ISSN 1948-5204 (online)

World Journal of *Gastrointestinal Oncology*

World J Gastrointest Oncol 2022 September 15; 14(9): 1604-1890

Published by Baishideng Publishing Group Inc

World Journal of Gastrointestinal Oncology

Contents

Monthly Volume 14 Number 9 September 15, 2022

REVIEW

1604 Advances in postoperative adjuvant therapy for primary liver cancer

Zeng ZM, Mo N, Zeng J, Ma FC, Jiang YF, Huang HS, Liao XW, Zhu GZ, Ma J, Peng T

1622 Immunotherapy for nonalcoholic fatty liver disease-related hepatocellular carcinoma: Lights and shadows

Costante F, Airola C, Santopaolo F, Gasbarrini A, Pompili M, Ponziani FR

1637 Emerging role of caldesmon in cancer: A potential biomarker for colorectal cancer and other cancers

Alnuaimi AR, Nair VA, Malhab LJB, Abu-Gharbieh E, Ranade AV, Pintus G, Hamad M, Busch H, Kirfel J, Hamoudi R, Abdel-Rahman WM

MINIREVIEWS

1654 Liquid biopsy to detect resistance mutations against anti-epidermal growth factor receptor therapy in metastatic colorectal cancer

Valenzuela G, Burotto M, Marcelain K, González-Montero J

1665 Implication of gut microbiome in immunotherapy for colorectal cancer

Koustas E, Trifylli EM, Sarantis P, Papadopoulos N, Aloizos G, Tsagarakis A, Damaskos C, Garmpis N, Garmpi A, Papavassiliou AG, Karamouzis MV

ORIGINAL ARTICLE

Basic Study

1675 Potential of six-transmembrane epithelial antigen of the prostate 4 as a prognostic marker for colorectal cancer

Fang ZX, Li CL, Chen WJ, Wu HT, Liu J

Case Control Study

1689 Inverse relations between *Helicobacter pylori* infection and risk of esophageal precancerous lesions in drinkers and peanut consumption

Pan D, Sun GJ, Su M, Wang X, Yan QY, Song G, Wang YY, Xu DF, Wang NN, Wang SK

Retrospective Cohort Study

1699 Prognostic impact of tumor deposits on overall survival in colorectal cancer: Based on Surveillance, Epidemiology, and End Results database

Wu WX, Zhang DK, Chen SX, Hou ZY, Sun BL, Yao L, Jie JZ

1711 Consolidation chemotherapy with capecitabine after neoadjuvant chemoradiotherapy in high-risk patients with locally advanced rectal cancer: Propensity score study

Sheng XQ, Wang HZ, Li S, Zhang YZ, Geng JH, Zhu XG, Quan JZ, Li YH, Cai Y, Wang WH

World Journal of Gastrointestinal Oncology
Monthly Volume 14 Number 9 September 15, 2022

Contents

Retrospective Study

1727 Efficacy and safety of computed tomography-guided microwave ablation with fine needle-assisted puncture positioning technique for hepatocellular carcinoma

Hao MZ, Hu YB, Chen QZ, Chen ZX, Lin HL

1739 Clinicopathological characterization of ten patients with primary malignant melanoma of the esophagus and literature review

Zhou SL, Zhang LQ, Zhao XK, Wu Y, Liu QY, Li B, Wang JJ, Zhao RJ, Wang XJ, Chen Y, Wang LD, Kong LF

1758 Endoscopic debulking resection with additive chemoradiotherapy: Optimal management of advanced inoperable esophageal squamous cell carcinoma

Ren LH, Zhu Y, Chen R, Shrestha Sachin M, Lu Q, Xie WH, Lu T, Wei XY, Shi RH

1771 Nomogram for predicting the prognosis of tumor patients with sepsis after gastrointestinal surgery

Chen RX, Wu ZQ, Li ZY, Wang HZ, Ji JF

1785 Efficacy and safety of laparoscopic radical resection following neoadjuvant therapy for pancreatic ductal adenocarcinoma: A retrospective study

He YG, Huang XB, Li YM, Li J, Peng XH, Huang W, Tang YC, Zheng L

Observational Study

1798 To scope or not - the challenges of managing patients with positive fecal occult blood test after recent colonoscopy

Rattan N, Willmann L, Aston D, George S, Bassan M, Abi-Hanna D, Anandabaskaran S, Ermerak G, Ng W, Koo JH

1808 Clinical implications of interleukins-31, 32, and 33 in gastric cancer

Liu QH, Zhang JW, Xia L, Wise SG, Hambly BD, Tao K, Bao SS

1823 Construction and analysis of an ulcer risk prediction model after endoscopic submucosal dissection for early gastric cancer

Gong SD, Li H, Xie YB, Wang XH

1833 Percutaneous insertion of a novel dedicated metal stent to treat malignant hilar biliary obstruction

Cortese F, Acquafredda F, Mardighian A, Zurlo MT, Ferraro V, Memeo R, Spiliopoulos S, Inchingolo R

EVIDENCE-BASED MEDICINE

1844 Prediction of gastric cancer risk by a polygenic risk score of *Helicobacter pylori*

Wang XY, Wang LL, Liang SZ, Yang C, Xu L, Yu MC, Wang YX, Dong QJ

META-ANALYSIS

1856 Dissecting novel mechanisms of hepatitis B virus related hepatocellular carcinoma using meta-analysis of public data

Aljabban J, Rohr M, Syed S, Cohen E, Hashi N, Syed S, Khorfan K, Aljabban H, Borkowski V, Segal M, Mukhtar M, Mohammed M, Boateng E, Nemer M, Panahiazar M, Hadley D, Jalil S, Mumtaz K

1874 Prognostic and clinicopathological value of Twist expression in esophageal cancer: A meta-analysis

Song WP, Wang SY, Zhou SC, Wu DS, Xie JY, Liu TT, Wu XZ, Che GW

LETTER TO THE EDITOR

1886 Nutrition deprivation affects the cytotoxic effect of CD8 T cells in hepatocellular carcinoma

Zhang CY, Liu S, Yang M

World Journal of Gastrointestinal Oncology

Monthly Volume 14 Number 9 September 15, 2022

Contents

ABOUT COVER

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AIMS AND SCOPE

The primary aim of *World Journal of Gastrointestinal Oncology* (*WJGO*, *World J Gastrointest Oncol*) is to provide scholars and readers from various fields of gastrointestinal oncology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

WJGO mainly publishes articles reporting research results and findings obtained in the field of gastrointestinal oncology and covering a wide range of topics including liver cell adenoma, gastric neoplasms, appendiceal neoplasms, biliary tract neoplasms, hepatocellular carcinoma, pancreatic carcinoma, cecal neoplasms, colonic neoplasms, colorectal neoplasms, duodenal neoplasms, esophageal neoplasms, gallbladder neoplasms, *etc*.

INDEXING/ABSTRACTING

The *WJGO* is now abstracted and indexed in PubMed, PubMed Central, Science Citation Index Expanded (SCIE, also known as SciSearch®), Journal Citation Reports/Science Edition, Scopus, Reference Citation Analysis, China National Knowledge Infrastructure, China Science and Technology Journal Database, and Superstar Journals Database. The 2022 edition of Journal Citation Reports® cites the 2021 impact factor (IF) for *WJGO* as 3.404; IF without journal self cites: 3.357; 5-year IF: 3.250; Journal Citation Indicator: 0.53; Ranking: 162 among 245 journals in oncology; Quartile category: Q3; Ranking: 59 among 93 journals in gastroenterology and hepatology; and Quartile category: Q3. The *WJGO*'s CiteScore for 2021 is 3.6 and Scopus CiteScore rank 2021: Gastroenterology is 72/149; Oncology is 203/360.

