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Original Article

MicroRNA-421 mediates immunosensitivity in late-stage human liver cancer

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Abstract: This report showed the novel clinical evidence to evaluate the pathological predisposition of microRNA-421 (miR-421) on immuno-insufficiency to cancer progression of human hepatocellular carcinoma. Some hospitalized patients with liver cancer were recruited when they were diagnosed by clinical parameters. As expected, all patients were subjected to routine biochemical analysis and histocytological inspection prior to receiving medical treatment. Clinical findings suggested that basal parameters of plasmatic aminotransferases and hepatitis B e antibody (HBeAb), alpha-fetoprotein (AFP) were elevated significantly. Further, immune cells and T lymphocytes in peripheral blood were abnormally altered in a time-dependent manner. More specifically, endogenous expressions of miR-421 level in liver cancer, immune-specific glycoprotein CD4 and CD8 were increased when compared to non-tumor control. Furthermore, intracellular immunocytochemical staining showed that proliferative marker of Ki-67, metastasized marker of CK19, as well as phenotypic markers of hepatocellular carcinoma for HBeAb and AFP were positively expressions with disseminated distribution. To sum up, these biochemical data and histopathological evidences demonstrate that miR-421-mediated immune hypersensitivity may function as a promising indicator for advanced liver cancer, even in further metastasis. Therefore, inhibition of intracellular miR-421 expression is an effective strategy for management of terminal hepatocellular carcinoma.

Keywords: Hepatocellular carcinoma, microRNA 421, immune hypersensitivity, proliferation

Introduction

In China, hepatocellular carcinoma (HCC) is one of the most lethal types of cancers. Although the detailed pathogenesis of HCC remains unknown, immune dysfunction and anti-apoptosis bioeffects are associated with uncontrolled growth of cancer cells [1, 2]. Pathologically, virus hepatitis is responsible for progression of HCC via an avenue of inducing own immune cells to destroy the liver cells, resulting in carcinogenesis over time [3]. If unmanaged, diseased liver will outgrow in weight and size gradually before subsequent metastasis [4]. In clinical practice, many therapeutic regimens can be provided for HCC patients, but adverse effects commonly occur [5, 6]. In order to lessen side effects in treatment, new strategy for combating cancer still needs to be designed and developed, especially in advanced HCC. In this study, we focus on exploring the miR-421-induced immune cell characterization and development of liver cancer when patient was received medical treatments, and discussing the possible underlying mechanism on uncontrolled growth of cancer cell in advanced stage.

Materials and methods

Clinical design

A group of 20 HCC patients were clinically diagnosed with HCC via biochemical tests and routine-/immune-stainings prior to further medical treatments. In brief, liver samples from cancer and non-cancer was isolated and stored immediately for subsequent bio-experiments. As declared, ethical guidelines were followed by the Declaration of Helsinki [7].

