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RAPID COMMUNICATION

Favorable prognostic role of IL-26 in HCC patients associated with JAK-STAT3-dependent autophagy

Interleukin-26 (IL-26), originally called AK155, is one of the cytokines of the IL-10 cytokine family, and its gene is located in human chromosome region 12q15. IL-26 signals through the receptors IL-20R1 and IL-10R2, which form a heterodimer, targets specific cells and induces activation of the JAK-STAT pathway, allowing the rapid phosphorylation of STAT1 and STAT3 and the initiation of their effects.[1] This signaling plays an important role in the regulation of host defense and inflammatory diseases. However, whether the role of IL-26 in the progression of hepatocellular carcinoma (HCC) occurs via autophagy remains unclear. Here, we analyzed the association between IL-26 levels and the clinicopathological characteristics of 115 patients with HCC and found that IL-26 levels were particularly associated with tumor stage and survival. We found that IL-26 levels were associated with the prognosis of HCC patients. Furthermore, we found that increased autophagy by IL-26 was dependent on the JAK-STAT3 signaling pathway, which could result in the inhibition of the migration and invasion abilities of these HCC cells. These findings suggest that the levels of IL-26 may be a useful prognostic marker for HCC patients and may offer insights into the clinical application of IL-26 in the future.

IL-26 is highly expressed in HCC patients. Among the 15 interleukins we selected, mainly those from the IL-10 family, we only found statistically significant differences in the expression of IL-26 mRNA in cancer and adjacent tissues ($P < 0.05$, Fig. 1A, $n = 60$ per group). In addition, we also found the higher IL-26 levels in patient serum ($P < 0.0001$, Fig. S1A) and tissues (Fig. 1B, Fig. S1B). Furthermore, through the chi-square test, we found that the IL-26 protein levels were significantly different according to tumor stage ($P = 0.010$) and HCC patient survival ($P < 0.0001$) (Table S1).

In solid HCC tumors, we directly found that IL-26 immunolabeling strongly overlapped with a known marker for the macrophage lineage, CD68 staining (Fig. 1C, Fig. S1C).

Next, we measured the proliferative and apoptotic effects of IL-26 on three HCC cell lines. And no effects on proliferation were observed at the indicated concentrations of IL-26 ranging from 0 ng/mL to 10 ng/mL (Fig. S1D–F). We also observed no apoptotic effects of IL-26 on HCC SK-hep-1 cell lines after 24 h (Fig. S1G–H).

Previous studies suggested that interleukins had effects on the JAK2-STAT3 signaling pathway, so we first investigated the effect of IL-26 on the JAK2-STAT3 pathway in two HCC cell lines using Western blotting. Interestingly, we found that the phosphorylation of STAT3 at the tyrosine 705 site decreased greatly as the IL-26 level increased gradually (Fig. 1D–E). Furthermore, the decreased phosphorylation at the tyrosine 705 site of STAT3 may activate downstream pathways, especially the autophagy pathway, to change the fate of cells. Therefore, the next step of this study will focus on autophagy changes.

After exposure to 20 μM hydroxychloroquine (HCQ) for 4 h, proteins related to autophagic flux signals were examined by Western blot in the SK-hep-1 (Fig. 1F) and HuH-7 (Fig. S2A) HCC cell lines treated with IL-26 at the indicated concentrations for 24 h. We observed that the expression of LC3-II was gradually increased as the IL-26 level increased combined with or without HCQ in the two cell lines.[2] However, the opposite trend was observed in the expression of SQSTM1/p62 and Bcl2 in the presence or absence of HCQ. Moreover, we investigated the changes in the numbers of autophagic vacuoles after IL-26 administration in HCC SK-hep-1 cells using transmission electron microscopy. We found that the number of autophagic vacuoles increased significantly after treatment with 10.0 ng/mL IL-26 compared with the control ($P < 0.001$, Fig. S2B, C).

