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Screening Anthraquinones by Fluorescent Ubiquitination Cell Cycle Indicators

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Abstract: Cancer is one of the diseases with the highest mortality rate in the world, and its occurrence and development are mainly related to the uncontrolled cell cycle. The cell cycle is the completion of a cell division from the end of the n1ext division, the cell cycle is divided They are interphase and division phase. Specifically, there's a DNA synthesis phase (G1 phase), DNA synthesis phase (S phase), and DNA synthesis phase (G2 phase) Phase) and division phase (M phase). Tumor cells can proliferate indefinitely, mainly with the cell cycle getting out of control, making it not subject to normal Growth regulation. The current methods for detecting cell cycle mainly include: Acridine Orange (AO) method, 5-Bromo-2-deoxy Uridine (BrdU) labeling method, Western blot and flow cytometry, etc., but these methods have certain limitations, such as the inability to make real-time observations in living cells. Therefore, we constructed the Cell Cycle Ubiquitination-based Cell Cycle Indicator (FUCCI) tool in liver cancer cells, hoping to provide a new technical means for the screening of anti-tumor drugs.

Keywords: Cell Cycle; FUCCI; Fluorescent Protein; Real-time Observation; Cell Cycle Factor; Tumor Cell.

1. Introduction

Hepatocellular Carcinoma (HCC) is the most common malignancy of the liver, ranking second in human cancer-related mortality in developing countries and sixth in developed countries [1]. Therefore, the prevention and treatment of liver cancer is imminent. The clinical treatment of tumor mainly includes surgery, radiotherapy and chemotherapy. Chemotherapy is currently one of the most effective ways to treat cancer. Cancer is caused by the uncontrolled proliferation of tumor cells due to the abnormal activity of various cyclins [2]. According to the relationship between drugs and cell cycle, drugs can be divided into cell cycle independent drugs and cell cycle dependent drugs. Therefore, the detection of tumor cell cycle and the study of cell cycle blocking drugs can provide useful information for screening potential chemotherapy drugs.

Fluorescent Ubiquitination-based Cell Cycle Indicator (FUCCI) is a new technology that fuses cell cycle factors with fluorescent proteins and transfers them to cells. As the cell cycle changes, labeled cells exhibit cell cycle-dependent fluorescent changes [3]. For example, SLBP (Stem Loop Binding Protein) is a protein that specifically characterizes the G1 and S phases, The cell cycle can be characterized by fusion of the orange fluorescent protein mKO2 (monomeric fluorescent protein Kusabira-Orange 2) with it [4]. When the cells appear orange fluorescence, it indicates that they are in G1 and S phases, and non-fluorescent cells are in G2 and M phases. Through changes in fluorescence, the cell cycle can be quantitatively observed in real time in living cells.

2. Method

2.1. Cell Resuscitation

Configuration of cell culture medium: Prepare the cell culture medium according to the ratio of DMEM: Double antibody: glutamine =10:1:1. Human liver cancer Bel-7402 cell line was removed from liquid nitrogen and quickly dissolved in a water bath at 37℃. After thawing completely, the cell line was centrifuged at 1000 rpm for 1 min, the supernatant was discarded, 5 mL of fresh culture liquid was added, and the cell suspension was transferred to the cell culture dish. It was placed in a cell incubator at 37℃ and 5% CO2 for static culture.

2.2. Cell Inoculation

When the cell growth density reached about 80%, the old culture medium was discarded, the cells were cleaned with 1 mL Phosphate Buffer Saline (PBS) and digested with 0.25% trypsin for 2 min. The cells were placed under the microscope for observation. When the adherent cells became round and bright, the old solution was discarded. Add 1 mL of fresh culture medium, blow, mix well, collect cells into 2 mL centrifuge tube, centrifuge at 900 rpm for 3 min, discard the supernatant, add fresh culture medium, mix well, transfer to cell culture dish according to the ratio of 1:3, and place in 37℃, 5% CO2 constant temperature cell incubator for static culture.

2.3. Cell Count

Discard the old culture medium, add 1 mL PBS to clean the cells, and use 0.25% trypsin to digest the cells for 2 min, then place the cells under the microscope for observation. When the adherent cells become round and bright, discard the old solution, add 1 mL fresh culture medium, blow and mix well, collect the cells into 2 mL centrifuge tube, centrifuge at 900 rpm for 3 min. Discard the supernatant, add 1 mL of fresh cell culture medium, suspend, mix well. Take 10 μL cell suspension to the counting gun and read the value.

