IL-33-expressing microvascular endothelial cells in human esophageal squamous cell carcinoma: Implications for pathological features and prognosis

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Published in: Microvascular Research
DOI: 10.1016/j.mvr.2023.104506

Available online: 13 Feb 2023

Citation:
Liu, X., Li, Z., Ren, J. & Cui, G. (2023). IL-33-expressing microvascular endothelial cells in human esophageal squamous cell carcinoma: Implications for pathological features and prognosis. Microvascular Research. 147. doi: 10.1016/j.mvr.2023.104506

Feb. 09, 2023

IL-33-expressing microvascular endothelial cells in human esophageal squamous cell carcinoma: implications for pathological features and prognosis

Subtitle: IL-33 in vascular endothelial cells

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Feltkode endret

Word count: 2406

All authors have read and approved this manuscript

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Abbreviations:

DIF: double immunofluorescence

ESCC: esophageal squamous cell carcinoma

IHC: immunohistochemistry

IL: interleukin

MVD: microvessel density

SEM: mean of standard error

TNM: tumor/node/metastasis

Abstract

Accumulating evidence suggests that interleukin (IL)-33 plays a critical role in regulating angiogenesis and cancer progression. In this study, we characterized the pathological importance of IL-33 deployed by tumor microvascular endothelial cells (ECs) in human esophageal squamous cell carcinoma (ESCC). The expression of IL-33 in microvascular ECs in 80 cases of ESCC was examined with immunohistochemistry (IHC) and double immunofluorescence. IHC results showed that strong IL-33-immunoreactivity (IR) in microvessels, which were confirmed to be ECs by double immunofluorescence staining with IL-33/CD31 antibodies. Moreover, high proliferative activity was shown in IL-33-positive ECs, and the IL-33 functional receptor ST2 was expressed in microvascular ECs. Clinicopathological analysis revealed that IL-33-positive microvessel density (MVD) was positively correlated with node involvement in patients with ESCC. A log rank test showed a highly significant inverse correlation between the densities of IL-33-positive MVDs and overall survival rate, and patients with higher IL-33-positive MVDs tended to have a lower survival rate (both $p < 0.05$). Therefore, we concluded that IL-33 deployed by microvascular ECs correlates with advanced pathological features and the long-term survival rate, which provides new insights into the regulatory mechanisms of tumor angiogenesis in the tumor microenvironment and might serve as a promising target in patients with ESCC.

Key words: Microvascular, Interleukin 33; Angiogenesis; Tumor, Esophagus

Introduction

Enhanced formation of new blood vessels (angiogenesis) has been recognized to be a key supportive factor for tumor cell proliferation and growth (Hida et al., 2018), whereas the blockade of angiogenesis through the administration of antagonists can remarkably suppress the tumor progression (Klein, 2018). Angiogenesis is regulated by proangiogenic factors and inhibitors within the tumor microenvironment (De Palma et al., 2017). Cytokines, e.g., interleukin (IL)-8, IL-6, and IL-33, produced in the tumor microenvironment, have been reported to stimulate tumor angiogenesis (Geindreau et al., 2022). Phenotypic analysis revealed that these proangiogenic cytokines can be identified in a variety of cell types, e.g., tumor cells, stromal cells, and immune cells, within the tumor microenvironment. Recently, several studies have demonstrated that proangiogenic factors produced by the tumor-associated microvascular niche play a critical role in the progression of cancers (Cao et al., 2014; Wang et al., 2018). We and others have previously shown that proangiogenic cytokines, such as IL-8, IL-17 and IL-33, are expressed in tumor-associated microvascular endothelial cells (ECs), suggesting that they might serve as additional cellular sources for proangiogenic factors within the tumor microenvironment and potentially participate in the self-regulation of tumor angiogenesis (Cao et al., 2018; Cui et al., 2023; Cui et al., 2018; Hida et al., 2018; Jou et al., 2022; Li et al., 2003; Li et al., 2020). More interestingly, functional receptors for these proangiogenic cytokines have also been expressed in target microvascular ECs (Cui et al., 2015; Heidemann et al., 2003), suggesting that a potential autocrine loop exists in tumor-associated microvascular ECs (Li et al., 2005). Thus, it is important to investigate the role of proangiogenic cytokines deployed in the tumor vascular niche in human tumors.