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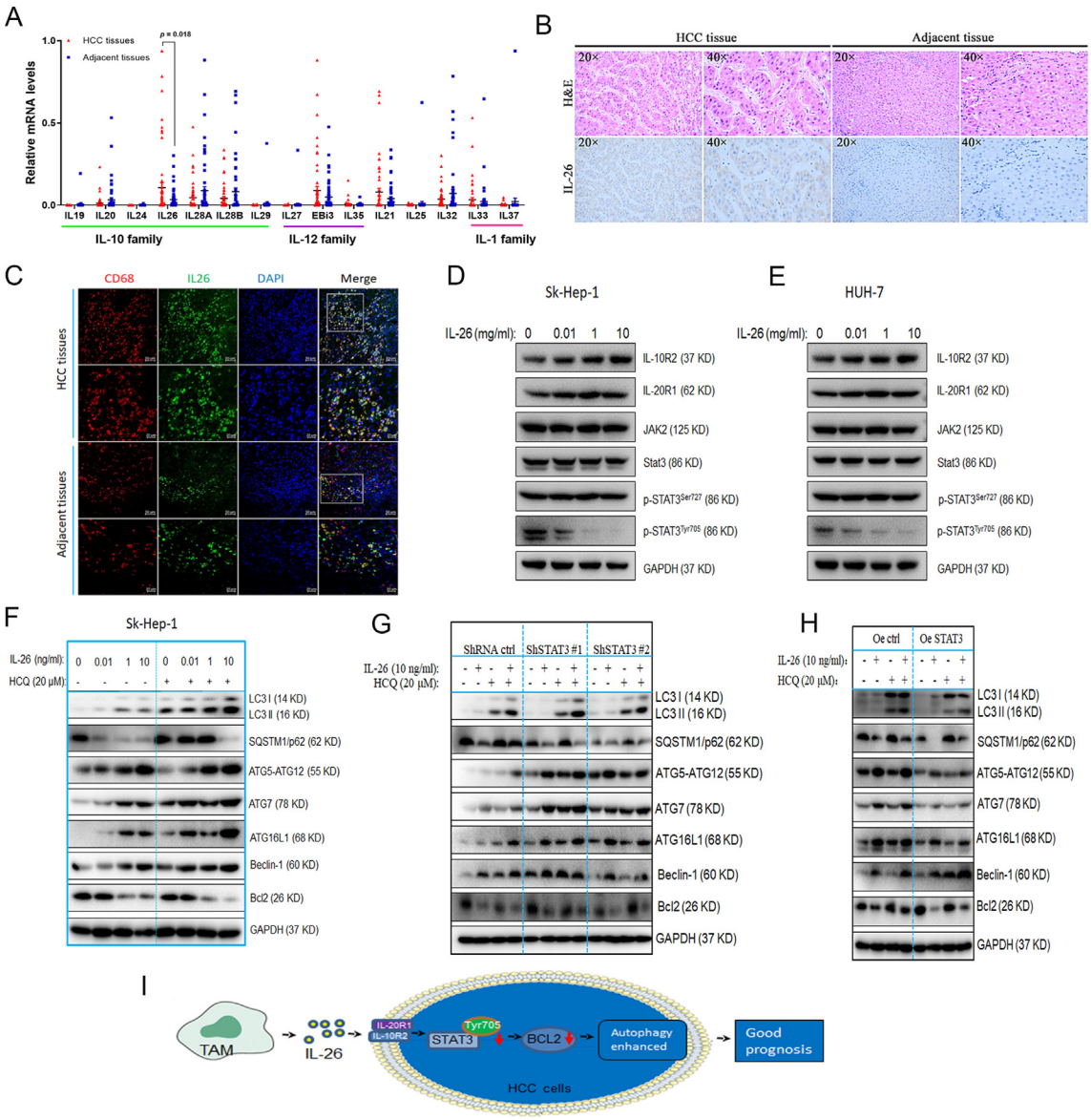

