Relationship between CD206 expression in macrophages and pathogenesis and prognosis of Hepatocellular carcinoma

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Abstract: Objective: to investigate the relationship between the expression of CD206 in macrophages and the pathogenesis and prognosis of Hepatocellular carcinoma (HCC). Methods: Human mononuclear macrophages (THP1) were cultured and stained with multi-labeling immunofluorescence staining based on tyrosine signal amplification technology using CD206 anti-proto and 4', 6- diamidino -2- phenylindomethacin (DAPI) markers simultaneously on 129 tissue microarray (TMA) containing HCC and its adjacent normal tissues. Immunohistochemistry (IHC) was used to detect the positive expression of the tumor-associated macrophage marker CD206 in HCC and the corresponding adjacent tissues on the tissue chip. Kaplan-Meier(log-rank test) and COX regression analysis were used to understand the correlation between the survival rate of patients and the expression of CD206 as well as each clinical index. Results: The number of CD68 positive macrophages (66.71±31.39) infiltrated into the carcinoma tissues was more than that of CD206 positive macrophages (30.24± 15.86) ($P < 0.001$), and the number of CD68 positive macrophages (79.33±22.74) in the paracancerous tissues was also more than that of CD206 positive macrophages (63.27 ±15.92) ($P < 0.001$). The average expression score of CD206 in carcinoma was higher than that adjacent to carcinoma (intra-carcinoma 3.68 ±1.02 points, adjacent to carcinoma 1.84 ±0.65 points, $P < 0.05$). The expression score of CD206 in the adjacent tissues was less than 6 points out of 84. The 5-year survival rate of HCC patients in the GP73 high expression group was not significantly different from that in the low expression group ($P = 0.8127$), but the survival rate of patients in the CD206 high expression group was significantly lower than that in the CD206 low expression group ($P = 0.0307$), and the difference was statistically significant. Conclusion: The mannose receptor CD206, which is closely related to TAM, is highly expressed in human primary hepatocellular HCC tissues as a whole, and the expression of CD206 in the adjacent tissues corresponding to each case is lower than that in the carcinoma. The high expression of TAM CD206 in cancer is an independent risk factor closely related to the prognosis of patients, which provides a theoretical basis for our subsequent intervention of CD206 in the treatment of HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is a pernicious tumor with high mortality in clinic, and its occurrence and development is a multi-stage and multi-step process. Studies have shown that under the action of some chronic inflammatory factors, liver cells activate hepatic stellate cells and macrophages to cause fibrosis and angiogenesis, further leading to the formation of abnormal nodules and malignant transformation [1]. With the deepening of research, people begin to pay attention to the effect of tumor microenvironment on the occurrence and development of tumors. Tumor microenvironment is mainly composed of macrophages, epithelial cells, immune cells, fibroblasts and some extracellular matrix, which plays an important role in the occurrence and development of tumors.

Tumor-infiltrating lymphocytes and tumr-associated macrophages (TAMs) are important matrix components of the microenvironment of HCC tissues [2–3]. CD68 is a specific molecular marker of macrophages, while CD206, mannose receptor 1 (MR1), is considered to be a marker of TAM, and its expression level can be used to determine the extent of TAM enrichment. On the other hand, anti-tumor therapy that inhibits tumor-associated macrophages is also effective. For example, CpG-

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ODN+IL-10 antibody therapy for breast cancer, STAT3, STAT6 inhibitor against melanoma, and CCL5/CCR5 antagonist against HCC have all achieved satisfactory results [4–5]. It is important to further find novel, efficient and economic TAM inhibitors [6].

There are few studies on the expression of CD206-positive macrophages in HCC and its impact on prognosis. In this study, the automatic MSI system and spectral resolution technology were used to accurately position and quantitate the expressions of the three proteins, aiming to analyze the expression of CD206 in HCC tissues and its impact on the prognosis of patients and provide some theoretical basis for the diagnosis and treatment of HCC.

2. Materials and methods

2.1. Cell culture

The human mononuclear macrophages (THP1 cells, HCC cells HepG2, and SMMC7721) were respectively inoculated into culture flasks, added with the complete medium, and cultured in a 37℃ 5% CO2 incubator until the logarithmic phase.

