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Biology of Hepatocellular Carcinoma are Restrained through PI3K/AKt Signaling Pathways and Targeted by Tumor Infiltrating Macrophages

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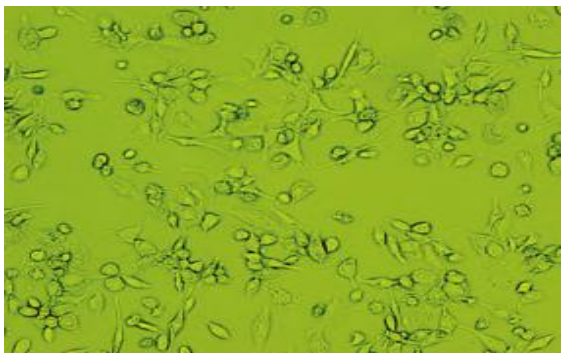
Abstract: **To study the biology of hepatocellular carcinoma are restrained through PI3K/AKt signaling pathways and targeted by tumor infiltrating macrophages.Methods: Mononuclear cells were isolated from healthy adult peripheral blood by density gradient centrifugation and induced with IL - 4 for selective activation of macrophagesin vitro.PI3K - siRNA and AKT – siRNA were transfected to hepatocellular carcinoma cell line HepG2 respectively by lipofectermine 3000, and then cultivate with selective activation of macrophages (M2) for 48 hour. PI3K and Akt mRNA expression level in liver cancer HepG2 cells were detected with real-time fluorescent quantitative PCR. Results: After transfection, PI3K, and Akt mRNA level were significantly decreased. In vitro microenvironment of liver cancer, M2 can significantly increase levels of PI3K, and Akt mRNA levels in HepG2 cell lines, and can significantly promote the HepG2 cell proliferation ability (P< 0.05) after co-culture with M2 for 24 hours. Conclusion: Tumor associated macrophages can promote liver cancer cell proliferation through PI3K/Akt signaling pathway. If changing the microenvironment of liver cancer or specific inhibition of PI3K/Akt signaling pathway can also control the malignant biological behavior of tumors.**



Keywords: Tumor associated macrophage; PI3K/AKt signaling pathway; Hepatocellular carcinoma (HCC)

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1. Introduction

Hepatocellular carcinoma (HCC) is a kind of invasive malignant tumor and lack of effective treatments. Alternative strategy is aimed at the target cells in HCC tumor microenvironment, such as tumor infiltrating macrophages [1]. The tumor associated macrophage (TAMs) is a source of mononuclear cells which can migrate to tumor microenvironmentin the action of tumor cytokines and chemokines, and prove proliferation and differentiationof cancer cells [2-3]. Under different micro environmental factors, macrophages had different activatedtrend and carrying out different functions. Many factors affect the prognosis of patients with liver cancer, including clinical factors, treatment, and molecular biology, etc., which the metastasis of tumor is closely related to the prognosis[4]. Further study the molecular mechanism of tumor associated macrophage in promoting malignant biological behavior of liver cancer, it can improve the effect of the treatment of liver cancer and guide clinical application.

2. Material and methods

2.1. Cell culture and groups

Cells culture in 1640 completely medium containing 10% fetal bovine serum, 2% glutamine, 100U/L streptomycin and100U/m L penicillin at 37 °C, 5% CO_2 incubator. When the adherent cells reached to 80%~90%, cells were passaged. Experiment is set to 5 groups, si PI3K group, the siAkt, Hep G2 cells separate training group (Control group), Hep G2 cells with unactivated macrophages (unactivated macrophages, Uagroup), Hep G2 cells and selective activation macrophages (alternatively activated macrophages, M2).