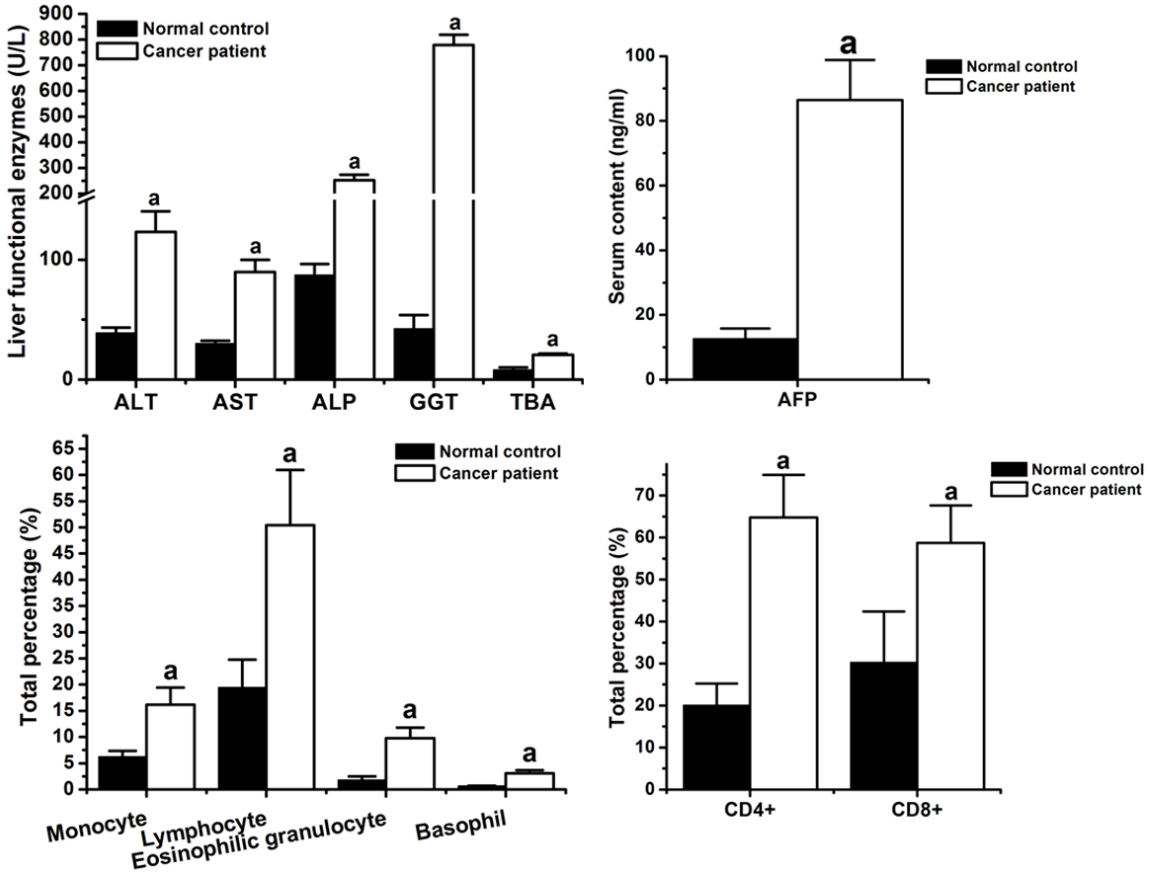

Figure 1. Clinical data showed that hepatic functional components (ALT, AST, ALP, GGT, TBA) and AFP concentration in sera were significantly upregulated in deseased liver cancer. Meanwhile, immune cells and lymphocyte types were abnormally changed with high levels. Note: cancer vs normal, $^{a}P < 0.05$.

Cytohistology staining

Firstly, HCC sample was harvested by surgical excision for further a group of biochemical analyses. The immunohistochemical protocols were conducted as following previous descriptions [8]. In brief, 5 μm section from liver cancer was subjected to rehydration and permeabilization through different concentrations of dimethylbenzene and ethyl alcohol. After being blocked with 5% non-fat milk solution, the liver slice was treated with diluted primary antibodies of AFP, HBeAb, Ki-67 (1:100, Fuzhou Maixin, China) at 4°C overnight. Subsequently, secondary antibodies (1:200, Fuzhou Maixin, China) were added to liver sample for 1 h incubation. And then specific 3, 3'-diaminobenzidine (DAB) was used as a developer, followed by counterstained with haematoxylin in cell nucleus. The indicated slice was mounted and imaged for further assessment.

As reported previously [9], paraffin-embedded liver cancer section was dewaxed and blocked with 5% BSA buffer (Beyotime Biotechnology, China) for 1 hour, followed by incubation of primary antibody of CK19 (1:100; Boster, Wuhan, China) overnight at 4°C, and reincubation of IgG H&L (Alexa Fluor® 488) (1:200; Abcam, UK) for 1 hour. Then, DAPI (Abcam, UK) was dyed to nuclear ahead of imaging and assaying.

RT-PCR assay

The liver samples were lysed with a TRIzol reagent (Gibco BRL, Grand Island, NY) to isolate total RNA. And then RNAs were reversely transcribed to cDNA with the First Strand cDNA Synthesis Kit (TIANGEN, Beijing, China) according the manufacturer's instructions. MiR-421 content was measured by using quantitative RT-PCR with targeting primers with commercially available SYBR Green qPCR Master Mix (Applied Biosystems, UK). Notably, MiR-U6 was used as an internal control to normalize the relative transcript of hepatocellular miR-421 [10].