IL-33 is a novel contributing factor for the process of tumorigenesis, and increased expression of IL-33 is observed in a variety of human cancers (Akimoto et al., 2016; Bergis et al., 2013; Cui et al., 2015; He et al., 2017). Extensive evidence has suggested that one of the underlying

mechanisms for IL-33 involvement tumor growth and progression is its potential proangiogenic effect (Cao et al., 2018; Choi et al., 2009; Kuchler et al., 2008; Stojkovic et al., 2014), in which, ST2 expressed in microvascular ECs is the main functional receptor and plays an essential role (Choi et al., 2009; Milosavljevic et al., 2016). These findings led to us to hypothesize that tumor-associated ECs could be an additional cellular source of IL-33 and that EC-derived IL-33 might significantly contribute to the process of angiogenesis and then to the progression and prognosis of human cancers.

We therefore undertook the current study to characterize the expression of IL-33 in the compartment of tumor-associated ECs and then evaluated its clinicopathological importance in affecting tumor progression and prognosis in patients with esophageal squamous cell carcinoma (ESCC).

Materials & methods

Patients and specimens

Eighty surgically resected ESCC and twenty nontumor esophageal tissues were collected from the paraffin tissue bank at the Department of Pathology, the Second Affiliated Hospital of Zhengzhou University between 2006 and 2010. Twenty nontumor esophageal tissues taken far from the ESCC tumor site served as controls. No patient received preoperative radiotherapy and/or chemotherapy before surgery. Routine histological diagnosis was conducted in the Department of Pathology. Patient information details are listed in Table 1. Informed consent was obtained from all participating individuals in this study, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008) as reflected in a priori approval by the Second Affiliated Hospital's Ethics Committee of Zhengzhou University.

Table 1. Clinical information of ESSC patients

	N	Gender	TNM			Lymph Node	
		Male/Female	I	II	III	Positive	Negative
ESCC	80	52/28	4	56	20	23	57
Control	20	13/7					

Immunohistochemistry (IHC) to characterize IL-33-positive and CD31-positive tumor-associated microvessels

IHC was performed with a Vectastain *Elite ABC* Kit (Vector Lab., Burlingame, CA, USA) according to the manufacturer's instructions and our published methods (Cui et al., 2015). The following primary antibodies were used: goat anti-IL-33 polyclonal (Lot# YYZ0611111, working dilution 1:100; R&D systems, Minneapolis, MN, USA) and CD31 monoclonal (Lot# M0823, purchased from Dako, Carpinteria, CA, USA) antibodies were incubated at 4 ºC overnight respectively, 3-amino-9-ethylcarbazole (*AEC*; Lot# SK-4200, Vector Laboratories, Burlingame, CA, USA) was used as the chromogen, and slides were slightly counterstained

with Mayer's hematoxylin. IL-33 immunoreactivity (IR) and CD31-IR located in tumor-associated microvessels were observed and counted.

Clinicopathological and prognostic value of IL-33-positive micovessel densities (MVDs) in patients with ESCC

Since previous studies have revealed that the expression level of IL-33 is correlated with the clinicopathological features in a variety of human cancers (Sun et al., 2011; Wang et al., 2016; Zhang et al., 2012), we therefore analyzed the clinical implications of IL-33-IR positive MVDs with clinicopathological variables of ESCC.

Overall survival data were available for forty-one patients with ESCC. Patients with ESCC were divided into two high and low groups according to the median IL-33-positive MVD values, and then, survival rates and differences in survival curves between ESCC patients with high or low levels of IL-33-IR positive MVDs were determined.

Double immunofluorescence (DIF) staining

CD31 is a commonly used histological biomarker for the identification of microvascular ECs in the tumor microenvironment (Miyata and Sakai, 2015). To confirm that the expression of IL-33 and its functional receptor ST2 in microvessels was microvascular ECs, DIF staining with IL-33 (Lot# Ab-11853, rabbit polyclonal antibody from Abcam, UK)/CD31 (Lot# M0823, monoclonal antibodies purchased from Dako, Carpinteria, CA, USA, to label microvascular ECs) antibodies according to the protocol described in our previous publications (Cui et al., 2019). After sections were incubated with primary antibodies at 4ºC overnight, IL-33-immunoreactivity (IR) was developed with Texas red-conjugated secondary antibody (Lot#705-076-147, Jackson ImmunoResarch Laboratories, West Grove, PA, USA), and CD31-

IR was developed with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Lot# 1030-02, Jackson ImmunoResarch Laboratories).

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Similarly, DIF staining with ST2/CD31 antibodies was performed. ST2-IR (labelled by rabbit anti-ST2 polyclonal antibody, Lot# PA5-23316, working dilution 1:100; Thermo Scientific., Rockford, USA) was developed with FITC, and CD31-IR was developed with Cy3 **(**Jackson ImmunoResarch Laboratories). Nuclear counterstaining was not applied.