2.2. Macrophages induced in vitro

50ng/ml PMA were used to stimulate THP-1 for undifferentiated macrophages M0 , and vaccinated in 6 well plates for culture 24h; Collect M0 Cells and detect the cell membrane protein expressionusingflow cytometry, and then it will be further induced M1 cells using 20ng/mL IFN -γand 100ng/m L LPS stimulation

training for 48h, and M2 type of macrophages were also induced using 100 ng/m L IL-4 stimulation training for 48h.TAMswereobtained with EC9706 and M0 co-culture.

Table 1. The PCR specificity primer sequences.

Designation	Primer sequences
PI3K	5′-ATAGGCAAGTCGAGGCAATGGA-3′
	5′-TGAGCAGGGTTTAGAGGAGACAGAA-3′
Akt	5′- CTTCTTTGCCGGTATCGTGTGG-3′
	5′- TGTCATCTTGGTCAGGTGGTGTG-3′
β-actin	5′-AATCTGGCACCACACCTTCTAC-3'
	5′- ATAGCACAGCCTGGATAGCAAC-3′

2.3. RT-PCR

Extraction of Total RNA with RNAiso Plus, Reverse transcription synthesis c DNA with 5x Prime Script RT Master Mix and PCR amplification with, SYBR Green method. β-actin genes as internal too. Repeat experiment for 3 times and three holes each time. qPCR reaction conditions: 95℃ degeneration for 3min, and then 95℃ for 10s, 55℃ for 30s, a total of 40 thermal cycle. Primer sequences are shown in table 1.

2.4. Statistical methods

Analyzed with SPSS 16.0 statistical software, data were sided to mean ± SD. The mean between the two groups were analyzed with independent samples T test, multiple groups comparison using one-way ANOVA, and using least significant difference methods (LSD methods)between groups. The difference had statistically significant when P less then 0.05.

3. Results

3.1. THP 1 macrophages cells induction and identification

THP 1 cells are round and suspended growth in the culture medium (Figure 1, ①), after PMA induced ,cells form irregular gradually, the cell body increase, Cell intracytoplasmic vacuoles appeared, cell surface extended pseudopodia, begin to stick to wall after 2h, and basic stick wall after 12h . It grow into macrophages (M0, Figure 1, ②) when completely adherent after 24h. Flow cytometry instrument testing shows that macrophage differentiation purity were above 99.57% (Figure 2).

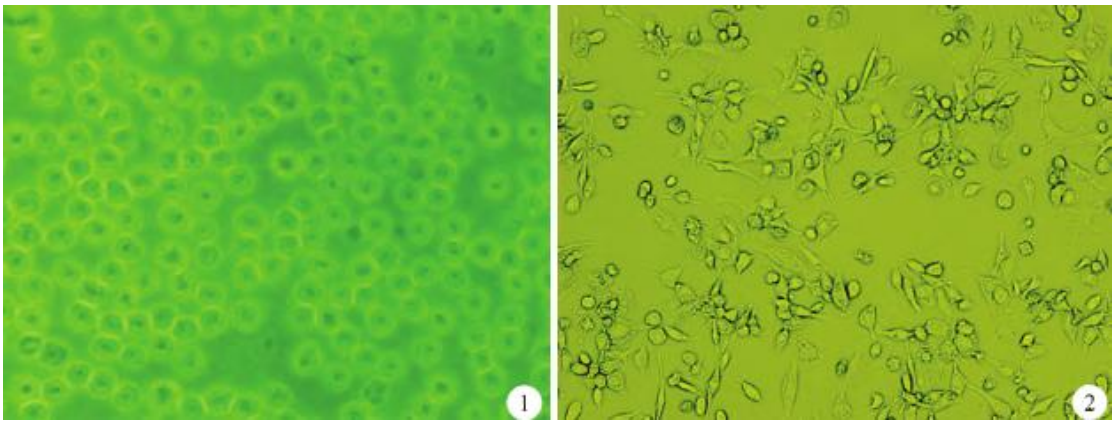

Figure 1. ①: THP 1 cells (not induced); ②: After PMA induced THP-1.

