Original Article

Anti-metastasis traditional Chinese medicine monomer screening system based on perinucleolar compartment analysis in hepatocellular carcinoma cells

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Abstract: Hepatocellular Carcinoma (HCC) lacks effective anti-metastasis drugs. Traditional Chinese Medicine (TCM) monomers have shown anti-proliferation activity in HCC, but few of them are specifically anti-metastasis. Therefore, further clarifying the indicators of HCC metastasis and screening TCM monomers based on the indicators, will effectively guide the development of novel anti-HCC drugs. The perinucleolar compartment (PNC), existing in the nuclear of tumor cells, is closely correlated with metastasis of several tumors. In this study, we found positive correlation between higher PNC prevalence and metastasis in HCC tissue of patients. The PNC prevalence was also positively correlated with the malignancy of HCC cell lines. On this premise, we established a PNC-based screening system for anti-metastasis TCM monomers and obtained Camptothecin (CPT), Evodiamine and Isoglycyrrhizin, the three most effective TCM monomers from a TCM monomer library to reduce the PNC prevalence in Huh7 cells. The anti-metastasis effect of these TCM monomers was positively correlated with their PNC inhibitor effect. Our data further revealed that CPT reduced metastasis of Huh7 cells possibly by inhibiting Epithelial-Mesenchymal Transition by upregulating the expression of ZO-1, E-cadherin and Claudin-1. The PNC-based screening system is effective and it may provide an effective technical platform for the development of anti-metastasis drugs.

Keywords: Hepatocellular carcinoma, perinucleolar compartment, traditional Chinese medicine monomers, metastasis

Introduction

Hepatocellular Carcinoma (HCC) is one of the most common tumors worldwide [1], and its incidence and mortality are on the rise [2]. Early focal HCC are generally treated with surgery combined with radiotherapy and chemotherapy [3]. However, HCC is difficult to diagnosis at the onset, and most of the HCCs are discovered at a late stage [4]. The sinus structure, abundant blood flow [5] and immunosuppressive tumor microenvironment [6, 7] of the liver tissue determines that the HCC cells are easy to spread via blood or lymphatic system. The metastasis of HCC cells is an important cause of the recurrence and even death of HCC. Advanced HCC lacks effective anti-metastasis drugs [8]. Traditional Chinese Medicine (TCM) monomers have shown anti-HCC activity [9, 10], but few of them are specifically anti-metastasis. Therefore, further seeking indicators of HCC metastasis and screening TCM monomers based on the indicators, will effectively guide the development of novel anti-metastatic drugs in HCC. Perinucleolar compartment (PNC) exists in the nuclear of tumor cells and is positively correlated with metastasis of various tumors such as breast cancer [11]. The PNC is mainly composed of RNA and RNA binding proteins [12], among which polyypyrimidine tract-binding protein (PTB) is one of the main protein components. The PNC structure can be detected by immunofluorescent assay or immunohistochemistry assay with PTB antibody [13]. We obtained 6-Methoxyethylamino-nunofamide (MEAN) that can disassemble PNC structure of HCC cell lines in our previous study, and we further validated its anti-HCC effects [14], indicating that
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PNC is an effective indicator for screening anti-HCC drugs. Another study confirmed the anti-metastasis effect of MEAN in Hela cell line [15]. Therefore, based on the analysis of PNC structure in HCC cell lines, we can establish a screening system for anti-HCC metastatic TCM monomers.

Materials and methods

Patients

Liver tissues were obtained from HCC patients during liver resection at the First Affiliated Hospital, School of Medicine, Zhejiang University. Fresh liver samples were snap frozen by liquid nitrogen and stored in liquid nitrogen. According to pathology reports and imaging reports of the hospital, the patients were divided into two groups, one group found focal HCC only, and the other group were diagnosed to have portal invasion or extrahepatic spread. Staging of HCC is based on the Barcelona Clinic Liver Cancer (BCLC) system [1]. Clinical data of HCC patients is shown in Table 1. The study followed the ethical guidelines of the Ethics Committee in First Affiliated Hospital of Zhejiang University. Informed consent was obtained.

Cell culture

PC-3M GFP-PTB, a PC-3M cell line stably expressing (Green Fluorescent Protein) GFP-fused PTB, was kindly gifted by Prof. Sui Huang (Department of Cell and Molecular Biology, Feinberg School of Medicine, Northwestern University Chicago, Illinois, USA), and human HCC cell lines Huh7, Hep3B, and HepG2 were maintained in our lab. PC-3M GFP-PTB cell line was cultured in RPMI medium modified (HyClone, USA) with 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin/streptomycin (Genom, China). Huh7, Hep3B, and HepG2 cells were maintained in 5% CO₂ atmosphere at 37°C.

