

肝细胞癌组织中FoxP3⁺调节性T细胞与 上皮间质转化和患者预后的关系

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[摘要] **背景与目的:** 肿瘤微环境中的调节性T细胞(tumor-infiltrating regulatory T cells, Tregs)在肿瘤的发生、发展中起重要作用, 上皮-间质转化(epithelial-mesenchymal transition, EMT)是恶性肿瘤获得转移和侵袭能力的重要生物学过程, 但对肝癌浸润性Tregs与EMT之间的关系尚不清楚, 本研究探讨原发性肝细胞癌(hepatocellular carcinoma, HCC)组织浸润的Tregs与EMT的关系及其临床意义。**方法:** 采用免疫组化和核酸原位杂交技术检测74例原发性HCC和20例癌旁组织中Tregs标志物FoxP3和EMT标志物(E-cadherin、Vimentin蛋白和Snail mRNA)的表达。**结果:** HCC组织中每高倍视野的FoxP3+Tregs数为9.7(3.8~20.2)个, 癌旁组织为1.7(0.5~4.2)个($P<0.01$), 有门静脉癌栓和镜下癌栓的HCC组织Tregs数高于无门静脉癌栓和镜下癌栓组($P<0.05$), 高Tregs组术后无瘤生存率、总体生存率均低于低Tregs组($P<0.05$, $P<0.01$)。Spearman相关分析表明, E-cadherin阳性率分别与另两种EMT标志物呈负相关(Vimentin: $r=-0.572$, $P<0.01$; Snail: $r=-0.597$, $P<0.01$)。Tregs数量与E-cadherin呈负相关($r=-0.513$, $P<0.05$), 与Vimentin和Snail呈正相关(Vimentin: $r=0.598$, $P<0.01$; Snail: $r=0.423$, $P<0.05$)。**结论:** HCC组织中浸润的Tregs有利于肝癌的侵袭、转移, 与EMT关系密切, 并且对患者预后判断有一定的参考价值。

[关键词] 肝肿瘤; 肝细胞癌; 调节性T细胞; 上皮-间质转化

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The relationship of the tumor-infiltrating FoxP3⁺ Tregs with epithelial-mesenchymal transition and prognosis of hepatocellular carcinoma XU Jun¹, WANG Jian¹, HU Yong¹, CHENG Lan-lan¹, YU Hong-yu² (1. Department of Pathology, the 105th Hospital of PLA, Hefei Anhui 230031, China; 2. Department of Pathology, Changzheng Hospital, Second Military Medical University, Shanghai 200003, China)

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[Abstract] **Background and purpose:** Tumor-infiltrating regulatory T cells (Tregs) play an important role in the development of various cancers, Epithelial-mesenchymal transition (EMT) is a key step toward cancer metastasis, but the interaction between Tregs and EMT has not yet been evaluated. We aimed to investigate the relationship of FoxP3+ Tregs and epithelial-mesenchymal transition (EMT) in hepatocellular carcinoma (HCC) and to determine the clinical significance of Tregs. **Methods:** Seventy-four sets of patient-matched tumors and twenty sets of peritumoral tissues were obtained during curative resection for HCC. The expression of FoxP3, E-cadherin, Vimentin protein and Snail mRNA was detected by immunohistochemistry and in situ hybridization histochemistry in formalin-fixed and paraffin-embedded tissues. **Results:** The average number of intratumoral Tregs was significantly higher than that in corresponding peritumoral tissues [9.7 (3.8-20.2) vs 1.4 (0.5-4.2), respectively; $P<0.01$]. The number of intratumoral Tregs was positively correlated with portal vein tumor thrombus and microvascular invasion ($P<0.05$). Increased number of intratumoral Tregs was significantly associated with decreased rates of recurrence-free and overall survival ($P<0.05$, $P<0.01$). There was a significant negative correlation between E-cadherin and the other EMT-related markers (Vimentin: $r=-0.572$, $P<0.01$, Snail: $r=-0.597$, $P<0.01$). Increased number of intratumoral Tregs was characterized by increased expression of EMT-related proteins (Vimentin: $r=0.598$, $P<0.01$; Snail: $r=0.423$, $P<0.05$) and loss of E-cadherin

expression($r=-0.513$, $P<0.05$). **Conclusion:** Intratumoral infiltration by Tregs is closely related to metastasis and EMT in HCC, FoxP3+ Tregs may be a potential prognostic indicator for HCC patients.