RESPONSIBLE EDITORS FOR THIS ISSUE

Production Editor: *Ying-Yi Yuan*; **Production Department Director:** *Xiang Li*; **Editorial Office Director:** *Jia-Ru Fan*.

NAME OF JOURNAL
World Journal of Gastrointestinal Oncology

ISSN
ISSN 1948-5204 (online)

LAUNCH DATE
February 15, 2009

FREQUENCY
Monthly

EDITORS-IN-CHIEF
Monjur Ahmed, Florin Burada

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/1948-5204/editorialboard.htm

PUBLICATION DATE
September 15, 2022

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INSTRUCTIONS TO AUTHORS
https://www.wjgnet.com/bpg/gerinfo/204

GUIDELINES FOR ETHICS DOCUMENTS
https://www.wjgnet.com/bpg/GerInfo/287

GUIDELINES FOR NON-NATIVE SPEAKERS OF ENGLISH
https://www.wjgnet.com/bpg/gerinfo/240

PUBLICATION ETHICS
https://www.wjgnet.com/bpg/GerInfo/288

PUBLICATION MISCONDUCT
https://www.wjgnet.com/bpg/gerinfo/208

ARTICLE PROCESSING CHARGE
https://www.wjgnet.com/bpg/gerinfo/242

STEPS FOR SUBMITTING MANUSCRIPTS
https://www.wjgnet.com/bpg/GerInfo/239

ONLINE SUBMISSION
https://www.f6publishing.com

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E-mail: bpgoffice@wjgnet.com https://www.wjgnet.com

World Journal of Gastrointestinal Oncology

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World J Gastrointest Oncol 2022 September 15; 14(9): 1808-1822

DOI: 10.4251/wjgo.v14.i9.1808

ISSN 1948-5204 (online)

ORIGINAL ARTICLE

Observational Study

Clinical implications of interleukins-31, 32, and 33 in gastric cancer

Qing-Hua Liu, Ji-Wei Zhang, Lei Xia, Steven G Wise, Brett David Hambly, Kun Tao, Shi-San Bao

Specialty type: Gastroenterology and hepatology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C
Grade D (Fair): D
Grade E (Poor): 0

P-Reviewer: Kawabata H, Japan; Yakar M, Turkey

Received: February 28, 2022
Peer-review started: February 28, 2022
First decision: April 17, 2022
Revised: April 21, 2022
Accepted: July 31, 2022
Article in press: July 31, 2022
Published online: September 15, 2022

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Abstract

BACKGROUND

Gastric cancer (GC) is one of the most common malignancies in China with a high morbidity and mortality.

AIM

To determine whether interleukin (IL)-31, IL-32, and IL-33 can be used as biomarkers for the detection of GC, *via* evaluating the correlations between their expression and clinicopathological parameters of GC patients.

METHODS

Tissue array (n = 180) gastric specimens were utilised. IL-31, IL-32, and IL-33 expression in GC and non-GC tissues was detected immunohistochemically. The correlations between IL-31, IL-32, and IL-33 expression in GC and severity of clinicopathological parameters were evaluated. Survival curves were plotted using the Kaplan-Meier method/Cox regression. Circulating IL-31, IL-32, and IL-33 were detected by ELISA.

RESULTS

We found that the expression levels of IL-31, IL-32, and IL-33 were all lower in GC than in adjacent non-GC gastric tissues (P < 0.05). IL-33 in peripheral blood of GC patients was significantly lower than that of healthy individuals (1.50 ± 1.11 *vs* 9.61 ± 8.00 ng/mL, P <0.05). Decreased IL-31, IL-32, and IL-33 in GC were observed in younger patients (< 60 years), and IL-32 and IL-33 were lower in female patients (P < 0.05). Higher IL-32 correlated with a longer survival in two

GC subgroups: T4 invasion depth and TNM I-II stage. Univariate/multivariate analysis revealed that IL-32 was an independent prognostic factor for GC in the T4 stage subgroup. Circulating IL-33 was significantly lower in GC patients at TNM stage IV than in healthy people ($P < 0.05$).

CONCLUSION

Our findings may provide new insights into the roles of IL-31, IL-32, and IL-33 in the carcinogenesis of GC and demonstrate their relative usefulness as prognostic markers for GC. The underlying mechanism of IL-31, IL-32, and IL-33 actions in GC should be further explored.

Key Words: Diagnosis and therapy; Gastric cancer; Immune cell interactions; Interleukin-31; Interleukin32; Interleukin-33

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Core Tip: Gastric cancer (GC) is one of the most common malignancies in China with a high morbidity and mortality. This study aimed to determine whether interleukin (IL)-31, IL-32, and IL-33 can be used as biomarkers for the detection of GC, *via* evaluating the correlations between their expression and clinicopathological parameters of GC patients. IL-31, IL-32, and IL-33 expression in GC was correlated with the severity of clinicopathological parameters. Circulating IL-33 was significantly low in GC patients. Our findings may provide new insights into the roles of IL-31, IL-32, and IL-33 in the carcinogenesis of GC.

Citation: Liu QH, Zhang JW, Xia L, Wise SG, Hambly BD, Tao K, Bao SS. Clinical implications of interleukins-31, 32, and 33 in gastric cancer. *World J Gastrointest Oncol* 2022; 14(9): 1808-1822
URL: https://www.wjgnet.com/1948-5204/full/v14/i9/1808.htm
DOI: https://dx.doi.org/10.4251/wjgo.v14.i9.1808

INTRODUCTION

Gastric cancer (GC) is one of the most common malignancies in China with a high morbidity (approximately 24%) and mortality (approximately 17%)[1] and is ranked third amongst malignant tumours[2]. Despite the more widespread use of recently developed diagnostic techniques, including endoscopic examination, many GC patients are diagnosed at advanced stages, resulting in a poor 5-year survival rate (< 20%). This emphasises the critical need for development of a reliable biomarker(s)[3] with high specificity and sensitivity, to improve the prediction of prognosis for more successful outcomes for GC patients. Endoscopic examination provides a useful approach in the early detection of GC and in reducing cancer-related mortality.

Immunity is critically important in inhibiting the development of malignancy[4], but the precise underlying mechanism concerning how host defence is involved in the oncogenesis of GC remains to be explored[5]. The role of pro-inflammatory and anti-inflammatory responses during the development of malignancy has been well established to be able to either stimulate or inhibit the growth of a cancer[4, 6]. The actions of the immune checkpoint molecules PD-1 and CTLA-4 have been elegantly demonstrated[7] to inhibit anti-cancer immunity during oncogenesis[8]. In addition, the molecular basis of carcinogenesis has also been studied within the gastrointestinal system[9]. Furthermore, a new classification of GC has recently been proposed based on subtype pathway clustering[10].

Helicobacter pylori (*H. pylori*), a spiral Gram-negative rod that infects and colonizes the human stomach in 50% of the human population, is a definite human oncogenic agent[11]. In addition, it has been suggested that *H. pylori* contributes to > 60% of all GCs, although the precise underlying mechanisms are complex[12]. It has been well illustrated by the Nobel laureate Barry Marshal that chronic gastric ulceration is caused by *H. pylori* infection, which can be eliminated by a cocktail of antibiotics[13]. It has been reported that the constitutive levels of interleukin 32 (IL-32) in both the gastric mucosa and GC tissue is upregulated in *H. pylori* infection[14]. Thus, it is reasonable to speculate that host immunity plays a critical role during the development of GC.

