

行政院國家科學委員會專題研究計畫 成果報告

肝細胞癌及非腫瘤肝組織肝浸潤 T 細胞的分析-著重在 CD25 的表現

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一、中文摘要

（一）計畫中文摘要。（五百字以內）

肝細胞癌（簡稱肝癌）是全世界常見的癌之一，自從 1984 至今，肝癌就高居台灣十大癌症死因的首位，每年大約有 6000-8000 國人死於肝癌，雖然目前以超音波及甲種胎兒蛋白定期追蹤檢查，可以早期篩檢出肝癌，然而肝癌的治療仍不十分的理想。預後不良的原因，除了發現太晚之外，肝癌復發也是一個很重要的原因。理想上的癌症治療方式，應該能夠有效的區分出腫瘤細胞與正常細胞的不同，同時能有效的清除轉移至全身的癌細胞。由於免疫治療具有上述這兩個優點，因此免疫療法也成為治療肝癌一個非常有潛力的方式。

過去的研究指出，肝癌組織的腫瘤浸潤淋巴細胞，與肝癌的預後可能有關。既然腫瘤浸潤淋巴細胞可以跑到肝癌組織，可是肝癌卻仍可以不斷生長，此表示腫瘤浸潤淋巴細胞型態上或功能上的缺陷。與腫瘤有關的免疫功能失調，包括腫瘤浸潤 T 細胞可能會變成不反應，可能分泌不利於抗腫瘤免疫的輔助 T 細胞第二型細胞泌素，可能在 T 細胞接受器的訊息傳遞有缺陷，可能 T 細胞比較容易產生自發性凋亡的現象。腫瘤本身也可能分泌能抑制免疫的細胞泌素，或者表現出 FasL，而 FasL 可以誘發 T 細胞的凋亡，或者腫瘤細胞也會產生 Fas 的訊息傳遞缺陷，腫瘤細胞調降第一型 HLA 分子及與抗原處理有關的分子的表現，這些都可能使得體內的免疫細胞無法殺死腫瘤細胞。

腫瘤浸潤淋巴細胞調降 CD25 (IL-2 接受器 α)的表現，是腫瘤細胞能逃過免疫系統監控的一個原因之一。有一些文獻指出，各式各樣的腫瘤都可能出現腫瘤浸潤淋巴細胞調降表現 CD25，CD25 的調降表現可能與腫瘤分泌 matrix metalloproteinase 有關。肝癌病人的非腫瘤肝組織，若不是肝硬化就是纖維化，再加上 matrix metalloproteinase 的活化與表現，與肝硬化及纖維化很有關係。因此，在非腫瘤肝組織的肝組織浸潤淋巴細胞，有可能也會調降 CD25 的表現。

因此，本研究的主要目的，乃是要研究肝癌病人腫瘤浸潤細胞其表現型及分子型態。我們收集 28 例肝癌組織及其非腫瘤肝組織，分離出浸潤淋巴細胞，以流式細胞分析儀來分析其表現型及活化情況。我們發現在腫瘤浸潤淋巴細胞及非腫瘤肝組織浸潤淋巴細胞，大部份會表現 HLA-DR，CD69 等活化標記，但 CD25 的表現將是調降的。在腫瘤浸潤淋巴細胞中，有較高的比例表現 CD4⁺ CD25⁺，CD4⁺ CD25⁺ 是一種免疫調控 T 細胞，因此較高比例的 CD4⁺ CD25⁺ 腫瘤浸潤淋巴細胞，可能與肝癌病人的免疫失調有關。

我們相信經由這個研究，我們可以對於肝癌病人其腫瘤浸潤淋巴細胞的失調及其機轉，以及非腫瘤肝組織對於肝浸潤淋巴細胞功能的影響，有進一步的瞭解，此結果將有助於日後發展出對於慢性肝病及肝細胞癌有效的免疫治療策略。