To examine the proliferation of IL-33-positive microvascular ECs, we stained ESCC sections with IL-33/Ki67 (Lot# 550609, 1:70; BD Pharmingen., San Jose, CA, USA) antibodies according to the method described above. IL-33-IR was developed with Texas red-conjugated secondary antibody and Ki67-IR with FITC-conjugated secondary antibody in IL-33/Ki67 DIF.

To evaluate a possible autocrine regulatory pathway of IL-33 (goat anti-IL-33 polyclonal antibody)/ST2 (rabbit anti-ST2 polyclonal antibody) in microvessels, we performed DIF with IL-33 with ST2 antibodies in ESCC sections according to the protocols describe above. IL-33-IR was developed with Texas red-conjugated secondary antibody and ST2-IR was developed with FITC-conjugated secondary antibody.

All stained sections were observed and photographed with an LSM-700 confocal microscopy (Carl Zeiss, Jena, Germany) under ×200 medium-power fields (MPF).

Morphometric evaluation

IL-33-positive MVDs and CD31-positive MVDs in both the control and ESCC sections were counted in well-oriented high-power fields (HPF) with abundant positive microvascular distribution (hot points) under ×400 magnification and average values of positive cells per slide were used for statistical analysis.

Statistical analysis

The data are presented as the mean ± SEM (standard error of the mean) unless otherwise stated. *P* values were evaluated by the Mann–Whitney test and Kruskal-Wallis test. Kaplan–Meier analysis was used to calculate survival rates and differences in survival curves in available ESCC patients with high or low IL-33 MVDs. A P value < 0.05 was considered statistically significant.

Results

IL-33-IR was highly expressed in tumor-associated microvessels

IL-33-IR was observed in tumor cells, stromal cells and tumor-associated microvessels in ESCC sections. Since the aim of this study is to evaluate the significance of IL-33-positive MVDs, we focused on the expression of IL-33-IR in tumor-associated microvessels in this study.

As shown in Fig. 1, images revealed that the majority of tumor-associated microvessels in the ESCC stroma were positive for IL-33-IR (arrow in Fig. 1B) compared with the nontumor controls (Fig. 1A). Ratios of IL-33-positive/CD31-positive MVDs/HPF were counted in both control and ESCC sections, data present in Fig. 1C showed that the IL-33-positive MVDs accounted for approximately 60% of the total MVDs labelled by CD31-IR in the ESCC stroma and 48% in the control stroma (ESCC vs. control, $P<0.05$, obtained from the Mann–Whitney test). Because MVDs in ESCCs were significantly higher than those in controls, total IL-33-positive MVDs in the ESCC might much higher than that in the control. Further quantitative data confirmed an increased density of IL-33-IR-positive MVDs/HPF in the ESCC stroma compared with the nontumor control ($P<0.01$, obtained from the Mann–Whitney test, refer to Fig. 1D).

Tumor-associated microvessels positive for IL-33 were CD31-positive DCs

The results in Fig. 2 show the colocalization of IL-33-IR (Fig. 2A) with CD31-labeled (Fig. 2B) vascular ECs in microvessls (merged image in Fig. 2C). This finding confirmed the expression of IL-33-IR in microvascular ECs. Similarly, ST2-IR (Fig. 2D) was observed in CD31-labeled (Fig. 2E) vascular ECs (merged image in Fig. 2F). Interestingly, ST2-IR was also observed in small arterioles (*pink* arrow in Fig. 2F). Further DIF with IL-33/SMA-alpha (to label smooth muscle in the arteriole wall) and ST2/SMA-alpha antibodies confirmed that

both IL-33- and ST2-IRs were expressed in small arterioles (refer to images in Supplementary Figure 1).

To evaluate whether an autocrine pathway exists in tumor-associated microvessels, we performed DIF with IL-33/ST2 antibodies. Images showed that IL-33-IR (arrow in Fig. 3A) was frequently colocalized with ST2-IR (arrow in Fig. 3B) in microvessels (merged image in Fig. 3C) within the ESCC microenvironment (refer to Fig. 3).

Proliferation in IL-33-IR positive ECs

As shown in Fig. 4, DIF images revealed that Ki67-IR (Fig. 4B) was observed in IL-33-IR positive (Fig. 4A) ECs (merged image in Fig. 4C). Furthermore, enlarged images confirmed that Ki67-IR was colocalized with IL-33-IR positive ECs in both ESCC (Fig. 4D-F) and control sections (Fig. 4G-I).