The cell-mediated immune response is extremely important in defence against tumour development, since compromised host immunity is known to contribute to the establishment, proliferation, and metastasis of malignant tumours[15]. Although high host inflammatory status has been reported in the tumour microenvironment, an incompetent inflammatory/immune response will lead to tumour progression[16].

IL-31, an immunoregulatory cytokine secreted mainly by activated Th2 cells, plays a major role in the process of chronic inflammation[17]. However, the involvement of IL-31 in the pathogenesis of cancer is unclear. Recent studies have shown that malignant Tcells produce IL-31, with an associated increase in serum levels of IL-31[18]. Additionally, in the advanced stages of cutaneous T cell lymphoma, improved pruritus in patients correlates with lower levels of IL-31[19].

IL-32, a pro-inflammatory cytokine, is highly produced in several autoimmune diseases, *e.g.*, rheumatoid arthritis, inflammatory bowel disease, and atopic dermatitis[20,21]. However, by contrast with autoimmune and inflammatory diseases, the role of IL-32 appears to differ amongst different forms of cancer, *e.g.*, IL-32 exhibits anti-tumour effects in human colon cancer and leukaemia[22,23], however, it promotes tumorigenesis in human pancreatic cancers[24]. The role of IL-32 in GC is controversial, *i.e.*, one study found that IL-32 expression is elevated in GC compared with normal stomach tissue[14], while another study reported that there is no significant difference between GC and normal stomach tissue[25]. The precise role of IL-32 in tumorigenesis of GC and other malignancies remains to be fully explored. An additional controversial finding, however, has also reported that there is substantially reduced IL32 expression in the GC tissue of patients with the diffuse type of GC[26]. These divergent observations concerning IL-32 expression in GC may be due to different races and/or different tumour microenvironments.

IL-33, a member of the IL-1 family, regulates innate and adaptive immunity as a potent inducer of pro-inflammatory cytokines. The involvement of IL-33 in non-small cell lung cancer is controversial, *i.e.*, high IL-33 has been found to be of diagnostic and prognostic value[27], but another group has reported no significant associations[28]. The possible role of IL-33 in GC remains to be explored. IL-33 promotes GC invasion and migration *via* stimulating production of MMP-3 and IL-6 *in vitro*, using the ST2ERK1/2 pathway[29], which has been confirmed in a GC animal model by ablation of the cognate IL-33 receptor ST2[30]. *IL-33* mRNA expression is significantly higher in GC tissue compared to that of non-cancer tissue[31], suggesting that IL-33 promotes the development of GC. However, another controversial report failed to demonstrate an association between IL-33 and the overall 5-year survival rate[32].

In this study, we specifically assessed the relationships among IL-31, IL-32, and IL-33 in GC utilising the same cohort of patients. We aimed to identify the expression of IL-31, IL-32, and IL-33 in GC and assess their inter-correlations and clinical significance. Our data may provide useful information for both basic understanding of tumour immunology and/or therapeutic targets for GC patients.

MATERIALS AND METHODS

Patients and samples

GC tissues and adjacent histologically normal gastric tissues (control) were obtained from 180 GC patients undergoing subtotal gastrectomy at the Affiliated Hospital, Xuzhou Medical University, China between 2015 and 2020. None of these patients had a total gastrectomy. These GC patients were comprised of 140 males and 40 females, aged from 23 to 85 years. No chemotherapy was administered to these patients prior to subtotal gastrectomy. There were no cases of local recurrences within the stomach after subtotal gastrectomy among the 180 GC patients included in the study. Non-cancer tissues were also collected (n = 159), but did not include cases without a mucosal layer present under microscopic examination (n = 21). This study was approved by the Human Ethical Committee, the Institutional Review Boards of Affiliated Hospitals of Xuzhou Medical University.

Immunohistochemistry

Sections (5 μm) from tissue microarray blocks were labelled with three antibodies, as described previously[33]. The antibodies used are: Rabbit anti-IL-31 polyclonal antibody (22859-1-AP, Proteintech, China), rabbit anti-IL-32 polyclonal antibody (11079-1-AP, Proteintech), and rabbit anti-IL-33 polyclonal antibody (12372-1-AP, Proteintech, China). The dilution for all three antibodies was 1:100. A horseradish peroxidase-conjugated secondary antibody (12127A07, Beijing Sequoia Jinqiao Biological Technology Co., Ltd.) was used. The specific target(s) were visualized with a DAB detection kit (Beijing Sequoia Jinqiao Biological Technology Co., Ltd.) and counterstained with hematoxylin.

Photomicrographs from each of the tissue arrays were taken with a fixed exposure time and colour balance to ensure consistency. IL-31, IL-32, and IL-33 production was quantified using ImagePro Plus9.1 (Media Cybernetic, Silver Spring, MD, United States), as described previously[34].

ELISA for IL-31, IL-32, and IL-33

To determine if there was a correlation between GC and circulating IL-31, IL-32, and IL-33, we enrolled prospectively ten GC patients prior to preoperative chemotherapy in the Affiliated Hospital, Xuzhou Medical University, China. Blood from ten healthy age and sex matched persons presenting for a routine health check-up were collected as controls. Consent was obtained from both GC patients and healthy controls. The circulating cytokine study was also approved by the Human Ethical Committee, the Institutional Review Boards of the Affiliated Hospitals of Xuzhou Medical University. Plasma samples were collected from subjects and stored at -80 °C until analysis. The concentrations of IL-31, IL-

32, and IL-33 were determined using an ELISA instrument (Bio-Rad 550, United States) at 450 nm, following the manufacturers' instructions for human IL-31 (KGEHC141, KeyGEN BioTECH, Nanjing, Jiangsu Province, China), IL-32 (SEB802Hu, Cloud-Clone Corp, Wuhan, Hubei Province, China) and IL-33 (KGEHC151, KeyGEN BioTECH). All samples were tested in duplicate.

Statistical analysis

GraphPad Prism 6.0 and SPSS 16.0 statistical software packages were used for the statistical analysis of the results of immunohistochemistry and ELISA. Comparison between two groups was performed *via* the Mann-Whitney *U*-test. Comparisons among multi-groups were performed *via* the Kruskal-Wallis test. Low and high cut-off values for cytokine expression were defined by receiver operating characteristic (ROC) curve analysis. Survival curves were plotted by the Kaplan-Meier method and compared by the log-rank test. Cox proportional hazards model was used to identify the prognostic factors that influenced survival. $P < 0.05$ was considered statistically significant[35].

RESULTS

Baseline characteristics of patients

The detailed patients' information is presented in Table 1. Notably, there were four early GC patients, specifically stage T1 patients, among the 180 GC patients involved (Table 1). The management of patients after gastric resection uniformly followed the 2018 Chinese guidelines for diagnosis and treatment of GC, the National Health Commission of The People's Republic of China[36]. All patients had complete clinical information. Among them, 77 had follow-up until their death or until their most recent contact. The other patients were lost to follow-up (Figure 1). There were 42 cancerrelated deaths among the 77 patients (54.5%). Thus, amongst the 77 cases, 6 were stage I, and 32 were stage II.