3.2. Selectively activated macrophages

To effectively inhibit the expression of PI3K and Akt, PI3K - siRNA and Akt - si RNA small molecules interference fragment were transfected respectively in Hep G2 cells. Collecting the corresponding cells after transfection for 4 h. At the same time, digesting and Centrifugal collecting Hep G2 including separate training group and groups cultured with Ua, M2 for 20h cells. Detected with RT-PCR for 5 groups respectively, experimental group Akt and PI3K mRNA levels were significantly lower than control group. But there were no statistical significance between control group and Ua group of PI3K.PI3K and Akt mRNA levels in M2 group were significantly higher than

Control group and Ua group. The results suggest that selective activation of macrophages can significantly promote liver Hep G2 cells of PI3K, and Akt-mRNA (Figure 3).

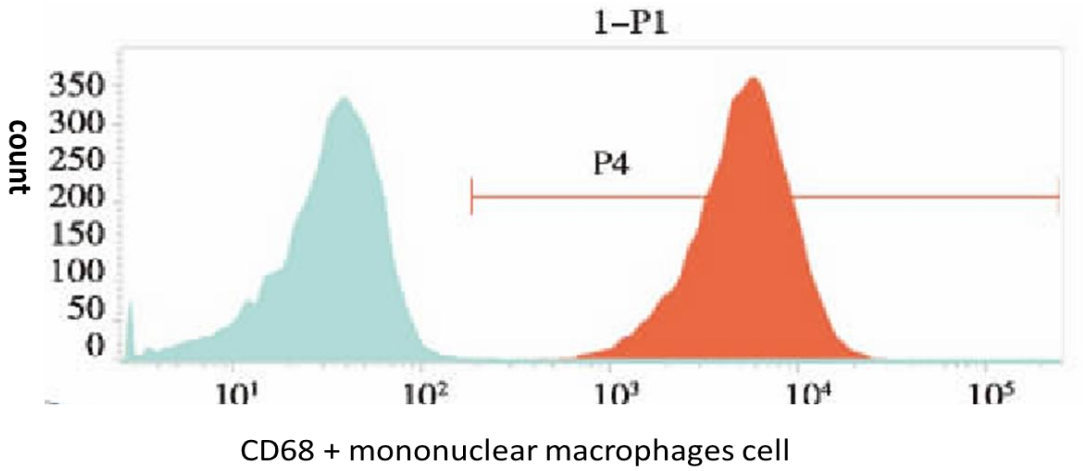

Figure 2. For CD68 macrophages identification tags. P4 representative indicators CD68, positive rate was 99.57%.

4. Discussion

Hepatocellular carcinoma is one of the common malignant tumor in the world, and China is a high incidence of hepatocellular carcinoma (HCC). In recent years, the treatment of liver cancer is increasing, but the treatment effect is not ideal, because of tumor recurrence and metastasis, patients survival rate reduce [5]. The occurrence of liver cancer, invasion and metastasis are the result of multifactor comprehensive. Research on liver cancer cells about the process of the mechanism and design corresponding intervention measures is particularly important. Academics have long argued that the tumor is not only composed of tumor cell mass lesions, but by the tumor cells, fibroblast cells, immune cells and inflammatory cells, glial cells and other cells to construct heterogeneous mixture [6-8], as well as the cells in the vicinity of including interstitial, microvascular and infiltration of many biological molecules [9]. Previous studies showed that many malignant tumors such as pancreatic cancer, prostate cancer, breast cancer around the tumor is present a very obvious interstitial reaction [10]. As early as in 1889, Stephen Paget based on organ specificity of breast cancer metastasis in clinical observation, put forward the concept of the famous "seed and soil" [11]. The Ioan - nides Anderson cancer research center and the university of Pittsburgh whitesides officially for the first time put forward "the tumor microenvironment (tumor microenvironment)" the concept of [12]. Tumor microenvironment is the main influence factors to decided tumor cell behavior in the development of tumor recurrence and transfer, and plays an important role .Tumor associated macrophage is the main immune cells in the tumor microenvironment, a large number of studies have shown that TAMs play an important role in the process of evolution of the occurrence of liver cancer [13].