2.2. Main story

Anti-human polyclonal antibody hnRNP L (Beijing bioss Co., Ltd.); Mouse anti-human polyclonal antibody CD68, rabbit anti-human polyclonal antibody CD206, and rabbit anti-human polyclonal antibody CDlle (Pro—teintechGroup, the US); DAB color reagent kit, hematoxylin, general goat anti-rat/rabbit secondary antibody, and β-actin (Beijing Zhongshan Jinqiao Co., Ltd.); TRIzol reagent, ECL hypersensitivity luminescence kit, and reverse transcription kit (Thermo, the USA); PCR primers (Shanghai Biological Technology Co., Ltd.);

2.3. Method

2.3.1. Multi-label immunofluorescence staining method

Dewax and rehydrating that tissue chip; The antigen was repaired by microwave heating of citrate repair solution, and endogenous peroxidase was inactivated by 3% hydrogen peroxide, followed by serum blocking. Finally, 4', 6- diamidino -2- phenylindole (DAPI) cell nucleus was stained, followed by gradient ethanol dehydration, and neutral gum sealing. At the same time, we prepared a tissue chip with single fluorescence labeling CD206 and DAPI and HCC tissue autofluorescence (only incubating secondary antibody and fluorescein staining), dehydrated with gradient ethanol, sealed with neutral gum, and finally used automatic MSI system for histopathological imaging analysis.

2.3.2. Immunohistochemical prediction of CD206 expression in human HCC

Freshly obtained HCC tissue (intra-cancer+para-cancer) fixed in neutral formaldehyde for at least 12 hours; The fixed tissue blocks were placed in gradient ethanol, and the tissue was gradually dehydrated from low to high concentration for 45min in each stage of ethanol dehydration, and the tissue blocks were made transparent in gradient xylene after dehydration; The tissue was taken out for paraffin infiltration and embedding, and sectioned with a microtome. Immersing the glass slide in citrate or 1*EDTA solution, and putting into a microwave oven for high-grade boiling; Downshift, keep about 92℃ non boiling state for 25 minutes; The cassette was taken out, allowed to stand, cooled to room temperature, and moistened and washed with PBS for 3 minutes. The slides were gradually immersed in gradient alcohol for dehydration, and put into xylene for sealing with transparent neutral gum. After drying, the slides were observed under the microscope and counted.

2.4. Result judgment

CD206 was expressed in the cytoplasm, and the positive cells were stained brown. For the sections stained by immunohistochemistry, the evenly stained areas were selected in the 400-fold view, and the ratio of positive cells in the total quantity of cells was calculated: 0 point for non-

staining; Less than 25% for 1 point; 26%~50% for 2 points; More than 50% for 3 points. Determination of color intensity: 0 point means no color; Light coloring is 1 point; Brown is 2 points; Dark brown is 3 points. The sum of the above two items was the final judgment standard, and it was negative: < 2 points; Positive: ≥2 points.

2.5. Statistical analysis

Data statistics were performed using SPSS 21.0 (IBM Inc.); The difference in expression of CD206 in carcinoma and adjacent tissues was examined by paired t test or nonparametric test. The chi-square analysis was used to analyze the association between high/low expression of CD206 and each clinical index subgroup. Kaplan-meier method and LogRank test were also used to analyze the correlation between clinical data and prognosis. Survival analysis of multiple prognostic factors was performed using COX regression analysis. Measurement data were expressed as mean standard deviation (x±s), and the specified test level was α=0.05.

3. Result

3.1. Distribution and density of CD206 positive macrophage in HCC tissue and paracancerous tissue

The immunohistochemical results of CD68-positive or CD206-positive macrophages in HCC tissues are shown in Figure 1. Among them, 100 cases of HCC tissues were used as the experimental group, and 93 cases of paracancerous tissues were used as the control group. Although CD68-positive and CD206-positive macrophage infiltration were observed in all HCC and paracancerous tissues in this study, both macrophages were highly expressed in the paracancerous tissues of HCC, and the expression in the carcinomatous tissues was weaker than that in the paracancerous tissues. At the same time, the number of CD68-positive macrophages (66.71 ±31.39) infiltrated into the carcinoma tissues was more than that of CD206-positive macrophages (30.24 ±15.86) (P < 0.001), and the number of CD68-positive macrophages (79.33 ±22.74) in the paracancerous tissues was also more than that of CD206-positive macrophages (63.27 ±15.92) (P < 0.001).

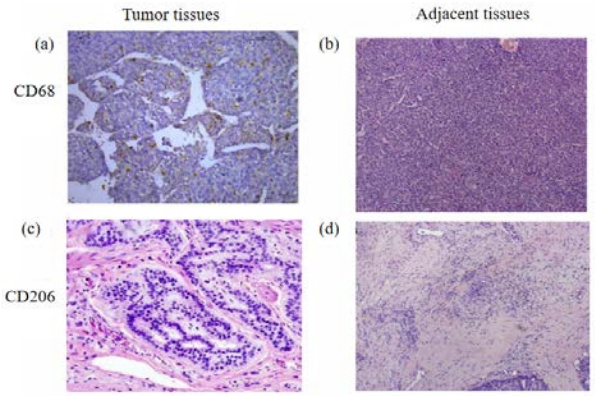

Figure 1 Expression of CD68-positive and CD206-positive macrophages in hepatocellular carcinoma and paracancerous tissues

The results of the Spearman grade correlation analysis showed that the intra-cancer tissues were significantly correlated with CD68-positive macrophages in the paracancerous tissues. There was also a significant correlation between CD206-positive macrophages and adjacent tissues within the cancer. In addition, the expression of CD68 in macrophages of HCC tissues is positively correlated with that of CD206, and the situation of adjacent tissues is similar.