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IL-33-IR-positive MVDs are associated with clinicopathological variables and the survival rate of patients with ESCC

Subsequently, we analyzed the clinical importance of IL-33-IR positive MVDs with clinical pathological variables of ESCC.

The analysis revealed that IL-33-expressing MVDs were associated with node involvement (node positive vs. node negative: 12.88±0.77 vs. 9.95±1.43, $P<0.05$. *P* value was obtained from the Mann–Whitney test) but not with TNM stages (TNM I vs. TNM II vs. TNM III: 13.20±2.27 vs. 13.75±0.96 vs. 11.29±0.96, $P>0.05$. *P* value was obtained from the Kruskal-Wallis test). Kaplan–Meier analysis indicated that IL-33-IR-positive MVDs (Fig. 4) were associated with the overall survival rate after surgery in forty-one patients with ESCC.

Discussion

Angiogenesis, which refers to the proliferation and sprouting of existing blood vessels in the tumor microenvironment, is crucial for tumor progression and metastasis. Previous studies have demonstrated that IL-33 is potent proangiogenic factor and can significantly enhance the process of angiogenesis (Choi et al., 2009; Fournie and Poupot, 2018; Zhang et al., 2017). Histological evaluation of the cellular types in human tumors has revealed that IL-33 can be expressed in a variety of cells including tumor cells, stromal cells and microvessels (Cui et al., 2015), suggesting that IL-33 is produced from a mixed cellular source. Our current results confirmed that IL-33-IR is highly expressed in ESCC tumor-associated microvessels with a high proliferative activity, and both IL-33 and its functional receptor ST2 are coexpressed in microvessels. Since tumor stroma has a higher density of microvascular vessels than normal tissue, it is not stranger that quantitative data shows higher IL-33-positive MVDs in ESCC than the nontumor tissue. Studies have revealed that the proangiogenic effect of IL-33 is possibly through an autocrine/paracrine pathway in human tumors (Choi et al., 2009; Cui et al., 2015; Kuchler et al., 2008; Milosavljevic et al., 2016), and current DIF images that showed a colocalization of IL-33-IR with ST2-IR in ESCC microvessels suggesting a potential autocrine mechanism. Further analysis revealed that increased IL-33-IR positive MVDs are associated with clinicopathological variables and overall survival rate in patients with ESCC. Our findings suggest that tumor-associated microvascular ECs are an important cellular source of IL-33 and EC-derived IL-33 might contribute to the process of angiogenesis and ESCC progression and prognosis. To the best of our knowledge, the current study is the first to examine the clinicopathological value of EC-derived IL-33 in patients with ESCC.

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Emerging evidence has suggested that EC-derived cytokines have diverse effects including enhancing angiogenesis, stimulating tumor proliferation and converting tumor cells to stem-like cells (Butler et al., 2010; Kim et al., 2018) and are involved in tumor progression, metastasis

and chemotherapy resistance (Maishi and Hida, 2017; Poulos et al., 2014). In this study, we showed that IL-33-IR was highly expressed in ESCC tumor-associated ECs in the ESCC microenvironment. Since previous studies have demonstrated a strong stimulatory effect of IL-33 on EC activation and angiogenesis (Cao et al., 2018; Choi et al., 2009; Choi et al., 2012; Milosavljevic et al., 2016), our current data suggest that the contribution of EC-derived IL-33 to enhanced angiogenesis and high MVDs in human ESCCs should not be ignored.

To examine the proliferative activity of IL-33-IR positive ECs, we performed double immunofluorescence staining with IL-33/Ki67 antibodies in ECSS sections. Images confirmed that IL-33-IR-positive ECs were with a high proliferation index, which reflects enhanced vascularization in the ESCC microenvironment.

Previous studies have shown that angiogenic activity correlates with clinicopathological parameters and prognosis in certain types of tumors (Chen et al., 2014; Cheng et al., 2014; Des Guetz et al., 2006; Kumagai et al., 2014; Uzzan et al., 2004), and MVD is associated with prognosis in cancer patients (Chen et al., 2014; Cheng et al., 2014; Choi et al., 2006; Des Guetz et al., 2006). We have therefore evaluated the clinicopathological and prognostic value of EC-derived IL-33 in patients with ESCC. Analysis demonstrated that IL-33-positive MVD was associated with node involvement. Moreover, Kaplan–Meier analysis revealed that the IL-33-IR positive MVDs were correlated with the overall survival rate in ESCC, and ESCC patients with higher IL-33-IR-positive MVDs tended to have a shorter overall survival time after surgery than those with lower IL-33-IR-positive MVDs. These data suggest that EC-derived IL-33 might be associated with the survival rate in patients with ESCCs after surgery.