[Key words] Hepatocellular neoplasms; Hepatocellular carcinoma; Tregs; Epithelial mesenchymal transition

肿瘤局部微环境中的免疫细胞在肿瘤的发生、发展中起重要作用^[1]。调节性T细胞(tumor-infiltrating regulatory T cells, Tregs)在肝癌、乳腺癌等肿瘤组织中浸润增多^[2-3],但其作用机制尚不完全清楚。上皮-间质转化(epithelial-mesenchymal transition, EMT)是指上皮细胞通过特定程序转化为具有间质表型的细胞,是上皮来源的恶性肿瘤获得转移和侵袭能力的重要生物学过程^[4],近年来EMT与肿瘤微环境中的免疫细胞之间的相互作用一直是研究热点。有研究报道利用Snail cDNA转染大鼠黑色素瘤细胞后,除了可以诱导出EMT外还伴随着Tregs增多引起免疫耐受,促进肿瘤转移^[5],由此设想肝细胞癌(hepatocellular carcinoma, HCC)组织中Tregs增多可能与EMT有一定关联。本实验采用免疫组化和核酸原位杂交技术检测肝细胞癌中FoxP3+ Tregs和EMT标志物(E-cadherin、Vimentin蛋白质和Snail mRNA)的表达,探讨HCC组织浸润的Tregs与EMT的关系及其临床意义。

1 资料和方法

1.1 临床资料

该研究经中国人民解放军第105医院伦理委员会审核通过。74例原发性HCC选自2005—2012年中国人民解放军第105医院普外科手术切除并经病理证实的组织,男性66例,女性8例,年龄11~85岁,中位年龄46岁,术前均未行放疗和化疗;Edmondson组织学分级I+II级53例,III+IV级21例,UICC分期I+II期39例,III+IV期35例。癌旁组织(对照组)取自距离癌灶边缘5cm以外的非癌组织共20例。术后患者每3~6个月行常规腹部B超或彩色多普勒、胸片和甲胎蛋白检查复发转移情况,必要时行CT或MRI检查,若出现转移复发,则为无瘤生存终止时间,随访采用门诊复查和定期电话回访,随访截止日期为2013年11月31日。

1.2 免疫组化染色

鼠抗人FoxP3单克隆抗体购自台湾Abnova公司(克隆号2A11G9, Cat: MAB5583),工作浓度1:400,兔抗人E-cadherin、Vimentin单克隆抗体购自福州迈新公司(即用型),免疫组化试剂盒Ultraview Universal DAB detection kit(D06808)为Roche公司产品。石蜡切片脱蜡至水,其余步骤均按在全自动免疫组化仪(BenchMark-GX, Roche公司)进行。EDTA抗原修复8 min,0.3% H₂O₂阻断内源性过氧化物酶,二抗为HRP multimer, DAB显色,苏木素复染,分别设阳性对照、阴性对照和空白对照。

1.3 原位杂交

人Snail mRNA原位杂交检测试剂盒购自博士德生物工程有限公司,寡核苷酸探针序列:5'-AGAGTTTACCTTCCAGCAGCCCTACGACCAGGCC-3',5'-AAGGCCTCAACTGC A A T A C T G C A A C A A G G A A T A -3',5'-TACCAGTGCCAGGCGTGTGCTCGGACCTTCTCCCG-3'。石蜡切片脱蜡至水,3% H₂O₂室温作用10 min,胃蛋白酶室温消化30 min,冲洗后每张切片滴加20 μL预杂交液,湿盒放入40℃恒温箱中4 h。滴加20 μL杂交液/片,38~42℃恒温箱中过夜。用37℃预温的2×SSC、0.5×SSC、0.2×SSC液先后冲洗,滴加封闭液37℃30 min,加生物素化地高辛37℃60 min,再加生物素化地高辛和SABC,37℃20 min,滴加生物素化过氧化物酶37℃20 min, DAB显色,苏木素复染,分别设阳性对照、阴性对照。