Local expression of IL-31, IL-32, and IL-33 in GC tissue and in peripheral blood of GC patients

The expression levels of IL-31 (Figure 2A and B), IL-32 (Figure 2E and F), and IL-33 (Figure 1I and J) in GC tissue were investigated using immunohistochemistry. The densities of IL31 (Figure 1C), IL-32 (Figure 2G), and IL-33 (Figure 2K) are presented as box plots, including medians and 25th and 75th percentiles. IL-31, IL-32, and IL-33 were decreased by 9.4%, 28.2% and 27.5%, respectively, in GC compared to histologically normal adjacent gastric tissues ($P < 0.05$).

There was no significant difference in IL-31 (Figure 2D) or IL-32 (Figure 2H) concentration in the peripheral blood between GC patients and heathy controls. However, the mean value for IL-33 levels in peripheral blood of GC patients was 1.50 ± 1.11 ng/mL, which was significantly lower than that of healthy individuals (9.61 ± 8.00 ng/mL; $P < 0.05$) (Figure 2L).

Correlation between IL-31, IL-32, and IL-33 expression in GC and clinicopathological parameters

Associations between clinicopathological parameters and IL-31, IL-32, and IL-33 expression are listed in Table 1, Figures 3 and 4, and Supplementary Figures 1 and 2. All three ILs were associated with the age of GC patients (Figure 3A-D, IL-31; Figure 3E-H, IL-32; Figure 3I-L, IL-33). There was significantly lower expression of IL-31, IL-32, and IL-33 in the group of GC patients aged ≤ 60 years compared to the patients aged > 60 ($P < 0.05$). Significantly lower IL-32 (Figure 4A-D) and IL-33 (Figure 4E-H) expression was also observed in female GC patients compared to male GC patients ($P < 0.05$). However, no significant difference was observed in IL-31 expression when GC patients were stratified by sex (Supplementary Figure 2). Additionally, there were no correlations observed among IL31, IL-32, and IL-33 and other parameters, such as tumour size, lymph node metastasis, tumour differentiation, tumour invasion depth (Supplementary Figure 1), and TNM stage (Supplementary Figure 2) of GC.

Prognostic cytokines for overall survival of GC patients

To evaluate whether decreased IL-31, IL-32, and IL-33 correlate with survival of GC patients, low and high cut-off points for IL-31 (Figure 5A), IL-32 (Figure 5B), and IL-33 (Figure 5C) were defined by ROC curve analysis (Figure 5). The cut-off values for the three ILs were determined to be: IL-31, 1486000 AU; IL-32, 64893 AU; IL-33, 166291 AU. Kaplan-Meier survival curves were constructed to compare the survival of GC patient with high and low expression of IL-31 (Figure 5D), IL-32 (Figure 5E), and IL33 (Figure 5F). The data revealed that there were no correlations between IL-31, IL-32, and IL-33 expression and the prognosis of GC patients (Figure 4). However, Kaplan-Meier analysis was applied to further compare overall survival according to IL-31 (Figure 5G), IL-32 (Figure 5H), and IL33 (Figure 5I) expression in different subgroups of GC (Figure 5). Figure 4 shows that decreased IL-32 staining correlated with a significantly worse survival of patients in the TNM I-II stage subgroup ($P = 0.006$) (Figure 5K) and in the tumour invasion depth T4 subgroup ($P = 0.004$). There were no significant differences in the other clinicopathological subgroups of GC for IL-31, IL-32, and IL-33 (Supplementary Figures 3-5). Furthermore, there was no significant differences in the combination of IL-31, IL-32, and IL-33 expression for the prognosis of GC patients (Supplementary Figure 6).

Table 1 Correlations between interleukin-31, interleukin-32, and interleukin-33 expression and clinical/pathological features in patients with gastric cancer (*n* = 180)

Characteristic	Patient number	IL-31 median	*P* value	IL-32 median	*P* value	IL-33 median	*P* value
All cancer	180	1.333×10^6		99245		125998	
Noncancer (non)	159	1.472×10^6	0.043	138164	0.001	173818	< 0.0001
Gender							
Male	140	1.344×10^6		106075		143830	
Female	40	1.208×10^6	0.329	81009	0.040	89697	0.029
Age							
≤ 60	79	1.082×10^6		74098		106857	
> 60	101	1.404×10^6	0.007	122682	0.001	148615	0.026
Tumour size (diameter)							
< 5 cm	87	1.325×10^6		98583		122572	
≥ 5 cm	93	1.335×10^6	> 0.999	101583	> 0.999	126415	> 0.999
Lymph node metastasis							
No	75	1.404×10^6		106075		143359	
Yes	105	1.267×10^6	0.284	93196	0.671	113657	0.3
Differentiation							
High	14	1.609×10^6	H/M > 1	114379	H/M > 1	171038	H/M: > 0.999
Moderate	78	1.393×10^6	H/L: 0.6	113024	H/L> 1	142850	H/L: 0.2
Low	88	1.146×10^6	M/L: 0.3	91551	M/L: 0.4	104570	M/L: 0.1
Invasion depth							
T1	4	2.072×10^6		218529	T1/T3: 0.5, T1/T4: 0.6	156096	
T2	27	1.600×10^6		110353		143582	
T3	75	1.208×10^6		98367		116081	
T4	74	1.318×10^6	All > 1	96542	T1/T2, T2/T3, T2/T4, T3/T4, all > 1	125941	All > 1
TNM							
I	12	1.355×10^6		98583		142117	I/IV: 0.8
II	70	1.414×10^6		113560	II/ IV: 0.6	147031	II/III: 0.3, II/IV: 0.1
III	92	1.288×10^6		87667		107919	III/IV: 0.7
IV	6	0.950×10^6	All > 1	54851	I/II, I/III, I/IV, II/ III, III/IV, all > 1	52195	I/II, I/III>1

IL: Interleukin.

Correlation of IL-32 with overall survival in subgroups of GC patients

Univariate and multivariate Cox regression analyses were used to examine whether IL-32 is an independent prognostic marker for subgroups of GC patients, including IL32 expression level, age, sex, tumour differentiation, lymph node invasion, tumour size, depth of tumour invasion, and TNM stage.

Data from patients within the T4 stage subgroup, analysed by univariate analysis, exhibited a correlation between the survival of GC patients and IL-32 expression and TNM stage. In multivariate analysis, IL-32 expression and TNM stage remained as significant independent prognostic factors for survival of GC patients (Table 2).

Furthermore, decreased survival of GC patients in the TNM I-II stage subgroup was found to correlate with lymph node metastasis and tumour size on univariate analysis, but not on multivariate analysis. However, both univariate and multivariate analyses revealed no significant correlations

Table 2 Univariate and multivariate analyses of clinicopathological factors affecting survival of patients with gastric cancer at T4 stage

Variables analysis	Univariate HR (95%CI)	*P* value	Multivariate HR (95%CI)	*P* value
IL-32 (low/high)	4.338 (1.450-12.980)	0.009	3.287 (1.024-10.555)	0.046
Tumour differentiation (low/moderate)	0.710 (0.225-2.237)	0.559		
TNM		0.008		0.037
IV (reference)	1		1	
II	0.034 (0.003-0.423)	0.008	0.069 (0.005-0.946)	0.045
III	0.203 (0.018-0.464)	0.004	0.127 (0.025-0.646)	0.013
Lymph node metastasis (no/yes)	0.441 (0.098-1.982)	0.285		
Diameter (< 5/≥ 5, cm)	0.475 (0.161-1.404)	0.178		
Female/male	0.912 (0.323-2.573)	0.862		
Age (≤ 60/> 60)	1.950 (0.688-5.529)	0.209		