PNC detection

Cell slides were incubated overnight and fixed with 4% paraformaldehyde (PFA). After disrupting cell membrane with 0.5% Triton X-100, anti-PTB mouse monoclonal antibody (1:500, B3-C8, Huabio, China) was added and incubated overnight at 4°C. Cell slides were then washed with phosphate buffer saline (PBS) for 3 times. Alexa Fluor 488 labeled fluorescent secondary antibody (Invitrogen, USA) was added and incubated at 37°C for 60 min, and rinsed with ddH₂O for 3 times. The slides were sealed with anti-fluorescence quencher (Beiyotime, China) and observed under confocal microscopy (FV1000, OLYMPUS, Japan). The PNC prevalence (the ratio of PNC positive cells) was determined in 3 randomly selected fields (400 ×).

Wound-healing assay

Huh7, Hep3B, HepG2 cell lines were cultured overnight in plates with a density of 5 × 10⁵/ml. Scratch vertically in the plate using a 200 μl pipette tip, with a space of 0.5 to 1.0 cm. Wash 3 times with PBS to remove delineated cells. Add serum-free medium and continue to incubate the cells in 5% CO₂ incubator at 37°C. Take photos under a microscope at 0 h and 48 h, respectively. The relative migration distance of the cells was analyzed according to the width of the scratched area. Gap ratio = width of scratch zone at 48 h/width of scratch zone at 0 h × 100%. The experiment was repeated 3 times and the results were averaged.

Transwell assay

100 μl of 12.5% Matrigel™ (BD, US) was added to the upper chamber of a transwell chamber (Corning Falcon, USA), and incubate at 37°C for 4-5 hours. After lightly washing the gel in serum-free medium, single cell suspension of Huh7, Hep3B, and HepG2 cells were added with a density of 1000/ml to the upper chamber, 200 μl per well, respectively. 600 μl of DMEM medium containing 10% FBS was added to lower chambers. After incubating for 24 hours at 37°C, the chambers were removed, and cells in the upper chambers were wiped off with a cotton swab. After being fixed with 4% PFA for 30 min at room temperature, the cells

| Table 1. Clinical data of HCC patients |
|-----------------|-----------------|-----------------|-----------------|
| Clinical Characteristics | Focal HCC | HCC with metastasis |
| Gender (Male/Female) | 9 (7/2) | 8 (7/1) |
| Age (Mean ± SD) | 61.78 ± 10.67 | 61.75 ± 7.74 |
| AFP (ng/ml) (Median) | 23.7 | 3335.85 |
| HBV-DNA (IU/ml) (Median) | 2.4 × 10³ | 4.95 × 10² |
| HBsAg (IU/ml) (Median) | 1382.75 | 556.05 |
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in the lower layer of Matrigel™ gel were stained with crystal violet staining solution (Beyotime, China). The photos were taken three non-overlapping fields to count the number of invasion cells penetrating into the lower layer of Matrigel™. The experiments were conducted in triplets and the results were averaged.

Drug treatment

The TCM monomers (Chengdu Herbpurify Co., LTD, China) were dissolved in DMSO, and stored in -20°C. GI99% concentrations of the drugs were determined by Cell Counting Kit-8 (CCK-8) assay (Dojindo, China). The highest dose of drug that does not cause significant inhibition of cell proliferation was also determined by CCK-8 assay.

qRT-PCR

RNA extraction kit (Takara, Japan) was used to extract total cellular RNA, and then reverse transcription was performed using reverse transcription kit (Takara, Japan), and detected by ABI Quantstudio™ DX (Thermofisher, USA); U6 was used as internal reference. Primers of U6, ZO-1, E-cadherin and Claudin-1 were synthesized in Tsingke (Hangzhou, China). 2^ΔΔCT method was used to analyze the relative expression levels of related mRNAs. The experiment was repeated 3 times and the results were averaged.

Western blot

RIPA lysis buffer (contained 1% PMSF; Beyotime, China) was used to extract total protein from Huh7 cells. Then denatured the protein using sodium dodecyl sulfate, and separate molecules by 4-20% SDS-PAGE gel (Genescript, China). The β-actin antibody and Epithelial-Mesenchymal Transition (EMT)-related primary antibodies (ZO-1, E-cadherin and Claudin-1) and HRP-conjugated secondary antibody were all purchased from Cell Signaling Technology (Danvers, USA).