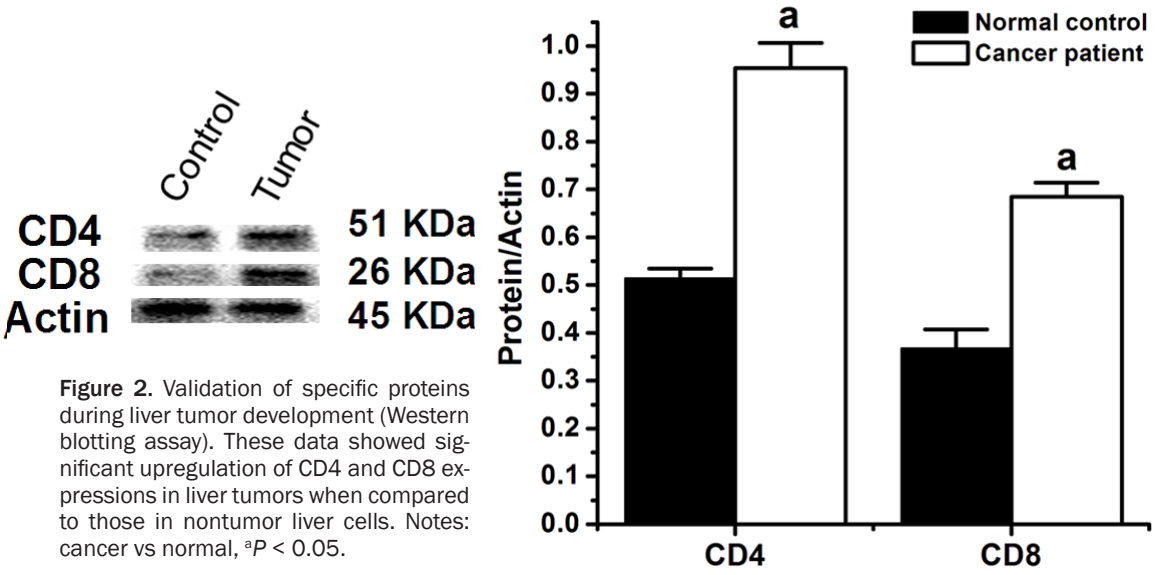

Figure 2. Validation of specific proteins during liver tumor development (Western blotting assay). These data showed significant upregulation of CD4 and CD8 expressions in liver tumors when compared to those in nontumor liver cells. Notes: cancer vs normal, $^{a}P < 0.05$.

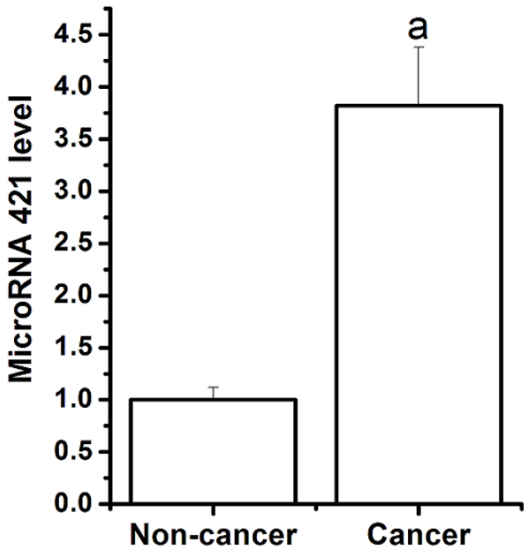

Figure 3. Intrahepatic miR-421 expression between non-cancer and liver cancer tissues (qRT-PCR testing). As results, liver cancer resulted in higher level of miR-421 than that in non-tumor tissue. Notes: cancer vs normal, $^{a}P < 0.05$.

Western blotting analysis

For immunoblotting analysis, equal amount of protein (40 μg/lane in each nontumor or tumor sample) was subjected to electrophoretic separation with SDS-PAGE, followed by targeting protein being blotted to membrane and incubated with antibodies against CD4 and CD8 (1:200, Boster, Wuhan, China). To validate protein loading, each membrane was re- incubated with Actin for final calculation [11, 12].

Statistical analysis

All final data are presented as means ± SD. Statistical analyses were conducted by using the Student's t test. Statistical significance was assessed when $^{a}P < 0.05$.

Results

Representative clinical parameters of HCC patients

As recorded in the hospitalized archive, blood plasma contents of liver functional enzymes (ALT, AST, ALP, GGT, TBA), and AFP content were markedly elevated when liver cancer development, which these changed results showed significant difference when compared to those in normal control ($P < 0.05$). Additionally, inflammation-related immune cells were time-dependently increased ($P < 0.05$), as well as the cell counts of $CD4^{+}$, $CD8^{+}$, and hepatic CD4/CD8 ratio were raised (**Figures 1** and **2**). More significantly, data from qRT-PCR showed that liver cancer had greater level of miR-421 than that in non-tumor tissue. And the expression level showed statistic difference when compared to control ($P < 0.05$, **Figure 3**).