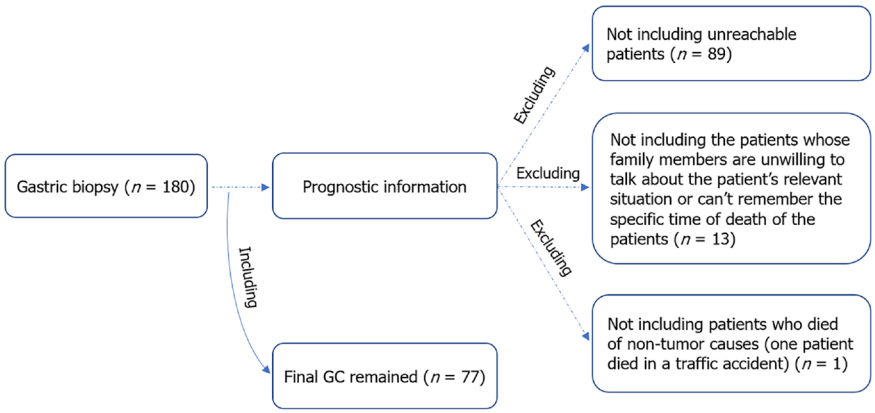

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Figure 1 Flow chart for recruitment of gastric cancer patients. GC: Gastric cancer.

between decreased IL-32 expression and survival of GC patients in the TNM I-II stage subgroup of GC patients (Table 3).

DISCUSSION

The current study demonstrated that the levels of expression of IL-31, IL-32, and IL-33 were all decreased in GC tissue compared to adjacent non-cancer gastric tissue and that the extent of these reductions in expression was higher in younger patients below the age of 60 years. Additionally, in the case of IL-32 and IL-33, their expression was found to be lower in females compared to males. However, the levels of expression of all three ILs amongst all the GC patients as a group did not correlate with a survival benefit, although subgroup analysis did reveal a survival benefit associated with higher levels of expression of IL-32 in the T4 stage and the TNM I-II stage subgroups.

H. pylori, a spiral Gram-negative rod that infects the human stomach in 50% of humans, is a definite human oncogenic agent[11], consistent with the previous finding that *H. pylori* contributed to > 60% of all GCs[12]. It has been clearly demonstrated by the Nobel laureate Barry Marshal that chronic gastric ulceration is caused by *H. pylori* infection[13]. The constitutive level of IL-32 is upregulated in both the gastric mucosa and GC tissue infected with *H. pylori*[14]. The cell-mediated immune response is extremely important in defence against tumour development, since compromised host immunity contributes to the establishment, proliferation, and metastasis of malignant tumours[15], a concept that is further supported by others who have shown that incompetent inflammation/immunity leads to tumour progression[16].

IL-31, an immunoregulatory cytokine secreted mainly by activated Th2 cells, plays a major role in the process of chronic inflammation[17]. However, the involvement of IL31 in the pathogenesis of cancer is unclear. Malignant Tcells produce IL-31, consistent with increased circulating IL-31[18]. Additionally, in the advanced stages of cutaneous T cell lymphoma, improved pruritus in patients correlates with lower

Table 3 Univariate and multivariate analyses of clinicopathological factors affecting survival of patients with gastric cancer in TNM I-II stage

Variable	Univariate HR (95%CI)	*P* value	Multivariate HR (95%CI)	*P* value
IL-32 (low/high)	0.180 (0.024-1.370)	0.098		
Tumour differentiation		0.947		
Low (reference)	1			
High	1.259 (0.258-6.133)	0.776		
Moderate	0.964 (0.324-2.871)	0.947		
Tumour invasion depth		0.546		
T4 (reference)	1			
T2	0.567 (0.059-5.491)	0.624		
T3	1.460 (0.187-11.379)	0.718		
Lymph node metastasis (no/yes)	0.307 (0.108-0.868)	0.026	0.490 (0.152-1.578)	0.232
Diameter (< 5/≥ 5, cm)	0.259 (0.092-731)	0.011	0.368 (0.112-1.165)	0.088
Female/male	0.522 (0.116-2.340)	0.396		
Age (≤ 60/> 60)	0.562 (0.192-1.646)	0.293		

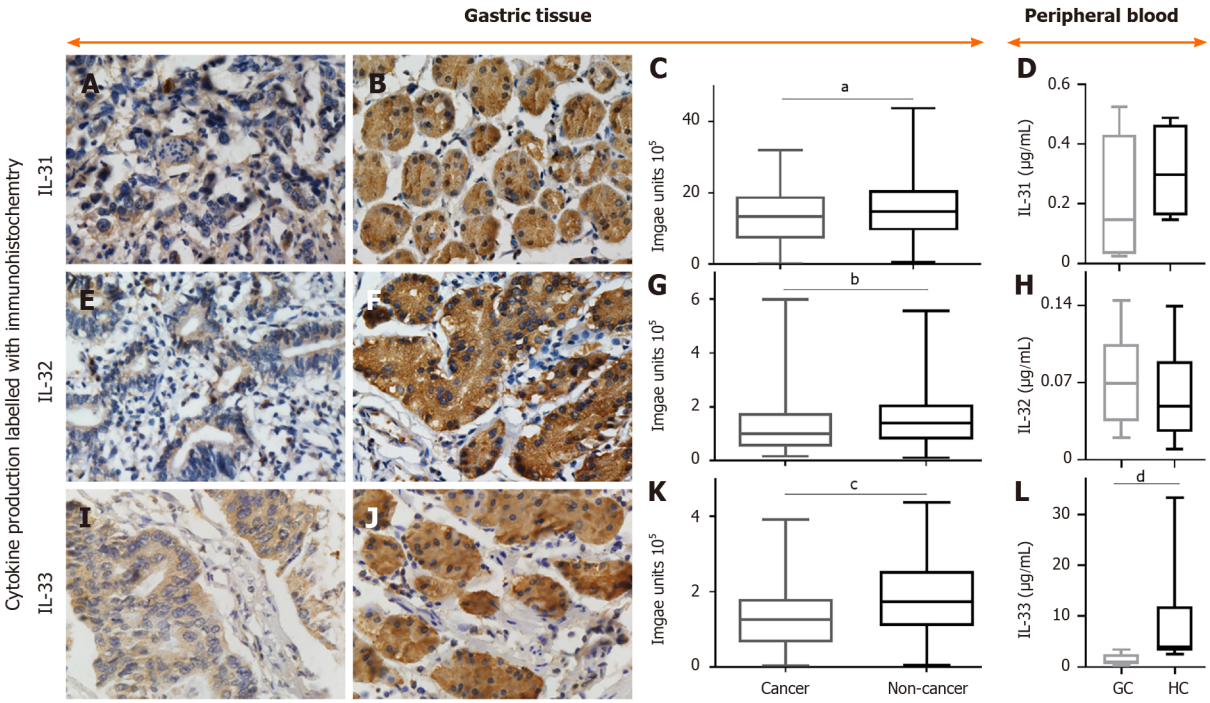

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Figure 2 Representative images of immunohistochemical staining for interleukin-31, interleukin-32, and interleukin-33 and their densities in non-cancerous and gastric cancer tissues, as well as their levels in peripheral blood of gastric cancer patients and healthy individuals. A-C: Positive (brown) interleukin (IL)-31 expression in gastric cancer (A) and noncancerous tissues (B) and quantified data (C); D, H, and L: IL-31 (D), IL-32 (H), and IL-33 (L) levels in peripheral blood from gastric cancer (GC) patients and healthy controls (HC); E-G: Positive IL-32 expression in gastric cancer (E) and noncancerous tissues (F) and quantified data (G); I-K: Positive IL-33 expression in gastric cancer (I) and noncancerous tissues (J) and quantified data (K). The densities of IL-31 and IL-33 were all decreased in GC compared to tumour-adjacent normal gastric tissues. Magnification, 600 ×. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.0001$; $^{d}P < 0.05$. GC: Gastric cancer; IL: Interleukin.

levels of IL-31[19].