1.4 结果判断

以FoxP3核阳性的淋巴细胞数量作为组织中Tregs数量,随机取5个高倍视野(×400)分别计数HCC组织和癌旁肝组织中Tregs绝对数,取平均数为每个样本的FoxP3+ Tregs数量。E-cadherin、Vimentin以细胞质或膜出现棕黄色颗粒为阳性,Snail mRNA以细胞核或质出现棕黄色颗粒为阳

性。计数方法为随机观察10个×400高倍镜视野, 取平均值为阳性细胞百分数, 0为0分, <10%为1分, 10%~50%为2分, 51%~80%为3分, >80%为4分; 阳性强度分为弱(1分)、中等(2分)、强(3分), 两者相乘得出总分值(0~12)。

1.5 统计学处理

所有数据均用SPSS 19.0软件处理, HCC和癌旁的Tregs数量以中位数和四分位数间距即M(Q_R)表示, 2组间比较采用Mann-whitney U检验, HCC组织中Tregs数量与临床病理参数之间的关系采用 χ^2 检验, Tregs数量与E-cadherin、Vimentin和Snail之间的关系采用Spearman等级资料相关分析, 采用Kaplan-Meier方法和Log-rank检验进行生存分析, $P<0.05$ 为差异有统计学意义。

2 结果

2.1 HCC及癌旁组织FoxP3的表达情况

FoxP3阳性细胞主要位于HCC组织和癌间质中, 为细胞核着色。HCC组织中每高倍视野(×400)的FoxP3⁺ Tregs平均个数为9.7(3.8~20.2)个, 癌旁组织为1.7(0.5~4.2)个, 差异有统计学意义($P<0.01$, 图1)。

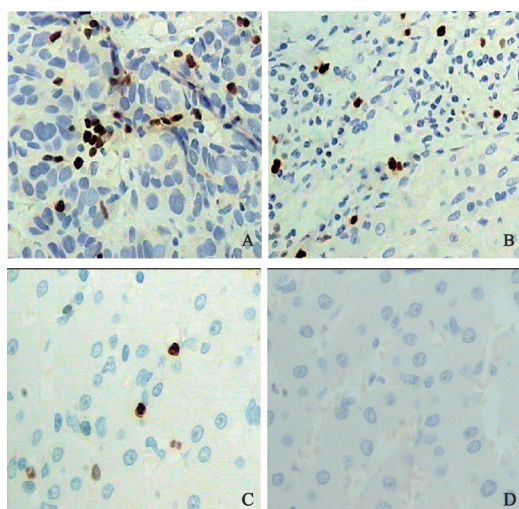


图1 FoxP3在HCC和癌旁肝组织中的表达

Fig. 1 Expression of FoxP3 in HCC and peritumoral tissues

(Elivision, ×400)
A. FoxP3 exhibited distinct nuclear staining in a surgical specimen of less differentiated HCC; B: FoxP3⁺ regulatory T cells in the stroma of HCC; C: Only a small number of FoxP3⁺ T cell were found in the peritumoral tissues; D: There were no FoxP3⁺ expression in the peritumoral tissues.