3.2. Expression of CD206 in human HCC and adjacent tissues detected by tissue microarray

One hundred 200-point human HCC tissue chips were detected with CD206 antibody at a concentration of 1/4000, and the expression of CD206 was obtained by Aperio automatic digital

pathology system scanning (Figure 2). It could be seen that the staining results at each point were satisfactory, with no peeling and peeling points.

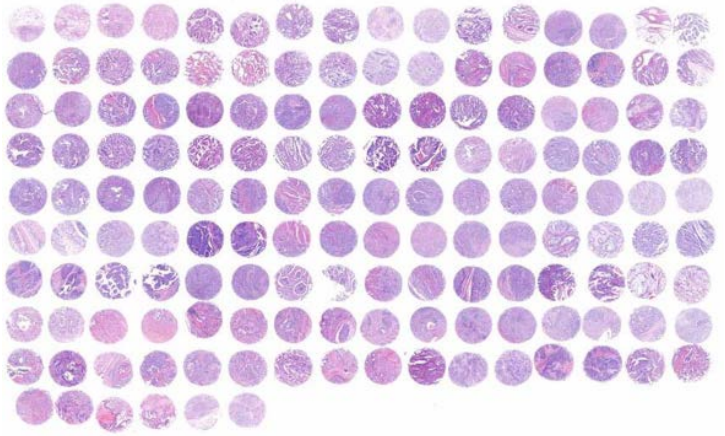

Figure 2 Expression of CD206 on tissue microarray in 200-point human hepatocellular HCC in 100 cases

The analysis revealed that the mean score of cd206 expression was higher in the cancer than in the adjacent area (3.68 ± 1.02 points in the cancer vs 1.84 ± 0.65 points in the adjacent area, $P < 0.05$).84 / 94 points were less than 6 points in the cd206 expression score of paracancerous tissues.

3.3. TAM enrichment in tumor microenvironment is closely related to the prognosis of patients with HCC

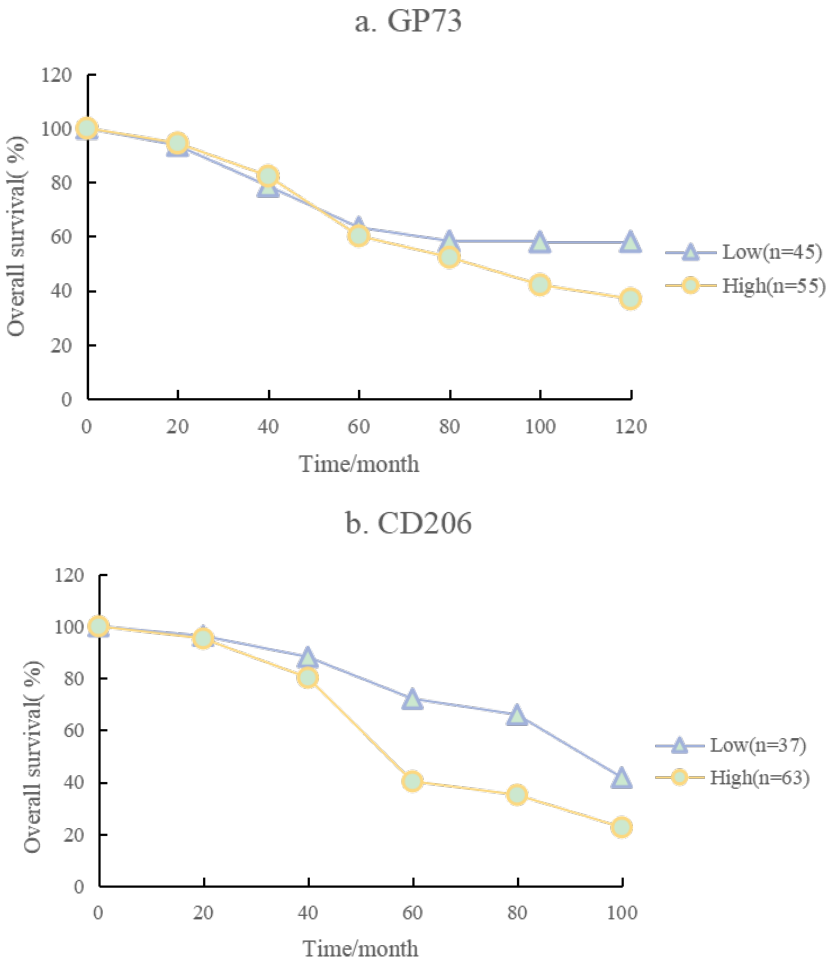

Figure 3 Survival rate of liver cancer patients

To further analyze the prognostic value of CD206 expression in HCC, we performed a statistical