Statistical methods

Data were analyzed by Graphpad Prism 7.00 software, and the results were expressed as mean ± standard deviation (SD). Data comparison between two groups was performed by Student’s t-tests. Data comparison between more than two groups was performed by one-way analysis of variance, and P < 0.05 was considered statistically significant.

Results

The PNC prevalence in metastatic HCC tissue was higher than that in non-metastatic tissue

To determine whether the PNC is associated with the malignancy of HCC, we performed PNC analysis in the liver tissue of HCC patients. In constant with the report that the PNC was rarely observed in normal primary cells [16], we rarely observed PNC in paracancer tissues (Figure 1A). We found that the PNC prevalence varies in different patients, and PNC prevalence in HCC tissues with metastasis was significantly higher than that in focal liver cancer without metastasis (Figure 1D). As shown in Figure 1, the liver tissue from patient burdened focal HCC (Figure 1B) exhibited fewer PNC positive cells than that from patient burdened poorly differentiated liver cancer with mesenteric metastasis (Figure 1C). We also correlated the PNC prevalence with the patients’ HCC stages, and found that the later the HCC stage, the higher the PNC prevalence (Figure 1E). The above results suggest the association between high PNC prevalence and the poor differentiation and easily metastatic traits of HCC.

The PNC prevalence is positively correlated with the migration and invasiveness of HCC cell lines

To further clarify the relationship between the PNC prevalence and the malignancy of HCC, we also detected the PNC in HCC cell lines (Table 2). The results of immunofluorescence assay showed that Huh7 cell line had the highest PNC prevalence, higher than Hep3B and HepG2 cell lines (77.03% ± 1.96%, 2.27% ± 0.88%, and 1.61% ± 1.45%, respectively) (Figure 2A, 2B). The metastasis ability of these three cell lines also varies. The results of wound-healing assay showed that, at 48 h, the gap ratio of Huh7 cell line was the lowest, lower than that of Hep3B and HepG2 cell lines (39.93% ± 3.66%, 52.07% ± 2.04% and 79.25 ± 3.91%, respectively) (Figure 2C, 2D), which indicated that the strongest migration ability of Huh7. As the transwell assay showed, invasion cells of Huh7 cell line were the most, indicating strongest invasion ability (Figure 2E, 2F). The results suggested that the migration and invasion ability of these three cell lines were positively correlated with their PNC prevalence.
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Based on the correlation between PNC prevalence and metastasis of HCC cells, we established an anti-metastasis TCM monomers screening system. The screening process is shown in Figure 3A. The primary screening used PC-3M GFP-PTB cell line as cell model, which enable us to observe the PNC directly under a fluorescence microscope. The PC-3M GFP-PTB cell line is commonly used as a cell model in primary PNC-based drug screening, owing to its particularly high PNC prevalence.
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According to the drug toxicity reported in the literatures, in the primary screening, we treated PC-3M cells with TCM monomers at concentrations vary from 25 μM to 200 μM. After 24 hours, the PNC prevalence was observed under the fluorescence microscope. Five TCM monomers were selected through the primer screening, named Camptothecin (CPT) (Figure 3B, 3C), Evodiamine (EVO) (Figure 3B, 3C), Isoliquiritigenin (ISL) (Figure 3B), Curcumin (Figure 3B) and Tanshinone (Figure 3E), which significantly reduced the PNC prevalence of PC-3M GFP-PTB cells.

In the secondary screening, Huh7 cell line was further used as a cell model to screen TCM monomers. Dosing Huh7 cells with TCM monomers at the GI99% concentration, CPT, EVO and ISL were demonstrated to reduce the PNC prevalence of Huh7 cells significantly (Figure 3F).

**TCM monomers reduce the PNC prevalence and inhibit the migration and invasion of Huh7 cells**

We determined anti-metastasis effect of CPT, EVO and ISO obtained through secondary screening via wound-healing assay and transwell assay performed on Huh7 cells. Treating Huh7 cells for 24 h with the highest dose of monomers that did not cause significant growth