Intrahepatic immunophenotype-labeled characteristics

In order to highlight the biocharacterization of cancer cells with immune supersensitivity, specific immunohistochemistry staining was used for further analysis. As results, liver cancer bio-

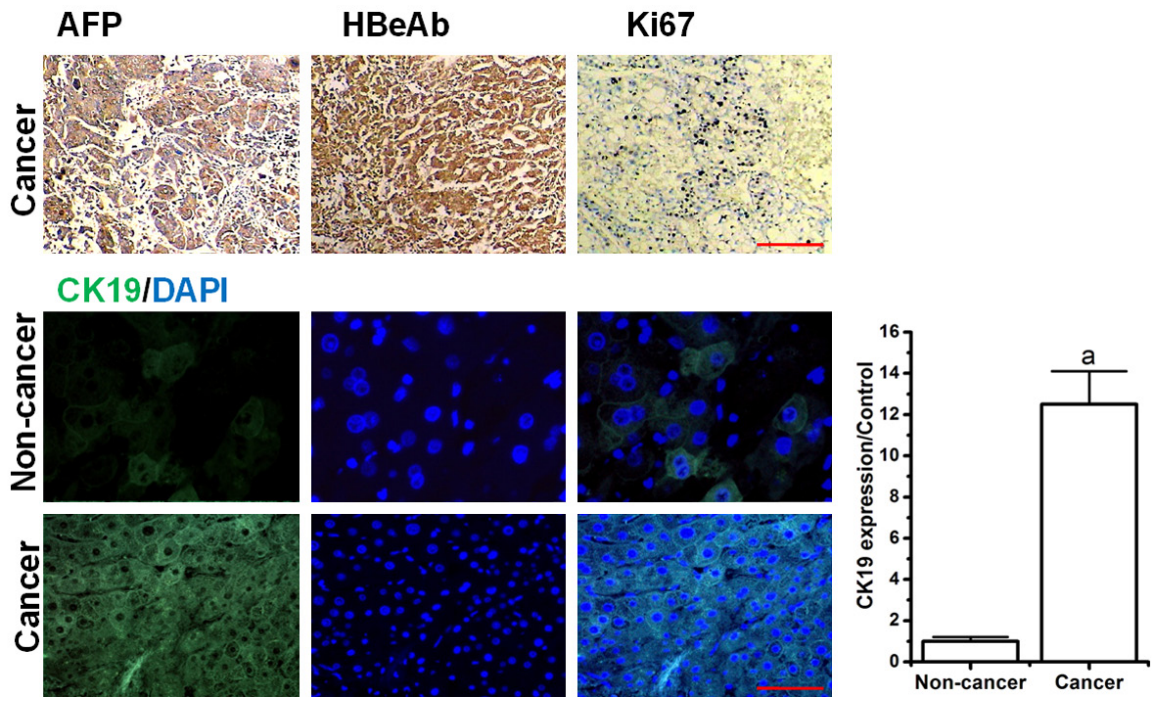

Figure 4. Microstructure pictures from representative HCC individual exhibited specific immunophenotypes for diagnosed markers for liver cancer progression (immunocytochemistrical staining; scale bar: 100 μm). More notably, CK19 implied the ability of epithelial differentiation and migration from the liver (immunofluorescence staining; scale bar: 200 μm).

markers for AFP and HBeAb were strongly expressed in the hepatocytes, with high area of positive endochylema-stained outcome. Significantly, compensatory cancer cell proliferation for ki67 immunoreactivity was observed around portal area and veniplex. Moreover, liver bile duct could be detected with plenty of CK19-positive cells which indicated metastasis tendency (**Figure 4**).

Discussion

Mounting scientific evidences suggest uncontrolled growth of cancer cells can be related to multifactorial coaction, including inflammation stress, immune deregulation [13]. In clinicopathological analysis, some representative biomarkers can be applicable for cancer diagnosis before making clinical prescription. Clinically, alpha fetoprotein (AFP) can serve as referenced biomarker to diagnose tumor in liver cancer, and abnormally high level of AFP in patients can be indicative for hepatocellular carcinoma [14]. Hepatitis B e antigen (HBeAg) is an infected hepatitis B viral protein that can be a strong induction factor for liver cancer development [15]. Notably, CK19, a cytokeratin, can reflect the ability of epithelial differentiation and migration, showing certain metastatic potentials [16]. Many existing miRNAs have links with cancer development, and microRNA has the potentials to be used as target for combating different cancers [17, 18]. Therefore, hepatocellular carcinoma management via reducing miR-421 level for regulating immune hypersensitivity can aid in suppression of tumor outgrowth in the liver.

As shown in clinicopathological observations, immunostaining and qRT-PCR indicate increased expressions of miR-421, AFP, HBeAg, Ki-67-, CK19-immunolabeled cells in the liver of HCC patient. These findings were matched for elevated levels of immune cells in whole blood and lymphocyte in peripheral blood during viral-derived liver cancer development. Taken together, miR-421-induced immune hypersensitivity in HCC patient should be normalized to inhibit progression of liver cancer with possible metastasis.

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Disclosure of conflict of interest

None.

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