2.2 HCC组织中Tregs数量与临床病理参数之间的关系

按Tregs中位数将样本分成高Tregs组和低Tregs组, 高表达36例, 低表达38例, 有门静脉癌栓和镜下癌栓的HCC组织FoxP3⁺ Tregs数高于无门静脉癌栓和镜下癌栓组($P<0.05$, 表1)。

表1 HCC中Tregs的数量与患者临床病理参数的关系

Tab. 1 Relationship of FoxP3⁺ Tregs and clinicopathologic characters of 74 HCCs

Clinical character	Tregs		χ^2	P value
	High	Low		
Gender			2.008	0.156
Male	34	32		
Female	2	6		
Age/year			0.196	0.658
≥46	17	16		
<46	19	22		
Tumor size/cm			3.409	0.065
≥5	20	13		
<5	16	25		
Multiple tumors			0.089	0.766
Yes	13	15		
No	23	23		
Grade			2.060	0.151
I+II	23	30		
III+IV	13	8		
UICC stage			3.425	0.064
I+II	15	24		
III+IV	21	14		
HBs antigen			2.154	0.142
Yes	24	31		
No	12	7		
Cirrhosis			0.571	0.450
Yes	23	21		
No	13	17		
Serum AFP/ng·mL ⁻¹			0.892	0.345
≥350	21	18		
<350	15	20		
Microvascular invasion			4.800	0.028
Yes	18	9		
No	18	27		
Major vascular invasion			6.504	0.011
Yes	21	11		
No	15	27		

2.3 Tregs数量与HCC预后的关系

74例HCC患者, 22例失访, 到随访截止期末复发未死亡6例、已复发未死亡5例, 记为截尾值。结果显示高Tregs组术后无瘤生存率、总体生存率均低于低Tregs组($P<0.05$, $P<0.01$, 图2)。

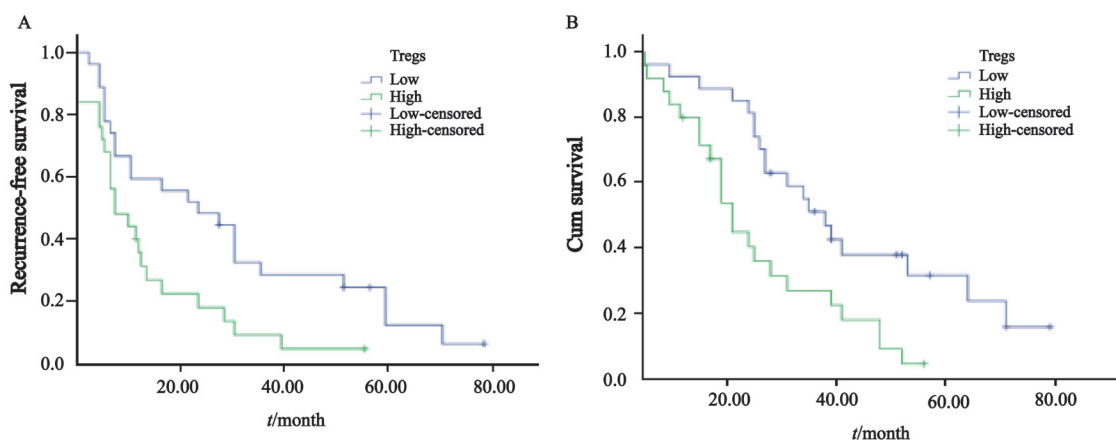


图2 高Treg组和低Treg组术后无瘤生存率、总体生存率的比较

Fig. 2 Correlation between Tregs and the rates of recurrence-free and overall survival

A: Increased number of intratumoral Tregs were significantly associated with decreased rates of recurrence-free ($\chi^2=6.251, P=0.012$); B: High level of Tregs were associated with decreased rates of overall survival ($\chi^2=8.001, P=0.005$).