We found decreased IL-31 in GC patients, particularly in younger patients. Our data are consistent with other studies that have shown that younger patients are more likely to have more poorly differentiated tumours compared to older patients with GC, suggesting that younger GC patients have more malignant types of GC[37]. The activity of IL-31 is mediated through the IL31 receptor A (IL-31RA) and

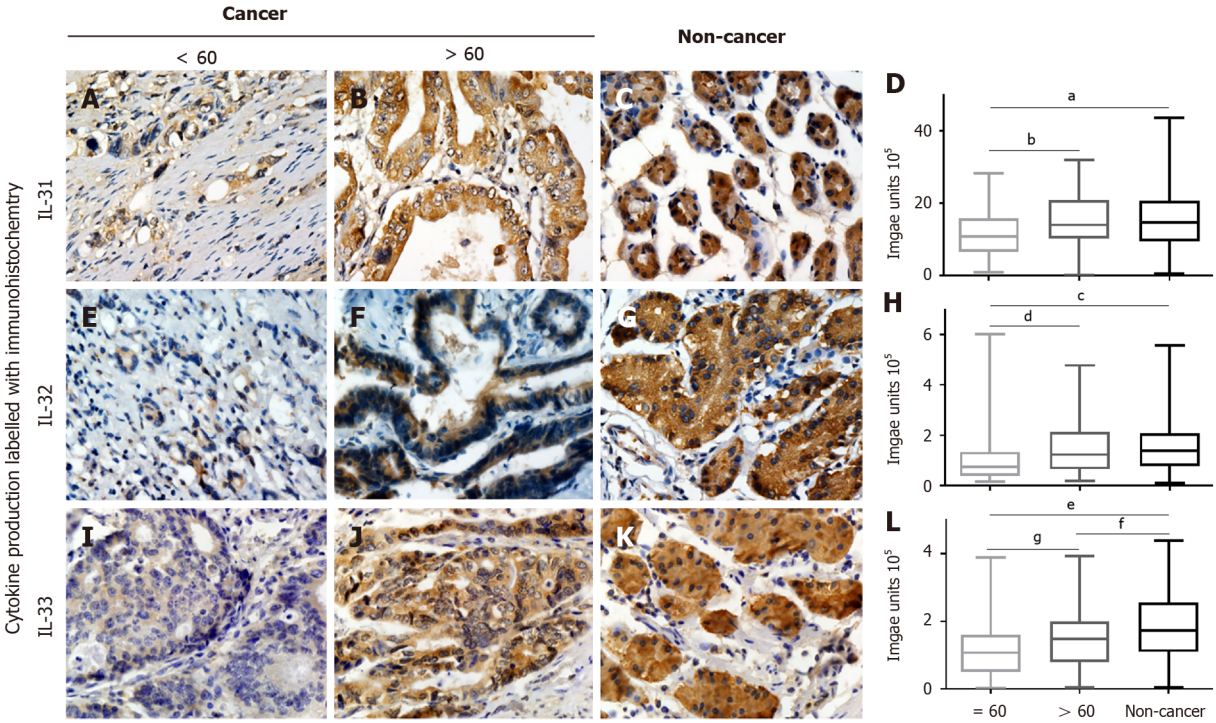

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Figure 3 Correlation of interleukin-31, interleukin-32, and interleukin-33 expression with age. A-D: Positive (brown) interleukin (IL)-31 expression in gastric cancer tissues from patients aged < 60 (A) *vs* > 60 (B) years and noncancerous tissues (C) plus quantified data (D); E-H: Positive (brown) IL-32 expression in gastric cancer tissues from patients aged < 60 (E) *vs* > 60 (F) years and noncancerous tissues (G), and quantified data (H); I-L: Positive (brown) IL33 expression in gastric cancer tissues from patients aged < 60 (I) *vs* > 60 (J) years and noncancerous tissues (K) plus quantified data (L). IL-32 and IL-33 were all decreased in the group of gastric cancer patients aged less than or equal to 60 years. $^{a}P < 0.01$; $^{b}P < 0.01$; $^{c}P < 0.0001$; $^{d}P < 0.01$; $^{e}P < 0.0001$; $^{f}P < 0.05$; $^{g}P < 0.05$. IL: Interleukin.

the oncostatin M receptor[38,39]. The two different isoforms of the IL-31RA consist of either long (745 residues) or short (560 residues) isoforms which may induce contrary functions[40]. Proliferation of follicular lymphoma is enhanced *via* the long IL-31RA isoform, whereas germinal centre-derived B-cell malignancy is inhibited *via* the short IL-31RA isoform[41]. There is no direct evidence available that identifies which isoform/s of IL-31RA are activated in GC *via* the IL-31 signalling pathway. However, our data are consistent with the hypothesis that IL-31 mediates an anti-cancer role in GC through the short IL-31RA isoform.

The involvement of IL-33 in non-small cell lung cancer is controversial, *i.e.*, high IL33 has been found to be of diagnostic and prognostic value[27], but another report shows no significant associations[28] between IL-33 and the overall 5-year survival rate[32]. IL-33 promotes GC invasion/migration *via* stimulating MMP-3 and IL6 *in vitro*[29], which has been confirmed in a GC animal model by ablation of the cognate IL-33 receptor ST2[30]. *IL-33* mRNA is significantly higher in GC tissue compared to that of non-cancer tissue[31], suggesting that IL-33 promotes the development of GC.

We observed similar levels of expression of IL-31 and IL-33 in GC, with decreased IL33 in both younger GC patients and in female GC patients, which is consistent with data from others, who have shown that female sex is a significant factor for predicting a higher likelihood of lymph node metastasis in mucosa-confined, poorly differentiated GC[42]. IL-33 is a multifunctional cytokine that can bind to the IL-33 receptor (ST2), to regulate immunity *via* activating Th1 cells, Th2 cells, $CD8^+$ T cells, and NK cells[43,44]. There are two forms of ST2: The transmembrane form ST2L that when bound to IL-33, is able to activate target cells[45], and the soluble, secreted form of ST2 (sST2) that acts as a decoy receptor and negatively regulates IL-33 signalling[46]. The possible role of IL33 in carcinogenesis has been demonstrated in an IL-33 transgenic mouse metastasis model, demonstrating inhibition of the growth and metastasis of B16 melanoma and Lewis lung carcinoma cells, *via* activating $CD8^+$ T cells and NK cells[47]. Thus, these data may be useful for future therapeutic design, utilising the anti-cancer role of IL-33 in GC.