2.4. FUCCI-7402 Tool Built

Transfection: 293T cells of logarithmic growth were collected and planted in a 6-well plate. When the cell fusion degree reached about 80%, appropriate amount of Opti-MEM and Lip 3000 were taken into the centrifuge tube and thoroughly mixed. mKO2-SLBP plasmid, pMD2.G plasmid

and pSPAX2 plasmid were thoroughly mixed in a centrifuge tube, and appropriate amount of P3000 and Opti-MEM were added for full mixing. Let stand at room temperature for 15 min. The above mixture was added to 293T cell suspension and cultured in an incubator at 37℃ and 5% CO2 for 12-18 h.

3. Result

3.1. FUCCI Tool Mode

mKO2 is an orange fluorescent protein, and SLBP is a protein that specifically indicates that the cell cycle is in the G1 and S phases. When the cell emits orange fluorescence, it indicates that this part of the cell is in the G1 and S phases (Fig.1). Since the wavelengths of orange fluorescence and green fluorescence overlap, the picture shows green fluorescence, which does not affect the conclusion.

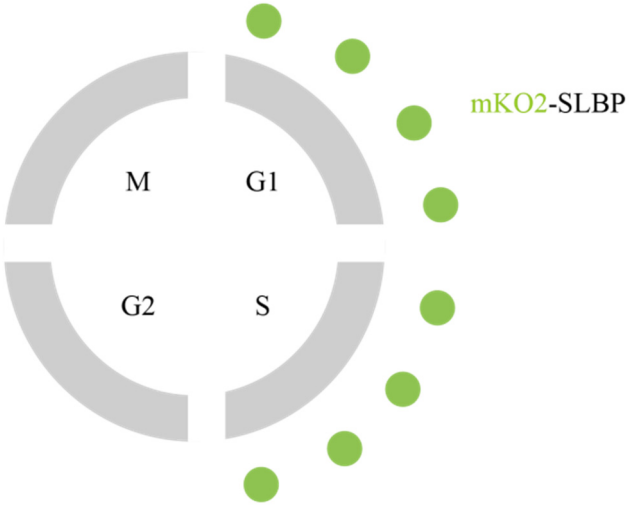

Fig 1. FUCCI-7402 Tool template diagram
(Modified to Bryce TB et al. Nat Methods. 2016.)

3.2. Infected with Hepatocellular Carcinoma

We integrated mKO2-SLBP into the genome sequence of liver cancer cells, which can stabilize inheritance with cell proliferation and division. As shown in Figure 2, liver cancer cells labeled with green fluorescence are in G1 and S phases, while cells without fluorescence are in G2 and M phases (Fig.2).

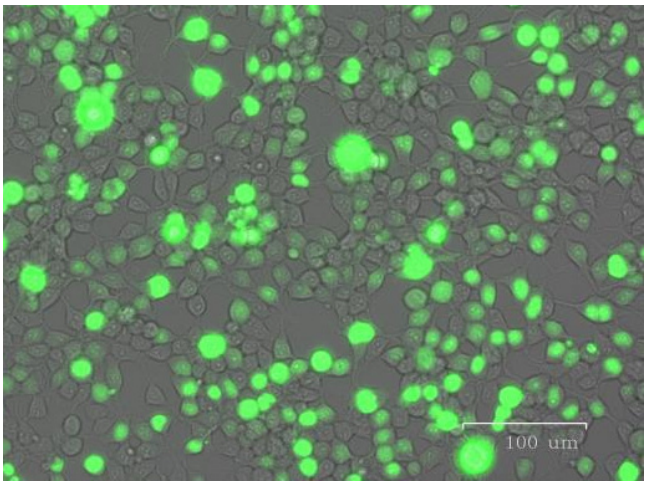

Fig 2. The FUCCI tool was successfully constructed in liver cancer cells

3.3. Screening of Anthraquinone Compound Library

We used the FUCCI-7402 tool to investigate 9 anthraquinone compounds: Aloe Emodin, Aloin A, Aurantio-obtusin, Purpurin, Rhein, Sennoside A, Sennoside B, Emodin and Hypericin were screened. The results showed that FUCCI-7402 cells were treated with 9 anthraquinone compounds for 24 h, compared with the control group, The fluorescence ratios of aloe emodin, aloin A, hesperidin, hesperidin, rubitin, sennoside A, sennoside B and emodin were not significantly changed, while the fluorescence ratios of hypericin and rhein were increased, indicating that hypericin and rhein could block the cell cycle of Bel-7402 in G1/S phase. As hypericin is a cellular fluorescent dye, no further study is undertaken here.