Taken together with previous findings of IL-33's regulatory effect on angiogenesis (Choi et al., 2009), our current interpretation of the data indicates that tumor-associated ECs are an additional cellular source for IL-33 in the ESCC microenvironment, and IL-33-IR-positive MVDs were associated with the overall survival rate after surgery in patients with ESCC. Future

277 studies will be required to explore the pharmacotherapeutic value of targeting EC-derived IL-
278 33 in this type of cancer in animal models.

Declarations

Funding: This project was financially supported by the grant from the Innovation Scientists and Technicians Troop Construction Projects of Henan Province (Program No. CXTD20150009), China.

Conflicts of interest/Competing interests: The authors declare no competing interests.

Ethics approval: Ethical approval was obtained by the Local Ethic Committee of the Second Affiliated Hospital of Zhengzhou University.

Consent to participate: Informed consent was obtained from human subjects.

Consent for publication: Not applicable

Availability of data and material: The data that support the findings of this study are available from our hospital but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of our hospital.

Code availability: Not applicable.

Authors' contributions: GC designed the project, analyzed data, XL participated in the immunohistochemistry and double immunofluorescence staining. JR and ZL did histological diagnosis and reviewed the immunohistochemical staining slides; All authors wrote, read, and approved the final manuscript.

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Legends

Figure 1 legend

Immunohistochemical examination of IL-33 expressed in ESCC microvessels.

In nontumor esophageal tissues, IL-33 immunoreactivity (IR) was observed in microvessels (*arrow* in Fig. 1A). In ESCC tissues, intensive nuclear positive IL-33-IR was observed in most microvessels (*arrow* in Fig. 1B).

The quantitative results of IL-33-positive/CD31-positive MVDs/HPF showed that the ratio of IL-33/CD31 MVDs in ESCC sections (*black* bar) was slightly higher than that in control sections (*white* bar) (refer to Fig. 1C, ESCC vs. control: 0.60 ± 0.04 vs. 0.48 ± 0.02, $P<0.05$). Further quantitative results also showed significantly increased IL-33-IR-positive (*black* bar in Fig. 1C) MVDs in the ESCC stroma compared to the controls (ESCC vs. control: 11.65 ± 0.65 vs. 4.24 ± 0.93, $P<0.01$, refer to *white* bars in Fig. 1D).

(A&B: IHC, counterstained with hematoxylin, original magnification 400×. *P* values in Fig. 1C & D were from the Mann–Whitney test).

Figure 2 legend

Double immunofluorescence staining to confirm that IL-33-IR and ST2-IR-positive cells were CD31-positive ECs

Double immunofluorescence results revealed the colocalization of IL-33-IR (*red* cells in Fig. 2A) with CD31-IR (*green* cells in Fig. 2B) in ESCC microvascular endothelial cells (merged image Fig. 2C). Similarly, the colocalization of ST2-IR (*green cells* in Fig. 2D) with CD31-IR (*red cells* in 2E) was observed in endothelial cells (merged image in Fig. 2F). Additionally, ST2-IR was observed in blood vessels with thickened smooth muscle (*pink arrow* in Fig. 2F), which had the morphology of a small arteriole.

(A- F: Double immunofluorescence images, original magnification 200×; counterstaining was not applied).

Figure 3 legend

Double immunofluorescence staining with confocal microscopy to evaluate the IL-33/St2 autocrine loop in ESCC microvessels

The results demonstrated that IL-33-IR (*red* cells in Fig. 3A) was frequently colocalized with St2-IR (*green* cells in Fig. 3B) in tumor-associated microvessels (merged image in Fig. 3C). (A-C: Double immunofluorescence images, original magnification 200×; counterstaining was not applied).

Figure 4 legend

Double immunofluorescence staining evaluating the proliferative activity of IL-33-IR-positive ECs in the ESCC microenvironment

Double immunofluorescence results revealed that both IL-33-IR-positive (*arrow* in Fig. 4A) ECs were positive for Ki67-IR (*arrow* in Fig. 4B) in the microvessels (merged images in Fig. 4C). Enlarged DIF images confirmed the presence of Ki67-IR in both ESCC (Fig. 4D-F) and control sections (Fig. 4G-I).