IL-32, a proinflammatory cytokine, is highly expressed in several autoimmune diseases, *e.g.*, rheumatoid arthritis, inflammatory bowel disease, and atopic dermatitis[20,21]. However, the role of IL-32 appears to vary amongst different forms of cancer, *e.g.*, IL32 has been reported to inhibit colon cancer and leukaemia[22,23], but promotes pancreatic cancer[24]. The role of IL-32 in GC is also controversial, *i.e.*, IL-32 is elevated in GC compared with normal stomach tissue[14], but other groups have found either substantially reduced IL32 expression in GC for the diffuse type of GC[26], or no significant difference has been observed between GC and normal stomach tissue[25]. These divergent observations

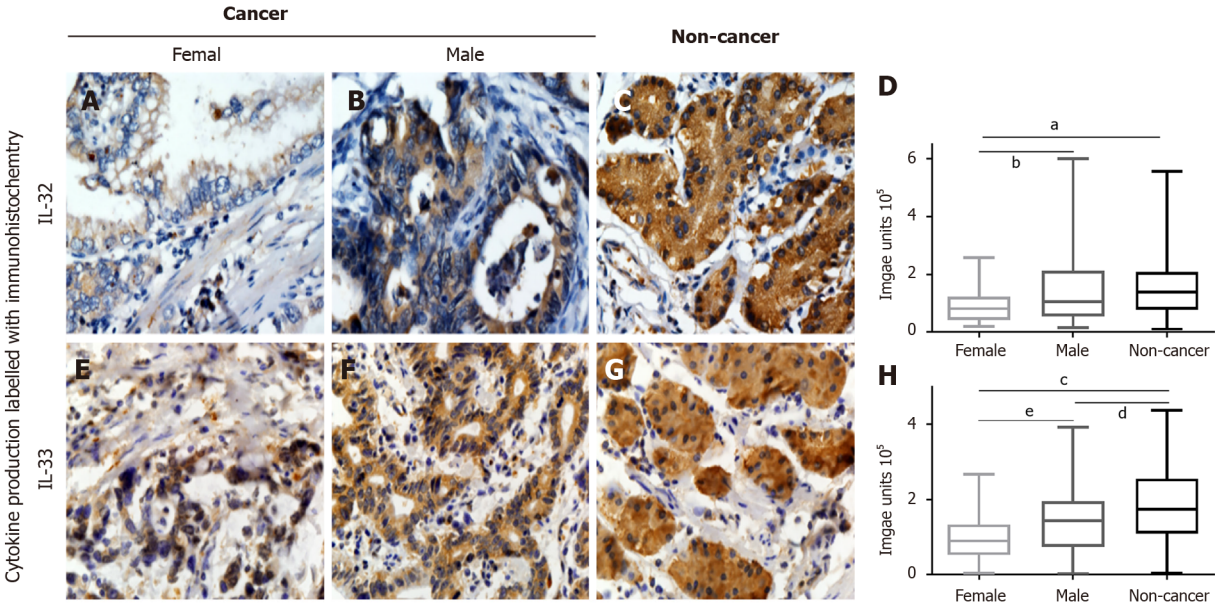

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Figure 4 Correlation of interleukin-32 and interleukin-33 expression with sex. A-D: Positive (brown) interleukin (IL)-32 expression in gastric cancer tissues from female (A) *vs* male (B) patients and noncancerous tissues (C) plus quantified data (D); E-H: Positive (brown) IL-33 expression in gastric cancer tissues from female (E) *vs* male (F) patients and noncancerous tissues (G) plus quantified data (H). IL-32 and IL-33 both decreased in female patients with gastric cancer. [a]*P* < 0.001; [b]*P* < 0.05; [c]*P* < 0.0001; [d]*P* < 0.01; [e]*P* < 0.05. IL: Interleukin.

concerning IL32 expression in GC may be due to different races and/or different tumour micro-environments.

We found that the expression of IL-32 was decreased in both younger patients and in female patients with GC, consistent with more severe forms of GC in younger and female patients, suggesting that IL-32 may mediate host defence against the development of GC. Furthermore, we found that high IL-32 expression correlated with a longer survival of GC patients, in the T4 stage and TNM I-II stage subgroups and that IL32 was an independent prognostic factor for survival in the T4 stage subgroup. Interestingly, the IL-32 positive rate in GC (12%) has been reported to be much lower than the rate in oesophageal squamous cell carcinoma (60%), but no comparison to non-cancerous tissues has been made[48,49]. Thus, we propose a hypothesis for the possible mechanism of IL-32 involvement in carcinogenesis as follows: Because IL-32 contributes to the host defence *via* enhancing differentiation of monocytes into macrophages[50], decreased IL-32 in GC tissue, seen particularly amongst the younger or female patients, may compromise host innate immunity, and subsequently contribute to poorly controlled development of cancer. Notably, macrophages are classified as either classical M1 macrophages that promote the inflammatory response against microorganism invasion and are thought to inhibit carcinogenesis, or as M2 macrophages that regulate host immunity and are thought to promote carcinogenesis[51]. It remains to be clarified whether tumourassociated macrophages in GC are derived from one subset or the other, which either promote the development of cancer (M2) or suppress cancer growth (M1), which is perhaps dependent on the tumour microenvironment[52]. For example, IL-32 can induce cell death in thyroid cancer cells through the induction of IL-8 and caspase-8[53], subsequently up-regulating the proinflammatory response.

IL-32 may also be able to inhibit tumour growth indirectly, hence it may be efficacious as a clinical anti-cancer therapy[54]. For example, the application of siRNA to inhibit IL-32 enhances angiogenesis in HUVECs[55] *via* up-regulation of VEGF and PDGF. Our current findings showed an inverse correlation between IL-32 and the development of GC, suggesting that IL-32 inhibits the development of cancer directly and/or indirectly, which will be further investigated in future experiments.

Finally, the levels of circulating IL-31, IL-32, and IL-33 were found to be consistent with their respective expression levels in GC tissue, further supporting the relevance of the potential role for these cytokines in mediating tumour-related immunity. However, we hypothesise that the host systemic and/or local inflammatory/immune response may be insufficient to inhibit the development of GC, among the GC cohorts studied, leading to tumour progression[16].

Unfortunately, no correlation with survival of GC patients was observed among any combination of IL-31, IL-32, and IL-33 expression, a similar result that we have reported previously for the relationship with IL-34 in GC[35]. The current observations are consistent with others, showing that there is no significant correlation between IL-33 expression and overall survival[32]. However, the advantage of our current data is the analysis for the combined IL-31, IL-32, and IL-33 data, to determine the correlation with GC patients from the same cohort. It remains to be explored why there is a discrepancy