關鍵詞：肝細胞癌，免疫治療，腫瘤浸潤 T 細胞，CD25，纖維化

Abstract

Background/Aims: Tumor may induce local immunosuppression and make the tumor-infiltrating lymphocytes (TILs) functionally inhibited and lose the ability to clonal proliferation and exert their cytotoxicity to tumor cells. Therefore, we investigated the phenotypic analysis of TILs in hepatocellular carcinoma (HCC) tissues. **Methods:** Lymphocytes were isolated from paired HCC tissues (TILs) and the corresponding non-tumor liver tissues (non-tumor-liver infiltrating lymphocytes, NILs) of 28 patients. These lymphocytes were subjected to flow cytometric analysis. **Results:** TILs had higher CD3⁺/CD56⁺ ratio than NILs. Around 70%-90% NILs or TILs expressed the antigen-experienced or memory phenotypes of CD45RA⁻ / CD45RO⁺ / CD62L⁻. Around 60%-70% CD4⁺ or CD8⁺ NILs and TILs expressed the activation markers CD69 and HLA-DR. However, we found that CD25 is under-expressed in both the CD8⁺ NILs and TILs. In addition, more CD4⁺ CD25⁺ regulatory T cells were detected in the TILs than in the NILs. **Conclusions:** Most of infiltrating lymphocytes from the HCC tissues and the corresponding non-tumor liver tissues were activated and expressed antigen-experienced phenotypes. However, the CD25 was underexpressed in the CD8⁺ TILs and the CD4⁺ CD25⁺ regulatory T cells were increased in the TILs. These factors might impair the antitumor immunity in HCCs.

Keywords: Tumor-infiltrating lymphocytes, liver-infiltrating lymphocytes, , regulatory T cells, hepatocellular carcinoma, activation

二、緣由與目的

Hepatocellular carcinoma (HCC) is one the most common malignancies in the world, especially in sub-Saharan Africa and Southeast Asia. Since 1984, it has been the leading cause of cancer death in Taiwan. About 6000-8000 people died of this cancer every year in Taiwan (1). Though regular sonographic examination can early detect small HCC (2) and there are many therapeutic modalities for HCC (3), the therapeutic results remains unsatisfactory because of the high recurrent rate.

Though cancer immunotherapies have been successfully demonstrated to be able to control the tumor growth in the murine models (4), ongoing clinical trials based on active immunotherapy often showed disappointing results. This can be explained by the fact cancer patients with large tumors may have a defective immune system which is secondary to their disease. In this regards, characterization of tumor-infiltrating lymphocytes (TILs) can greatly help us understand the immune responses and immunosuppression in cancer patients. The rationale to investigate TILs instead of peripheral blood mononuclear cells (PBMCs) in cancer patients is that certain local microenvironmental factors can suppress the functions of immune cells (5).

Limited studies have investigated the TILs in HCCs. However, these studies have a common drawback: the activation status and CD25 expression in the non-tumor-liver infiltrating lymphocytes, (NILs) were not explored. This point is very important because the majority of intrahepatic T cells express markers of activation, such as CD45RO, CD25 and CD69 (6). Comparing the activation status of TILs with PBMCs may just reflect the differences between intrahepatic T lymphocytes and PBMCs. Such comparison cannot reveal the true impact of HCC environment on the activation of TILs. Therefore, the ideal comparison should be done by comparing the TILs with NILs. Thus, in this current study, we investigated the phenotypes of TILs in HCC tissues, and compare the data with the NILs instead of PBMCs.

三、結果與討論

More T cells than NK cells infiltrated into HCC tumor tissues

Compared with NILs, TILs had higher CD3⁺/CD56⁺ ratio (6.1 vs. 2.3). The TILs also had higher CD3⁺CD56⁺/CD3⁺CD56⁺ (classical T/NTK) ratio (11.6 vs. 4.0) than NILs. This meant that more T cells than NK or NKT cells infiltrated into the HCC tumor tissues.

More CD4⁺ CD25⁺ T cells detected in TILs than in NILs

To understand the activation status of TILs, we analyzed the expressions of surface activation markers CD69, HLA-DR and CD25 in both CD4⁺ and CD8⁺ subpopulations. As shown in **Table 1**, around 70% and 60% of CD8⁺ NILs and TILs expressed the activation markers CD69 and HLA-DR respectively. In contrast, the expressions of CD25 in CD8⁺ NILs or TILs are much lower compared with the expressions of CD69 and HLA-DR. Only 1.0% of the CD8⁺ NILs and 7.7% of CD8⁺ TILs expressed CD25.

Similarly, around 60% of CD4⁺ NILs and TILs expressed CD69 and HLA-DR. The CD4⁺ TILs had marginally significant higher percentage of CD69 expression than the CD4⁺ NILs. Though the CD4⁺ TILs or NILs had lower CD25 expressions compared with the expressions of CD69 and HLA-DR, we found that the CD4⁺ TILs expressed higher levels of CD25 than NILs (28.2% vs. 11.2%) (**Table 1**).

