. The outcome of metastasis is to a large extent dependent on a selection process that favors the survival and growth of a special subpopulation of cells. Isolation of clonal populations of cells that differed from the parent neoplasm in their metastatic capacity supported the hypothesis that not all the cells in a primary tumor could successfully disseminate^[18]. The liver is the first and primary site affected by the hematogenous spread in colorectal carcinoma. In the present study, although only two rats were injected variant HT-29c cells with three selections *in vivo* into the mesenteric vein, still they showed a higher metastatic potential. One of the two rats developed extensive hepatic metastases, and the other developed synchronous multiple organ metastases. Both rats developed mesenteric metastases. The variant HT-29d with four-cycle selections had a higher metastatic efficiency in nude rats. The taking rate of liver metastases was increased as compared with parental HT-29 cells (70 % versus 50 %), and variant HT-29b cells with two-cycles selections (70 % versus 60 %)^[16]. Three of the ten rats developed lung metastasis, and extensive organs were synchronously involved in metastases. The results showed that HT-29d cells had more extensive metastatic potential than parental HT-29, variant HT-29b and HT-29c. The present study supported the hypothesis of metastatic heterogeneity. Pre-existing tumor cell subpopulations with heterogeneous metastatic capacity could be isolated from their parental neoplasms by means of cycles of selection *in vivo*. The availability of selected human colorectal cell lines and the *in vivo* model of metastasis in rats allows to search for the molecular determinants of human colorectal cancer metastases and for the establishment of relevant models to test novel therapeutic agents against colorectal cancer metastasis.

uPA is a serine protease with multiple actions that could enable it to play a role in cellular migration, tissue remodeling, and cancer spread^[19]. Some investigators reported that patients with a high uPA or high PAI-1 level had a poorer prognosis for relapse than those with a low uPA or low PAI-1 antigen level. It was concluded that uPA and PAI-1 antigen levels in tumor tissue were an independent and useful prognostic marker in a variety of malignancies^[20,21], especially breast cancer^[22,23]. However, there have been no studies concerning the relation between uPA as well as PAI-1 level of high-metastatic cells and low-metastatic cells or non-metastatic cells. We analyzed the difference of uPA and PAI-1 level produced by WiDr (non-metastatic), HT-29 (low metastatic) and variant HT-29d cells (highly metastatic) in their supernatants by ELISA. It showed that variant HT-29d cells produced higher a levels of uPA and PAI-1 as compared with non-metastatic WiDr cells and low metastatic HT-29 cells. HT-29 cells produced higher levels of uPA and PAI-1 as compared with WiDr cells. uPA might act as an offensive mechanism for tumor cell invasion and

metastasis. Although the functional role of PAI-1 in tumor biology has been unknown, PAI-1 might play a role in enhancing tumor spread by mechanisms involving in angiogenesis or promote tumor cell division^[24]. Since PAI-1 is present in endothelial cells and platelets of normal tissues, increased PAI-1 levels may reflect a high degree of angiogenesis, which offers the possibility of tumor spread and metastasis. Our results showed that levels of uPA and PAI-1 in variant HT-29d cells were higher than that in HT-29 cells, suggesting that the process of metastasis might depend on the selection of tumor cell clones with increased expression of plasminogen activator, which was positively correlated with the metastatic potential of colorectal carcinoma cells in this study.

CEA has been implicated in the development of hepatic metastases from colorectal cancers, and has been described as an adhesion molecule either by Ca²⁺-independent homophilic binding or heterophilic binding. The present results showed that CEA levels on cell surfaces *in vitro* were not different among WiDr, HT-29 and HT-29d cells. In CEA immunostaining, no difference was observed among the cell lines, s.c. tumor and liver metastases of WiDr, HT-29 and HT-29d cells, respectively. These results showed that CEA surface expression might not be correlated with the metastatic potential in this model. However, the secretion of CEA was significantly altered in metastatic cells. Moreover, other members of the CEA family might also be differently expressed in metastatic cells.

PI3-kinase is a heterodimer consisting of an 85-kDa regulatory subunit (p85), and a 110-kDa catalytic subunit (p110). The role of PI3-kinase in growth factor signaling pathways has been studied intensively^[25]. Several reports have shown PI3-kinase was involved in signal transduction pathways associated with cell growth regulation, cell cycle progression, and cell survival^[26]. Some studies showed increased cellular content of PI3-kinase products and also physical association of the enzyme with transmembrane receptor tyrosine kinases following stimulation by various growth factors, including hepatocyte growth factor^[27,28] and insulin-like growth factor^[29]. A recent research indicated the importance of PI3-kinase in increased cell motility stimulated by growth factors such as PDGF and hepatocyte growth factor^[30]. However, these data do not indicate whether expression of PI3-kinase is associated with the metastatic potential of colorectal carcinoma cells. In this study, we investigated the PI3-kinase expression in colorectal carcinoma cell lines WiDr, HT-29 and HT-29d *in vitro* and in s.c. tumors and liver metastases of rats immunohistochemically. *In vitro*, non-metastatic WiDr cells, metastatic HT-29 cells and highly-metastatic variant HT-29d cells all expressed PI3-kinase. No significant differences were found in positive cell ratio and staining intensity. In s.c. tumors, highly metastatic variant HT-29d cells showed a stronger PI3-kinase expression in staining intensity and a higher ratio of positive cells compared to WiDr and HT-29 cells. In liver metastases, variant HT-29d cells showed a stronger staining intensity and a higher positive ratio than that of parental HT-29 cells. These results suggest metastatic potential may positively correlate with synthesis of PI3-kinase in this *in vivo* model. The highly-metastatic HT-29d variant highly expresses PI3-kinase. The expression of PI3-kinase correlates with tumor development and metastasis, and it may be a late event of tumor development of colorectal carcinoma and may correlate with an invasive stage of cancer.

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