(A-I: Double immunofluorescence images, original magnification 200×; counterstaining was not applied)

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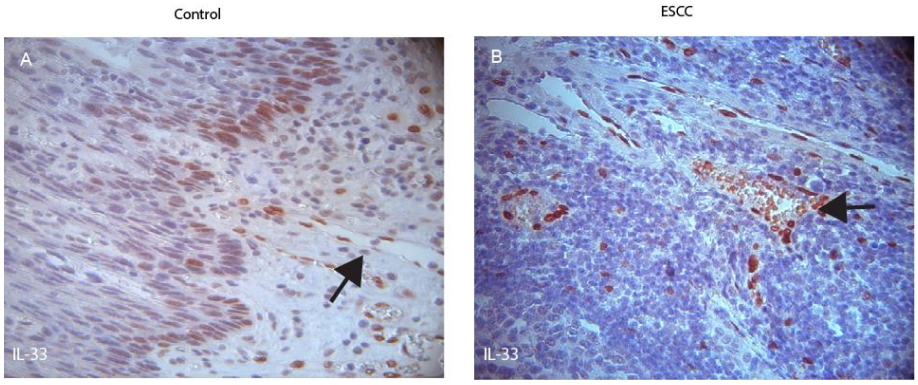
Figure 5 legend

Kaplan–Meier curve of overall survival rate differences among ESCC patients with different IL-33-IR-positive MVDs

Kaplan–Meier analysis showed that the IL-33-IR positive MVD may predict the overall survival in patients with ESCC, and ESCC patients with high IL-33 MVDs tended to have a shorter survival rate after surgery than those with low IL-33 MVDs (P=0.0056, obtained from the log-rank test).

448 **Figure 1 A&B**



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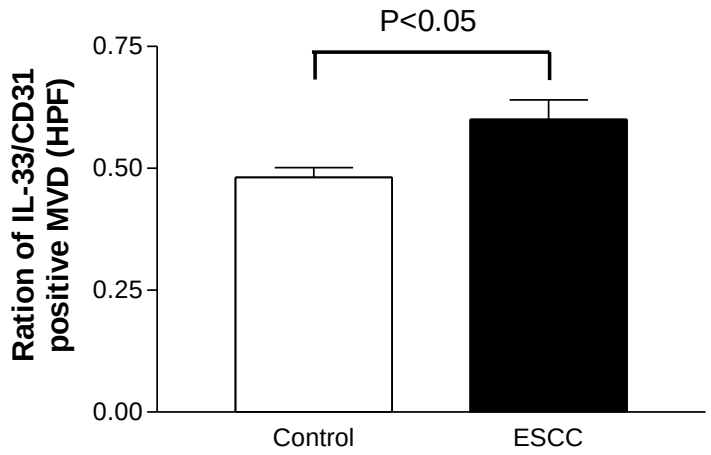
452 **Figure 1 C**