2.4 Tregs数量与EMT的相关性

E-cadherin表达于部分癌细胞和正常肝细胞，细胞膜阳性，Vimentin细胞质阳性，Snail mRNA定位于细胞质或细胞核。Spearman相关分析表明，E-cadherin阳性率分别与另两种EMT标志物呈负相关(Vimentin: $r=-0.572, P<0.01$; Snail: $r=-0.597, P<0.01$)。Tregs数量

与E-cadherin呈负相关($r=-0.513, P<0.05$)，与Vimentin和Snail呈正相关(Vimentin: $r=0.598, P<0.01$; Snail: $r=0.423, P<0.05$ ，图3)。图3A、B、C、D均为同一例HCC组织，Tregs数量高(29个/HPF)，Vimentin和Snail表达显著增加，而E-cadherin显著下降。

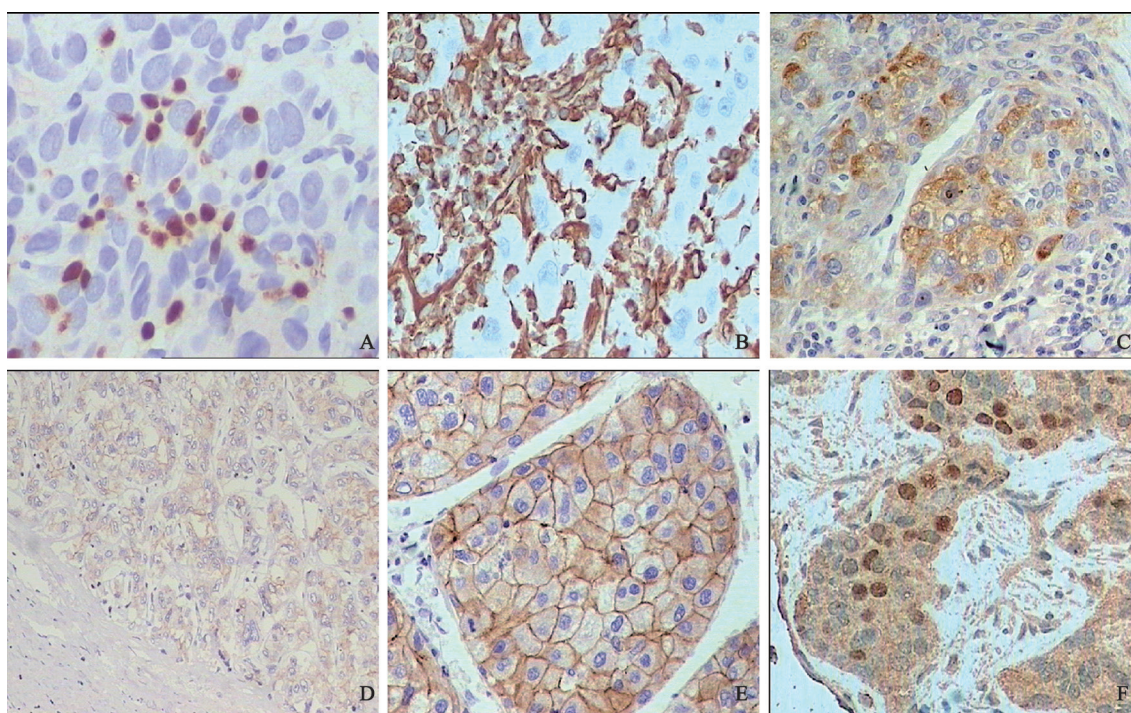


图3 EMT相关标志物在HCC组织中的表达

Fig. 3 Expression of EMT-related markers (E-cadherin, vimentin and Snail) in HCC

A: FoxP3 (Elivision, $\times 400$); B: Vimentin (Elivision, $\times 200$); C: Snail (ISH, $\times 200$); D and E: E-cadherin (Elivision, $\times 200$); F: Snail (ISH, $\times 200$).