analysis of survival in patients with high or low CD206 or GP73 expression. The results showed that although the 5-year survival rate of HCC patients in the high-expression group of GP73 was not significantly different from that in the low-expression group (P = 0.8127, Figure 3 a); However, the survival rate of patients in the CD206 high-expression group was significantly lower than that in the CD206 low-expression group (P = 0.0307, Figure 3 b), and the difference was statistically significant. The survival rate of patients in the group with high expression of both GP73 and CD206 was significantly lower than that in the group with low expression (P = 0.0338). These results suggest that high expression of both GP73 and CD206 may predict poor prognosis.

4. Discussion

HCC is a common inflammatory-related malignancy with an extremely high mortality rate. The recurrence and metastasis of HCC are the main causes of the persistently high mortality rate in patients with HCC. Although some progress has been made in the study of HCC in recent years, many key issues remain to be resolved. There is a class of macrophages with unique phenotype in the tumor microenvironment: tumor-associated macrophages, which are important constituent cells in the tumor microenvironment. TAMs acquire unique phenotypic characteristics under the action of local microenvironment, and thus can be divided into inflammation-related macrophages (M1 type) and tumor-related macrophages (M2 type) according to their phenotypes. Among them, inflammation-related macrophages with high expression of CD197 and HLA-DR [7] mainly mediate the inflammatory response of the body. Tumor-associated macrophages have high expression of CD163 and CD 206 on the surface [8], which are mainly involved in the occurrence and development of tumors [9].

CD206, mannose receptor 1, has been demonstrated to play an momentous role in promoting macrophage activation, antigen presentation, and immune response [10]. There are reports of high-density enrichment of CD206 in breast cancer, suggesting that high expression of CD206 in breast cancer may be related to its poor prognosis. To verify the intrinsic relationship between CD206 and HCC, and focus on whether CD206 can become a prognostic indicator of potential HCC, we collected clinical data and pathological specimens from 100 cases of human primary hepatocellular carcinoma and used tissue microarray to verify. Pre-experimental results clearly showed that CD206 was localized to macrophages in the human hepatic sinusoids. The tissue microarray showed high expression of CD206 in carcinoma, while the expression of CD206 adjacent to carcinoma was lower than that in carcinoma, and the difference in expression scores between the two had statistical significance.

There were more CD68-positive macrophages in both the intratumoral and paracancerous tissues in this study than in the CD206-positive macrophages, consistent with the fact that there were more abundant total macrophages than M2-like macrophages. In addition, some studies have found a significant correlation between CD68-positive macrophages in HCC and the number of CD163-positive macrophages; Similar experimental result have been obtained in our study: There was also a significant correlation between CD68-positive macrophages in cancerous tissues and the number of CD206-positive macrophages, as well as between adjacent tissues. The expression levels of CD68 and CD206 in the paracancerous tissues of HCC are significantly higher than those in the carcinomatous tissues, and their expressions on macrophages of carcinomatous tissues are positively correlated.

More and more evidences show that the activity of TAM can affect tumor progression, metastasis and chemotherapy drug resistance [11], and its activation is regulated by such signaling pathways as NF-κB, STAT3, and TLR4/IL-10 in the tumor microenvironment. Thus, enhanced secretion of GP73 before exacerbation or in malignant cells may be conducive to the progression of HCC. In fact, the correlation between GP73 and poor prognosis of HCC has always been controversial [12]. This study establish that high GP73 show had no significant correlation with patient survival rate, but up-regulation of both GP73 and CD206 could be used as a predictor of poor prognosis of HCC. In addition, CD206 is increased with the increase of GP73 expression. Whether GP73 can directly regulate the expression of CD206 and the mechanism by which GP73

plays a regulatory role are not known. Therefore, henceforth, we will conduct further studies on the molecular mechanism of GP73 exerting its biological function in the microenvironment of HCC.

5. Conclusions

In summary, we have found that the CD206 polymorphism is associated with the genetic susceptibility to HCC in the Chinese population. The GG genotype at this locus may be a hazard element for HCC, and the carrying of the GG genotype is an independent risk factor for postoperative recurrence of HCC. However, there is no significant correlation between the polymorphism at this locus and the main clinical features of HCC. The discovery of CD68 and CD206-related SNPs can further complement and enrich their involvement in the molecular biology and genetic mechanisms of HCC, and may become biological markers for early screening, early diagnosis and prognosis monitoring of HCC.

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