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455 **Fig. 1D**

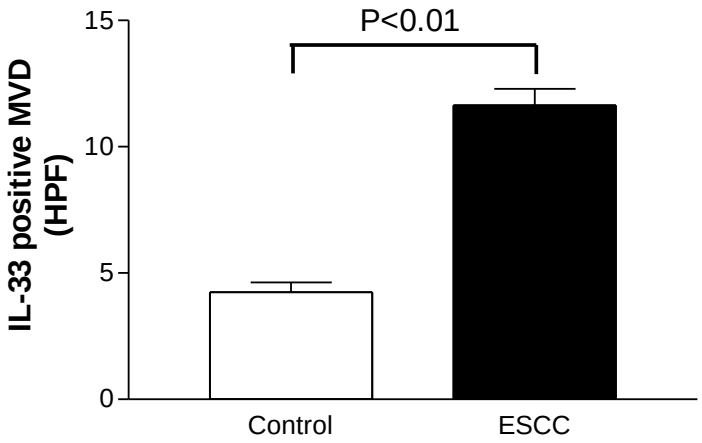

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Figure 2

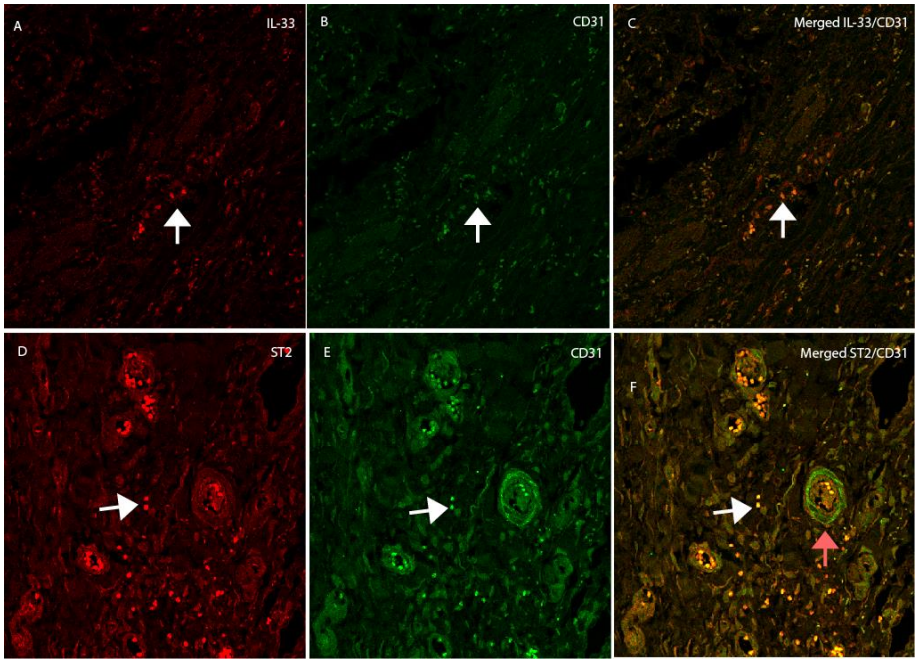


Figure 3

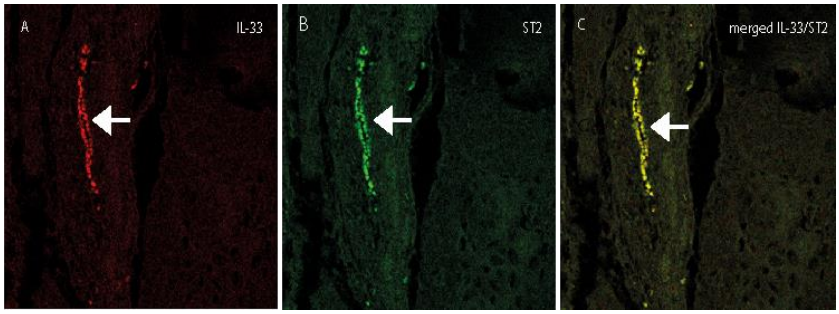
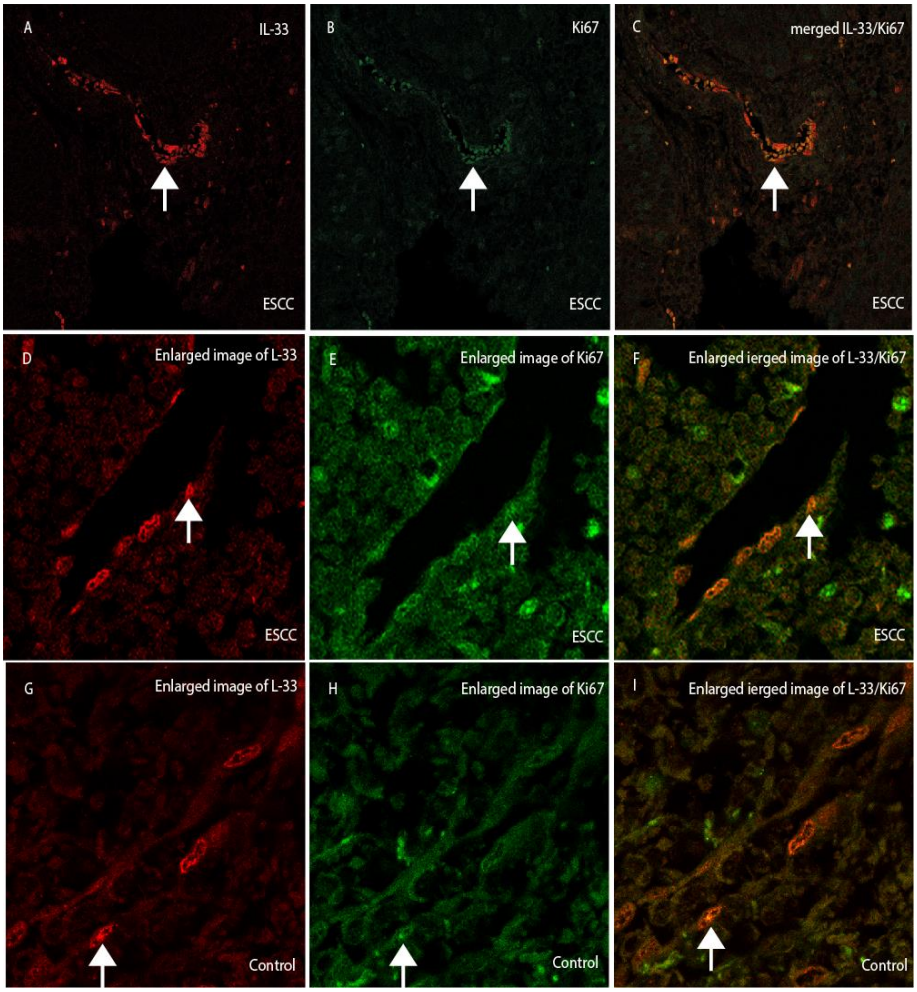
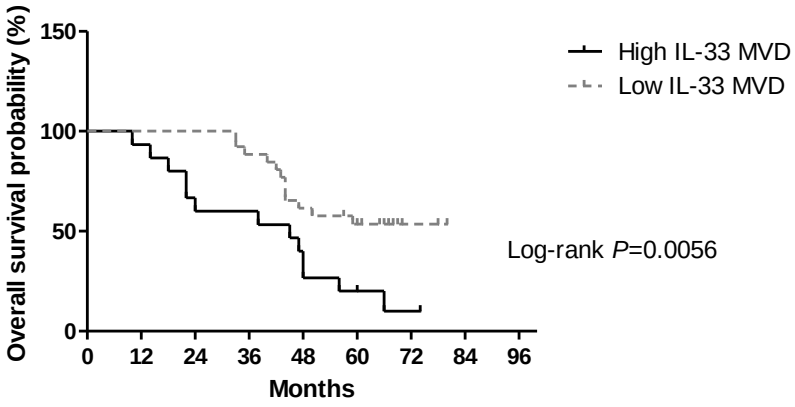