Figure 1 The increased autophagy by IL-26, which was highly expressed in HCC patients and was partly derived from tumor-associated macrophages, was dependent on the JAK-STAT3 signaling pathway. **(A)** Relative mRNA expression levels of IL-26 and some other interleukins in HCC tissues ($n = 60$) and adjacent HCC tissues ($n = 60$) from HCC patients were determined with qRT-PCR. **(B)** Representative images of protein levels of IL-26 in HCC tissues ($n = 115$) and peri-HCC tissues ($n = 115$) using immunohistochemical staining, along with their images using H&E staining at 10 × or 40 × magnification for each row. **(C)** Confocal microscopy analysis of the colocalization of IL-26 and CD68 in double-immunolabeled human peri-HCC tissue sections and HCC sections. The nuclei were stained with DAPI. The scale bar is 50 μm in the upper representative images of adjacent tissues and HCC tissues and 20 μm in the lower representative images of adjacent tissues and HCC tissues. JAK2-STAT3 signals were in Sk-hep-1 cells **(D)**, HuH-7 cells **(E)** and the autophagic signals in Sk-hep-1 cells **(F)** were analyzed by immunoblotting after treatment with the indicated concentrations of IL-26 for 24 h. With STAT3 knockdown (ShSTAT3) or STAT3 overexpression (Oe STAT3), the changes in the autophagic pathway (G and H) after treatment with IL-26 (10 ng/mL) were measured using western blotting. The western blotting experiments were conducted in triplicate. **(I)** Schematic diagram of the role of IL-26 in the prognosis of patients with HCC via JAK-STAT3-dependent autophagy.

The previous results in the present study showed that the effects of IL-26 were related to the activation of STAT3, so we knocked down and overexpressed STAT3 in HCC SK-hep-1 cells using lentiviral transfection. After confirmation of the mRNA and protein expression of STAT3 (Fig. S2D, E), we first investigated cell migration and invasion using a wound healing assay (Fig. S3A, B) or Transwell migration (Fig. S3C, D) and invasion assays (Fig. S3E, F) after treating

the cells with 10 ng/mL IL-26. We found that IL-26 inhibited migration and invasion in a STAT3-dependent manner.

We first used an online liver RNA-seq analysis system to analyze the relationship between IL-26 mRNA levels and patient prognosis (http://kmplot.com/analysis/). High mRNA levels of IL-26 (gene ID: 55,801) were found to be positively correlated with good prognosis in this cohort of 364 HCC patients (log rank $P = 3.6 \times 10^{-8}$, Fig. S4A). Moreover, Kaplan–Meier log-rank (Mantel–Cox) analysis showed that the protein levels of IL-26 exhibited strong significant differences with the survival of HCC patients ($P < 0.0001$, Fig. S4B and Table S1), which was consistent with the trends of good prognosis in IL-26 mRNA expression and survival.

After knocking down STAT3, we examined the autophagic pathway and found that the expression of LC3 II increased greatly after treatment with 10 ng/mL IL-26, while the expression of p62 decreased after treatment with 10 ng/mL IL-26 compared with each control (Fig. 1G). The expression of Bcl2, an anti-apoptosis protein, decreased in response to 10 ng/mL IL-26 (Fig. 1G) compared with each control. Combined with the administration of HCQ, we found more direct changes in the autophagic signals after STAT3 knockdown.

Once STAT3 was overexpressed, we observed that the expression of LC3 II also increased in response to 10 ng/mL IL-26 (Fig. 1H). The expression of SQSTM1/p62 and Bcl2 decreased greatly after treatment with 10 ng/mL IL-26 compared with the respective controls (Fig. 1H). Consistent with the previous results in the present study, we found that the expression of phospho-STAT3^{Tyr705} (Fig. S4C, D) decreased in response to 10 ng/mL IL-26 compared with the respective controls in both groups, but with the differences in degree. So, the effects of IL-26 on autophagy are linked to the JAK-STAT3 pathway. The schematic diagram of the present study is summarized in Figure 1I.

Generally, autophagy is thought to prevent the origination of cancers, but once cancers are established, increased autophagic flux often favors tumor cell survival and growth.[3] Interestingly, by both activating autophagy and inhibiting it in advanced cancers, this paradoxical, contradictory suggestion has been used as a therapeutic strategy.[4,5] From this study, the survival or growth of tumor cells is only a part of the consequences of autophagy, and the other important part is the ability of tumor cells to migrate and invade. This step could be the key that affects the prognosis of patients. In addition, rather than the activation or inhibition of autophagy in HCC patients, the levels of the factors that cause the activation or inhibition of autophagy in patients may play a decisive role.

Conflict of interests

The authors declare no potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2021.08.002.

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