3 讨 论

Tregs在稳定自身免疫环境中发挥重要作用,但在抗肿瘤免疫应答中,Tregs则主要发挥抑制效应,通过抑制CD4⁺和CD8⁺T淋巴细胞白介素2基因的转录和表达来抑制T淋巴细胞的活化增殖,从而营造免疫抑制微环境^[6]。Foxp3是CD4⁺CD25⁺Tregs的一个特征性标志,是免疫抑制的关键调节分子^[7]。目前认为肿瘤局部微环境中的免疫细胞在肿瘤的发生、发展中起重要作用^[1]。有研究显示,HCC实验小鼠模型的脾组织内Tregs数目较正常小鼠显著增加^[8],肿瘤患者外周血及肿瘤微环境中Tregs数量较正常人明显增多,并且多种肿瘤包括乳腺癌、结肠癌等组织中Tregs数量较癌旁组织增多^[7,9]。本研究显示,HCC组织中FoxP3⁺Tregs数明显高于癌旁组织,与上述研究结果基本相符,提示Tregs可以促进HCC的发生。本实验结果还显示,有门静脉癌栓和镜下癌栓的HCC组织FoxP3⁺Tregs数高于无门静脉癌栓和镜下癌栓组,提示Tregs在HCC的进展和转移中发挥作用。可能因Tregss具有免疫抑制作用,与相应癌旁组织相比,癌组织Tregs浸润明显增多,使HCC微环境处于免疫抑制状态,从而使肝癌细胞逃脱自身免疫监视,促使肝癌的发生、发展和转移。

本研究结果显示,高Tregs组术后无瘤生存率、总体生存率均低于低Tregs组,癌组织Tregs浸润越多,患者生存时间越短,提示肿瘤浸润的Tregs数量增多不仅预示肿瘤播散、转移的危险,还能够预示患者的预后,与徐原林等^[11]在弥漫大B细胞淋巴瘤、Bates等^[12]在乳腺癌、Huang等^[1]在肝癌、Salama等^[13]在结直肠癌中的研究结论一致。通过检测术后HCC标本Tregs的数量可能对肿瘤的转移、预后做出判断和预测。如果能去除或减少HCC患者体内的Tregs或调节其对各种免疫活性细胞的免疫抑制功能,有可能提高HCC的免疫治疗效果,从而提高HCC患者的无瘤生存率和总体生存率。但该

结果是建立在组织病理学上,必须提供肝穿或手术切除标本用于检测,下一步需检测外周血Tregs水平并研究其与患者预后的关系,以便全面探讨Tregs的预后价值。

EMT在癌症浸润转移过程中发挥着重要作用。有研究显示,在肿瘤侵袭前沿的细胞发生EMT后可获得移动、抗凋亡能力以及保持干细胞样特性等^[14]。近年来,EMT与肿瘤微环境中的免疫细胞之间的相互作用一直是研究热点,但对HCC浸润性Tregs与EMT之间的关系尚不清楚。研究报道利用Snail cDNA转染大鼠黑色素瘤细胞和脾细胞共培养可以诱导出EMT,另外还伴随着Tregs增多引起免疫耐受,促进肿瘤转移^[5]。Snail是一种含锌指结构的DNA结合蛋白,可以结合E-cadherin启动子部位的E-box连接基序,抑制E-cadherin基因的表达及上调Vimentin的表达,引起上皮细胞向间质细胞表型转化,产生EMT,是关键的EMT调控因子^[15]。本研究通过检测EMT标志物E-cadherin、Vimentin蛋白和Snail mRNA在HCC组织中的表达,结果显示E-cadherin表达与Vimentin和Snail表达呈负相关,证实了HCC组织中EMT现象的存在。本研究结果还显示,Tregs的数量与E-cadherin呈负相关,与Vimentin和Snail呈正相关,说明HCC组织中浸润的FoxP3⁺Tregs对EMT有重要影响。综上所述,HCC组织中浸润的Tregs有利于肝癌的侵袭、转移,与肝癌细胞的EMT关系密切,并且对患者预后判断有一定的参考价值,可作为治疗靶点成为控制肿瘤转移的策略之一。今后有必要通过体外实验进一步明确,在HCC中,是Tregs诱导了EMT,还是EMT激发了Tregs的免疫活性。

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