Table 1. Phenotypic analysis of liver-infiltrating lymphocytes

Percentage of positive surface markers in CD8 ⁺ T cells						
	CD69 ⁺	CD25 ⁺ *	HLA-DR ⁺	CD45RO ⁺	CD45RA ⁻	CD62L ⁻ *
NIL	72.6±17.6	1.0±0.8	62.0±20.4	62.3±22.6	74.3±18.5	98.3±1.8
TIL	72.8±22.3	7.7±14.6	64.3±20.9	70.9±25.7	77.3±24.0	89.4±19.9
Percentage of positive surface markers in CD4 ⁺ T cells						
	CD69 ⁺ *	CD25 ⁺ *	HLA-DR ⁺	CD45RO ⁺	CD45RA ⁻	CD62L ⁻
NIL	56.3±13.3	11.2±8.4	50.6±16.4	87.5±13.1	85.2±12.4	90.3±17.8
TIL	65.6±21.2	28.2±16.7	58.3±14.5	89.3±13.8	83.8±21.9	85.9±22.4

Data expressed as mean ± standard deviation * p < 0.05 between NIL and TIL

Most TILs and NILs expressed antigen-experienced or memory phenotypes

To understand the antigen-experienced or naïve status of TILs, we analyzed the expressions of CD45RA, CD45RO, and CD62L in both CD4⁺ and CD8⁺ subpopulations. As shown in **Table 1**, around 70% of CD8⁺ NILs or TILs expressed CD45RO⁺/CD45RA⁻. Similarly, around 80%-90% of CD4⁺ NILs or TILs expressed the antigen-experienced phenotypes. In addition, around 90% of either CD4⁺ or CD8⁺ TILs or NILs were of the CD62L⁻ phenotypes. These results showed that most of the NILs or TILs expressed the antigen-experienced or memory phenotypes.

Thus, compared with CD69 and CD45RO, the percentage of CD25 expressions in both CD8⁺ TILs and NILs were much lower. The downregulation of CD25 in the TILs of HCCs was consistent with those found in other cancers (7-11). As the CD25 is the Interleukin-2 receptor, the downregulation of CD25 makes the TILs functionally inhibited and lose the ability to clonal proliferation. The exact mechanisms leading to CD25 downregulation on activated TILs are unknown. One of the causes for CD25 downregulation in TILs is that tumor-derived matrix metalloproteinases (MMPs) can induce the proteolytic cleavage of CD25 on activated T cells (12). The lower expression of CD25 in the NILs may also be explained by the MMPs, since accumulating data demonstrated that the MMPs play important roles in the hepatic fibrogenesis (13, 14). However,

the interaction between MMPs and immune system is very complicated and not quite well understood (15). This hypothesis needs to be confirmed in the future.

We found that the CD4⁺ TILs expressed higher levels of CD25 than NILs. An increasing body of evidence indicates that the CD4⁺ CD25⁺ T cells may be the regulatory T cells, which are powerful inhibitors of T cell activation both in vivo and in vitro (16-18). The CD4⁺ CD25⁺ regulatory T cells may be good for the maintenance of transplantation tolerance (17, 19), however, it might impair the antitumor immunity in cancer patients. Several studies demonstrated that regulatory T cells were increased in the PBMC or TILs of cancer patients (20, 21). These regulatory T cells may inhibit the antitumor immunity (16, 22, 23). However, it needs further studies to answer whether the higher expressions of CD4⁺ CD25⁺ T cells in the HCC tissues lead to impaired antitumor immunity and whether depletion of these regulatory T cells might be beneficial for the HCC immunotherapy.

Correlation between the phenotypes of TILs and NILs with clinicopathological parameters

The percentage of CD69 expression in CD4⁺ TILs was positively correlated with tumor size ($r=0.393$, $p=0.038$). The percentage of HLA-DR expression in CD8⁺ NILs was positively correlated with ALT levels ($r=0.455$, $p=0.022$). The percentage of negative CD45RA expression in CD8⁺ TILs was positively correlated with ALT levels ($r=0.434$, $p=0.021$). However, except the above items, there is no correlation between other phenotypes of TILs or NILs with the clinicopathological parameters.

四、計畫成果自評

We finished the project as scheduled in the original grant. We found something interesting in HCC TILs. However, the underlying mechanism for the observed phenomena is not clearly yet. We are currently investigating the influence of hepatic fibrogenesis and CD25 expression of liver-infiltrating lymphocytes.

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