![Figure 2](image-url). The PNC prevalence is positively correlated with the migration and invasiveness of HCC cell lines. A and B: The PNC structure of Huh7, HepG3B and HepG2 cell lines were displayed in immunofluorescence assay; and the PNC prevalence was determined and analyzed; C and D: The migration ability of HCC cell lines was assessed by wound-healing assay, and the gap ratio was determined and analyzed; E and F: The invasion ability of HCC cell lines was assessed by transwell assay, and the invasion cells were calculated and analyzed. (Data is presented as mean ± SD. Data comparison between Huh7, Hep3B and HepG2 was performed by one-way ANOVA, and P < 0.05 was considered statistically significant, *P < 0.05, ***P < 0.001, ****P < 0.00001, n=3).
inhibition, we observed decreased PNC prevalence under CPT, EVO, and ISL treatment (Table 3; Figure 4A, 4B). CPT reduced the PNC prevalence in Huh7 cells most. In wound-healing and transwell assays, all three of these TCM monomers increased the gap ratio of Huh7 cells (Figure 4C, 4D) and reduced the number of invading Huh7 cells (Figure 4E, 4F), that is,
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inhibited the migration and invasiveness of Huh7 cells. Among them, CPT exerted the strongest anti-metastasis effect. The anti-metastasis abilities of these three TCM monomers were positively correlated with their ability to disassemble PNC.

**CPT inhibited Huh7 cell metastasis possibly by inhibiting EMT**

Considering EMT is a crucial event in HCC metastasis, we examined EMT-related molecules at both mRNA and protein level. Dosing Huh7 cells with gradient concentrations (10 nM, 30 nM, 100 nM and 200 nM) of CPT for 24 h, we observed upregulation of ZO-1, E-cadherin and Claudin-1 at mRNA level (Figure 5A-C) and protein level (Figure 5D) in dose-dependent manner, indicating inhibited EMT and decreased metastatic ability.

**Discussion**

Metastasis is a major challenge in the treatment of cancer [19]. However, there is currently no clinically well-defined anti-metastasis drug.
TCM has been practiced for a long time. TCM monomers and their derivatives have been widely studied and applied to the treatment of cancers [20]. In the field of HCC, several TCM monomers exhibit anti-HCC activity, such as S-allylmercaptocysteine [21], Bufalin [22], Narciclasine [23], Bruceine D [9] and Melittin [24]. But only a few of them was proved to have significant anti-metastasis activity.

The pathogenesis of cancer metastasis consists of a series of steps [10, 25-28]. At present, international teams have carried out research and developed anti-metastasis drugs targeting key molecules during metastasis, such as growth factor receptors, cell adhesion molecules, matrix metalloproteinases, and angiogenic factors [29, 30]. Among them, some new anti-metastasis drugs have entered clinical trials, such as Licartin [31], Bevacizumab [32], Brivanib [33], Cixutumumab [34], and Sorafenib [35] etc. However, the clinical effects of some drugs are disappointing [34]. There are also some targeted therapies based on genes that are uniquely expressed in primary tumors, but the heterogeneity of tumor cells [36-38] and the increased genomic instability in metastatic cells [39, 40] make the identification of genetical targets impractical. In fact, homogeneously targeting these genes failed to produce long-lasting responses in most HCC patients [3, 8, 38, 41]. Therefore, the establishment of a novel HCC metastasis marker and the screening system for anti-metastasis drugs based on this marker are hot spots in development of anti-HCC drugs.

Many clinical studies have shown that the PNC prevalence is positively correlated with metastasis of multiple malignant tumors such as breast cancer [11], prostate cancer [42] and ovarian cancer. PNC prevalence corresponds to metastatic capacities in several cancer cell lines [42], and PNC prevalence in distant metastases is also higher than that in the primary tumors [11]. As a component of PNC, the PTB has been reported to be involved in metastasis of bladder cancer [43] and colorectal cancer [44]. Our research in HCC tissues also suggested the correlation between PNC prevalence and the malignancy of HCC (Figure 1). Higher PNC prevalence may indicate a more malignant HCC and a greater metastatic potential. Therefore, PNC can be used as an important indicator of tumor metastasis assessment. A PNC-based anti-metastasis drug screening system is of great value for the development of novel anti-metastasis drugs.

PNC is a special structure adjacent to the nucleolus, with an irregular morphology and a dense electronic structure [45]. It is rarely detected in normal cells, but is ubiquitous in immortalized or transformed cells as well as in tumor cells in vivo. The PNC consists of newly synthesized RNA polymerase III transcripts and RNA-binding proteins, such as PTB and CUG binding protein 1 [12, 16, 46]. PTB has been
used as an effective and intuitive detector of PNC [13]. In this study, we applied an anti-PTB monoclonal antibody to visualize PNC via immunofluorescence assay and we confirmed the existence of PNC in HCC tissue and HCC cell lines.