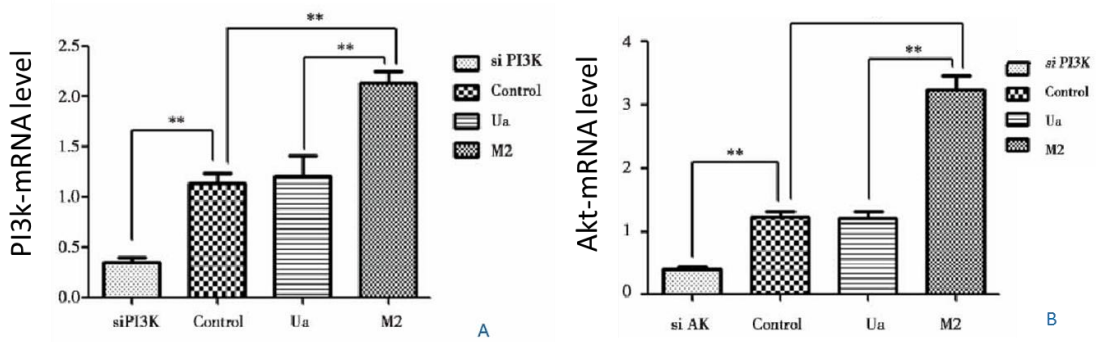

Figure 3. PI3K and Akt mRNA expressed can be promoted in Hep G2 cells M2 after co-culture with M2 and selective activation of macrophages. ***Note: * represents P < 0.05, * * P < 0.01.Figure 3A: compared with Control group, siPI3K -mRNA levels were significantly lower in siPI3K group (P = 0.0072). There were no significant statistical significance difference between Ua group and Control group (P= 0.1730). Compared with Control group and Ua group, PI3K mRNA were***

significantly increase in M2 (P = 0.0041, P = 0.0056).Figure 3B: compared with Control group, Akt-mRNA were significantly lower in siAkt group (P = 0.005) .There were no significant statistical significance difference between Ua group and Control group (P= 0.146). Compared with Control group and Ua group, Akt mRNA were significantly increase in M2 (P = 0.006, P = 0.0074).

This experiment adopts the density gradient centrifugation, isolated from healthy adult peripheral blood mononuclear cells, again with IL - 4 or LPS in vitro to simulate and differentiated respectively to different activated macrophages for forming a real tumor microenvironment. The experimental results show that the M2 can significantly increase liver Hep G2 cells PI3K and Akt mRNA level, which,AMs is the largest number of inflammatory cells in the tumor stroma, account for 30% ~ 50% of the total number of inflammatory cells, famous for its significant phenotypic heterogeneity and functional diversity of. M1 mainly secreted inflammatory substances such as IL-1, IL-6, IL-12, IL-23, TNA - a, CXCL - 10, ROI. M1 can Present antigen, participate in the positive immune response, immune surveillance function. And M2 is only the weaker antigen presenting ability, which produce a high level of suppression of inflammatory substances such as IL - 10, CCL - 17, 18, CCL CCL - - 22, it can repair tissue, inhibit inflammation and promote tumour [14]. In tumor microenvironment, under the action of stimulating factors such as GM-CSF, M - CSF, IFN - gamma, IL - 4, IL - 13,lactic acid, macrophages is selectively activated and form of tumor associated macrophages. A large number of studies have shown that M2 is associated with tumor which is similar to the phenotype and function of macrophages. Now vitro experiment were going by M2 simulation entity tumour TAMs [15]. The experimental results showed that the M2 co-culture stimulation can significantly increase the PI3K, and Akt mRNA expression level in liver cancer cells .

To sum up, we supplementary interpret the TAMs molecular mechanism on promoting the development of liver cancer.

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