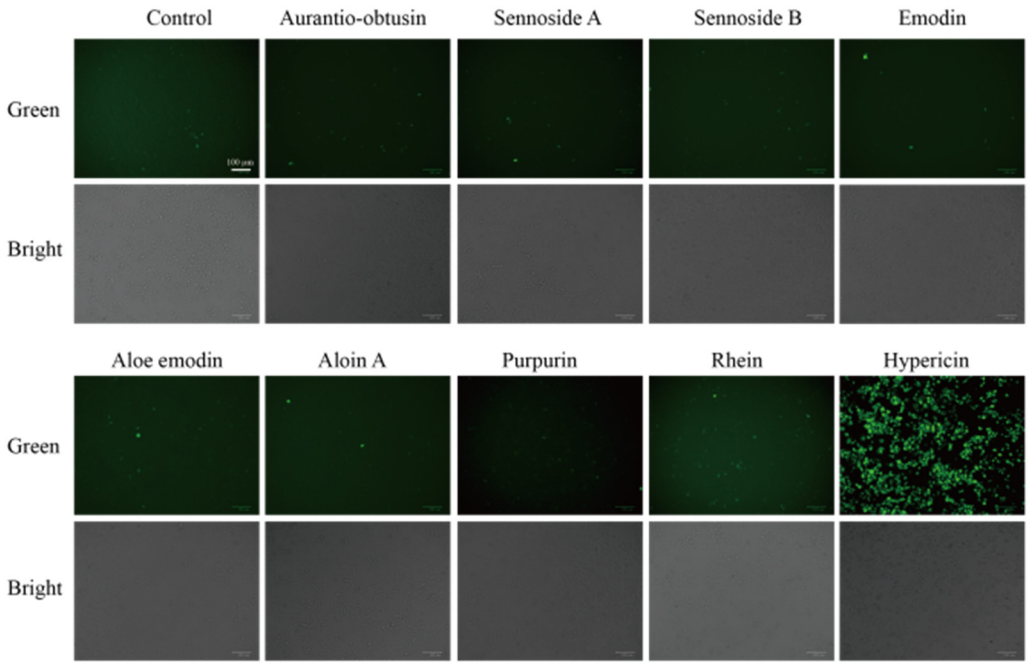

Fig 3. The FUCCI-7402 tool was used to screen anthraquinone compounds

4. Discussion

Ubiquitination-based cell cycle indicator (Fucci) is a technology that labels the cell cycle by binding fluorescent proteins to cell cycle-specific proteins. Depending on the cell cycle, the fluorescence displayed varies. Therefore, FUCCI has the characteristics of tracking cell cycle in living tissues, and has been widely used in various cell cycle studies, including tumor cells

Ubiquitination-based cell cycle indicator (Fucci) is a technology to label cell cycle by binding fluorescent protein to cell cycle specific protein, which was first proposed by Sakaue-Sawano et al. Fucci's initial probe was constructed by specifically binding the ubiquitination domains of two cell cycle regulators Geminin and Cdt1 to Azami Green monomer (mAG) and Kusabira Orange2 monomer (mKO2) [5]. Prior to the emergence of Fucci, the cell division from M to G1 in a single cell was morphologically observed, whereas the G1 to S phase transformation was observed mainly by staining with 5-bromodeoxyuridine (BrdU) or synchronizing the cell cycle with drugs. Traditional cell cycle indicators, such as BrdU or proliferating cell nuclear antigen (PCNA), cannot track proliferating cells throughout the organism due to cell immobilisation prior to immunofluorescence. Fucci provides a powerful tool for the visualization of cell cycle in the multicellular state of living tissues, and has shown great potential in nervous system development and cancer research from the very beginning, and has been widely used in the construction of various biological models and cell cycle tracking [6].