In our study, we detected the PNC prevalence, migration and invasion ability in HCC cell lines, which showed that the PNC prevalence in Huh7, HepG2 and Hep3B cell lines were positively correlated with their migration and invasion ability (Figure 2). Huh7 cells were turned out to have the highest PNC prevalence and strongest migration and invasion ability than the other two HCC cell lines. Therefore, we used Huh7 cells as a model for anti-metastasis drug screening in the follow-up study.

In the primary screening, we screened a variety of TCM monomers, among which CPT, EVO, ISL, Curcumin and Tanshinone were able to reduce PNC prevalence in PC-3M GFP-PTB cells. In the secondary screening, we measured the TCM monomers’ ability to reduce PNC prevalence in Huh7 cells at GI99% concentration. CPT, EVO and ISL turned out to reduce PNC prevalence of Huh7 cells. Among them, CPT exerted the strongest ability to disassemble PNC. Further functional studies (wound-healing assay and transwell assay) showed that the above three TCM monomers could effectively reduce the migration and invasiveness of Huh7 cells at concentrations without significantly inhibiting cell proliferation. And CPT exhibited the most prominent anti-metastasis activity. CPT was first discovered from Camptotheca acuminate six decades ago [47]. It is a natural alkaloid with inhibitory effect on DNA topoisomerase I. CPT and its analogs have approved antitumor activity by inhibiting proliferation, promoting tumor cell apoptosis, antagonizing angiogenesis, etc. Since early in 1990 s, clinical trials have been adopted with analogues Topotecan [48] and Irinotecan [49], in combination with Chemotherapy [50] or not. Besides mentioned antitumor effects above, CPT itself has been reported inhibiting tumor cell invasion in melanoma and in prostate cancer. We obtained CPT via our anti-metastasis screening system and we found CPT negatively regulated tumor cell migration and invasion in constant with previous studies [51, 52]. These data suggest the possible anti-metastasis effects of the selected TCM monomers and their positive correlation with their ability to reduce PNC prevalence. However, the anti-metastasis effects of these TCM monomers still need to be further verified by animal models. The PNC-based anti-metastasis TCM monomer screening system has been also confirmed practical and feasible.

We have further studied the mechanisms by which these TCM monomers inhibit metastasis. EMT is associated with a variety of tumor functions, including tumor initiation, malignant progression, cancer stem cells, tumor cell migration, metastasis, and resistance to treatment [4]. E-Cadherin’s transcriptional repression has long been considered a key step in EMT. E-cadherin is a key component of adhesion junctions, and down-regulation of E-Cadherin promotes the EMT and the invasion of HCC cells [53]. Expression of E-cadherin in HCC tissues was negatively correlated with HCC grades, and E-cadherin was more weakly expressed in metastatic HCC than in non-metastatic HCC [54, 55]. In our study, we chose CPT which reduced the PNC prevalence and inhibited metastasis in Huh7 most to further study its anti-metastasis mechanisms. Treating Huh7 cells with gradient concentrations of CPT, we found that CPT could increase E-Cadherin at both mRNA and protein level in a dose-dependent manner. We also tested EMT-related molecules such as ZO-1 and Claudin-1, and it was found that they were also up-regulated by CPT at mRNA and protein level. These molecules have been found to be involved in tumor progression in many studies [55, 56]. Therefore, we concluded that CPT inhibits the metastasis of Huh7 cells possibly by inhibiting EMT. Our study briefly explained the anti-metastasis mechanism of CPT, further supporting the effectiveness of the PNC-based anti-metastasis TCM monomers screening model.

In summary, our study finds the correlation between PNC and malignancy of HCC, and provides an effective technology platform for the development of new anti-metastasis candidates for HCC. The TCM monomer screening model based on the PNC analysis provides an effective screening platform for the development of anti-metastasis drugs (or prodrugs), and is expected to shorten drug screening period and avoid unnecessary investment.

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Disclosure of conflict of interest

None.

Abbreviations

HCC, Hepatocellular Carcinoma; TCM, Traditional Chinese Medicine; PNC, perinucleolar compartment; CPT, Camptothecin; PTB, Polypyrimidine tract-binding protein; MEAN, 6-Methoxyethylamino-numonafide; GFP, Green Fluorescent Protein; PFA, paraformaldehyde; PBS, phosphate buffer saline; CCK-8, Cell Counting Kit-8; EMT, Epithelial-Mesenchymal Transition; EVO, Evodiamine; ISL, Isoliquiritigenin.

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