Figure 4


A
IL-33
ESCC
B
Ki67
ESCC
C
merged IL-33/Ki67
ESCC
D
Enlarged image of L-33
ESCC
E
Enlarged image of Ki67
ESCC
F
Enlarged ierged image of L-33/Ki67
ESCC
G
Enlarged image of L-33
Control
H
Enlarged image of Ki67
Control
I
Enlarged ierged image of L-33/Ki67
Control

470 **Figure 5**



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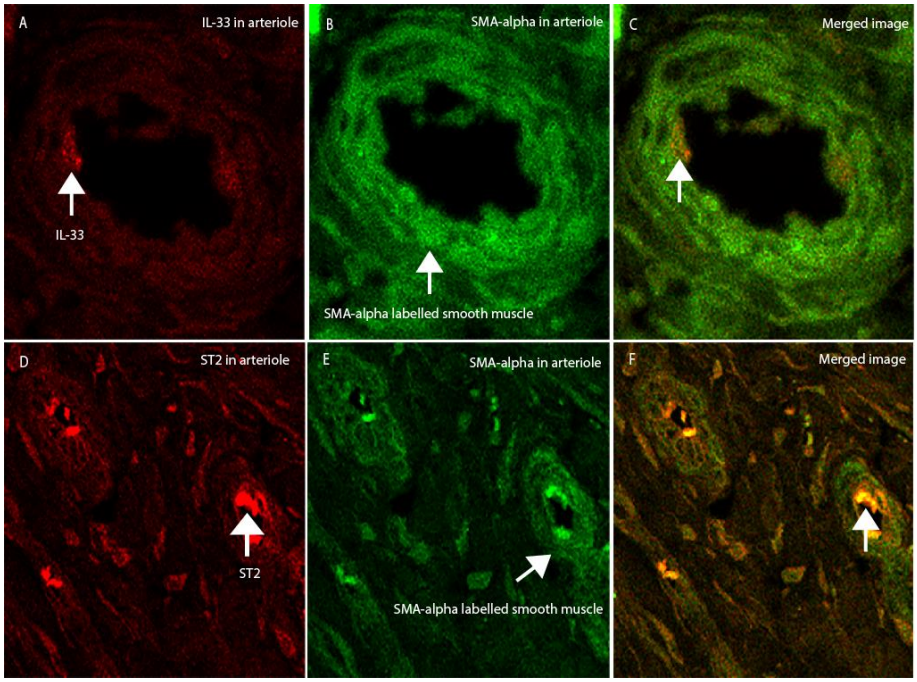
Supplementary Figure 1



Legend: Double immunofluorescent images showed that both IL-33-IR (*red* color in A) and ST2-IR were observed in small arteriole with thicken smooth muscles (labelled by SMA-alpha antibody, mouse-anti human monoclonal antibody from Dako Cor, Lot# M085, *green* color in B & F), which suggested that both IL-33 and ST2 were also expressed in small arterioles (merged images in C & F).