As a cancer with the fourth highest incidence and the third highest mortality rate in the world, colorectal cancer has always been a research hotspot in the fields of medicine and biology. Receptor interaction protein 140 (RIP140) is a core suppressor of various transcription factors and nuclear receptors. Marion and her team used the FUCCI system to determine that RIP140 plays an important role in maintaining intestinal homeostasis and inhibiting the development of colorectal cancer. Meanwhile, RIP140, as a new regulator of APC gene transcription and an inhibitor of Wnt/APC/β-catenin pathway, has been found to be a potentially valuable biomarker in the diagnosis, prognosis and treatment of colon cancer at the clinical level [7].

In the studies related to cervical cancer, Wang et al. used the FUCCI system to study the effect of Haspin protein expression silence on the cell cycle of cervical cancer, and found that the lack of Haspin would cause the prolongation of the S/G2 phase of Hela cells, which confirmed that Haspin has the ability to regulate mitosis and has extensive potential in anticancer cell proliferation [8]. Shinji Miwa, et al. used FUCCI to conduct univariate and multivariate analyses of chemotherapy resistance and sensitivity of cervical cancer cells, and the results showed that both doxorubicin (DOX) and cisplatin (CDDP) could induce cell cycle arrest in most cells at S/G2/M stage. However, some cells enter mitosis. DOX treated cells were apoptotic after mitosis, while CDDP treated cells were apoptotic both before and after mitosis. Multifactor survival analysis of single-cell FUCCI imaging data showed that G1/S transition was significantly associated with increased cell survival [9].

For most patients with gastric cancer, cancerous ascites is an unavoidable problem during treatment, and OBP-301 telomerase-dependent adenovirus can induce quiescent cells to enter the cell cycle to reduce the avoidance of G0 stage tumor cells from cell cyck-related therapeutic drugs, thus extending the survival of patients [10]. Tatsiana et al. analyzed the expression of MYC in neuroblastoma and its therapeutic response to DNA damage, and found that in cells with high MYC levels, cycle checkpoints failed and cells were forced to proliferation, but new cells would avoid proliferation and apoptosis when DNA was damaged by stopping cell cycle in G1 phase and conducting DNA repair. It shows that tumor cells can resist non-hereditary cytotoxic therapy and stop proliferating during favorable cell cycle phases [11]. In another FUCCI breast cancer study, Battuya's team found that G1 stage breast cancer cells are the most aggressive and therefore likely to metastasize. However, many anti-proliferative drugs can block cell proliferation in G1 phase, which may lead to accelerated metastasis of tumor cells. On the contrary, targeting aggressive cells may also promote cell proliferation and the growth of metastases. Therefore, research on the relationship between cell cycle and tumor cell migration is of great significance for cancer treatment [12].

Bleomycin (BLM) is a classic anti-tumor drug, which can selectively induce single or double strand DNA break, inhibit the synthesis and replication of tumor cell DNA, promote the degeneration and necrosis of tumor cells, and its anti-tumor activity may be related to cell cycle arrest. Soojin Jang et al. found that bleomycin can induce G2/M block of pulmonary epithelial MLE-12 cells, promote cell apoptosis, and cause pulmonary toxicity [13]. But the large adverse reactions of western drugs, it is easy to cause poor compliance and tolerance of patients.

Chinese herbal medicine has a long history, little side effects and significant anti-tumor effect, so it is widely used in clinical treatment of tumor. In polygonaceae, rhubarb, polygonum and Polygonum are commonly used Chinese medicines in clinic. Emodin is one of the main effective components of rhubarb, which has a wide range of pharmacological effects, including anti-inflammatory, anti-tumor, anti-diabetic nephropathy, antibacterial, antiviral and other functions [14]. The extraction, quality analysis and screening of the active components of natural medicinal plants is a complicated process. The monomer compound library involves a wide range of active ingredients, and preserved in the form of single products or 96-well plates. Therefore, we purchased natural plant-derived anthraquinone compound monomers and successfully constructed the FUCCI tool in liver cancer Bel-7402 cells to observe live cell cycle in real time through changes in fluorescence ratio before and after administration, focusing on the effect of natural compounds on tumor cell cycle.

However, at present, this study only detected liver cancer and did not screen for more compounds, so it has certain limitations. In the future, we will continue to use the FUCCI tool to screen more compounds, hoping to provide more options for anti-tumor drug screening and mechanism research.

5. Conclusion

We successfully constructed the FUCCI tool for fluorescein ubiquitination cell cycle indication in liver cancer cells, and used this tool to screen 9 anthraquinones, and successfully found that rhein can block the cycle of liver cancer cells in the G1/S phase, which may be a potential anti-

tumor drug.

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