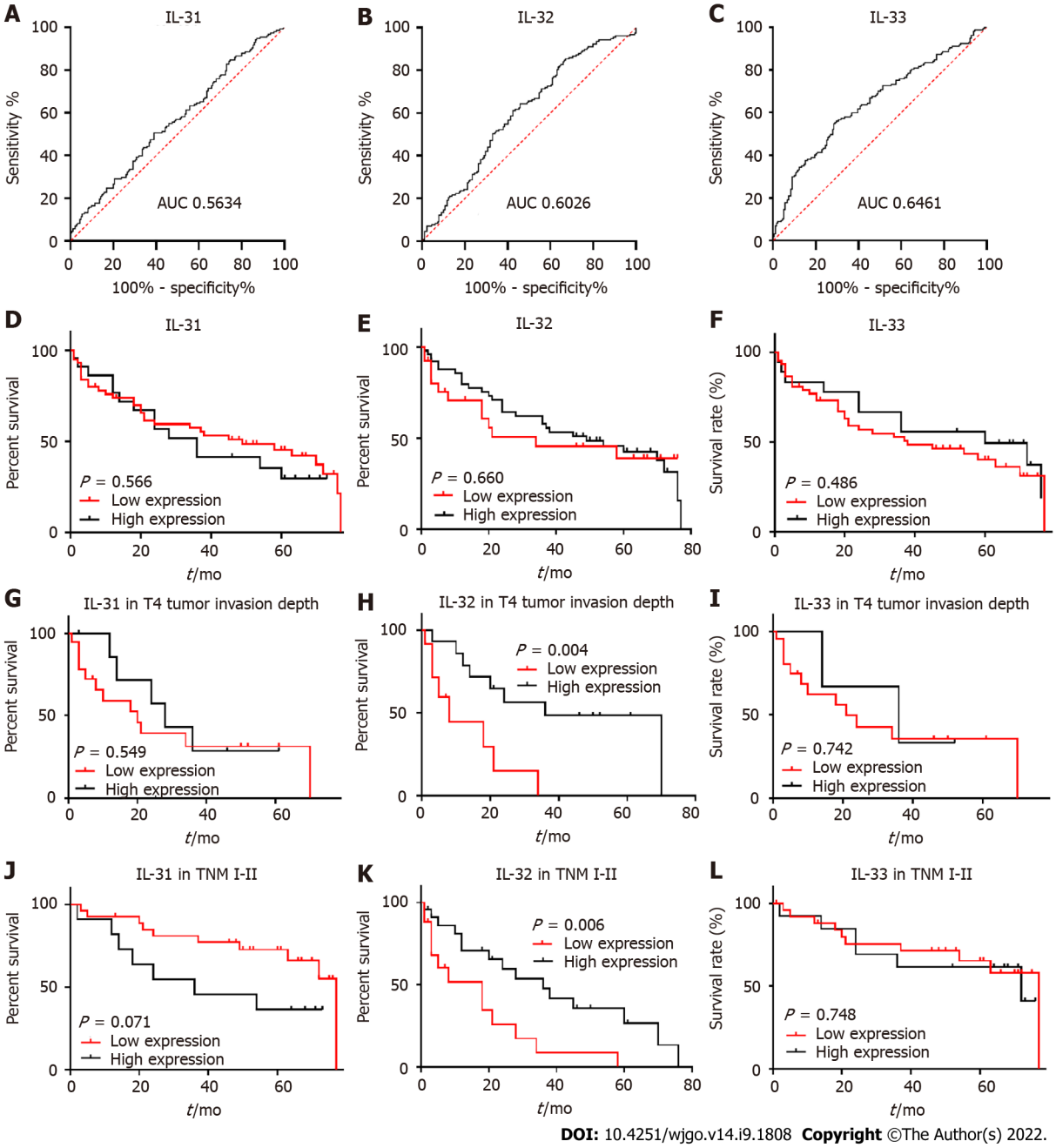

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Figure 5 Receiver operating characteristic curves, correlation of interleukin-31, interleukin-32, and interleukin-33 with prognosis of gastric cancer, and subgroup analysis for patients at T4 stage and TNM stage I-II. A-C: The specificity (X-axis) *vs* sensitivity (Y-axis) of interleukin (IL)-31 (A), IL-32 (B), and IL-33 (C); D-F: Comparison of 5-year survival rate between patients with high and low IL-31 (D), IL-32 (E), and IL-33 (F) expression; G-I: Comparison of 5-year survival rate between patients with high and low IL-31 (G), IL-32 (H), and IL-33 (I) expression in the T4 subgroup; J-L: Comparison of 5-year survival rate between patients with high and low IL-31 (J), IL-32 (K), abd IL-33 (L) expression in the TNM stage 2 subgroup. ROC curves analysis displayed the poor diagnostic potential of IL-31, IL-32, and IL-33 expression for GC. The cut-off point and area under the curve were: IL-31: 1.486 × 10^6, area under the curve (AUC) = 0.563; IL-32: 64893, AUC = 0.603; IL-33: 166291, AUC = 0.646. Kaplan-Meier survival analysis of GC patients showed that decreased IL-32 expression correlated with a poor survival of GC patients in the T4 and TNM I-II subgroups. IL: Interleukin; AUC: Area under the curve.

among IL-31, IL-32, IL-33, and IL-34 during the development of GC, which may be due to different receptors and/or signalling pathways, which will be clarified in the conditioning knockout mice in future studies.

There are some limitations for the current study. First, the number of GC patients and normal individuals who were sampled was rather small for the evaluation of circulating cytokines, using ELISA. However, this pilot study was undertaken to simply provide proof of concept that a systemic response is involved compared to only local cytokine expression in the affected gastric tissues, as well as to support our immunohistochemistry findings. A study with a larger sample size and a range of different backgrounds will be performed in the future.

Second, the stomach tissue of normal healthy people would be the ideal control for GC for comparison, and would offer more convincing evidence. However, we were unable to collect any normal healthy stomach tissue due to ethical issues. We are applying for human ethics approval for the collection of normal healthy stomach tissue from organ donors in the future.

The GC patient cohort recruited for this study was initially set at a reasonable size, *i.e.*, 180 in total, to establish sufficient power to detect clinically relevant differences in the expression levels of the ILs that we examined. Regrettably, more than half of the patients were lost to follow-up during the course of the study, and only 77 GC patients had complete followup data (Figure 5). The data in relation to expression levels were based on all 180 patient samples that were initially recruited to ensure that the study was sufficiently powered to detect the potential role of IL31, IL32, and IL33 during the development of GC. If we had only selected the 77 GC patients with complete follow-up data for all aspects of this study, we would be highly likely to lose some important information and/or statistical power in exploring the correlation of these cytokines with clinical presentations. However, the survival analysis could only be performed on the adequately followed sub-cohort of 77 patients. We are currently collecting more samples with a full history and complete follow-up data in collaboration with other institutes, *i.e.*, a larger number of samples for more convincing information for our future studies.

Because there was no local recurrence of GC within the current cohort, we cannot explore the potential role of these cytokines in the prediction of local recurrence of GC. We are currently searching for both primary and recurrent GC cases for future study.

CONCLUSION

In summary, our data demonstrate that IL-31, IL-32, and IL-33 expression in GC is all decreased, which correlates with younger age of the GC patients. IL-32 and IL-33 also correlate with the sex of the GC patients. Decreased IL-32 correlates with a poorer survival of GC patients in the T4 stage and TNM I-II stage subgroups. Downregulation of IL-32 is an independent prognostic factor for survival of T4 GC patients. Finally, low IL-33 in peripheral blood may be considered as an objective predictive marker for the development of GC. However, further studies are required to investigate the mechanism of action of these ILs in GC.

ARTICLE HIGHLIGHTS

Research background

Gastric cancer (GC) is one of the most common malignancies in China with a high morbidity and mortality. Despite the more widespread use of recent diagnostic techniques, including endoscopic examination, many GC patients are diagnosed at advanced stages, resulting in a poor 5-year survival rate, emphasizing the critical need for development of a reliable biomarker(s) with high specificity and sensitivity to improve the prediction of prognosis for more successful outcomes for GC patients. Endoscopic examination provides a useful approach in the early detection of GC, and in reducing cancer-related mortality.

Research motivation

The cell-mediated immune response is extremely important in defence against tumour development, since compromised host immunity is known to contribute to the establishment, proliferation, and metastasis of malignant tumours. Although high host inflammatory status has been reported in the tumour microenvironment, an incompetent inflammatory/immune response will lead to tumour progression.

Research objectives

We aimed to identify the expression of interleukin (IL)-31, IL-32, and IL-33 in GC and assess their inter-correlation and clinical significance.

Research methods

GC tissues were obtained from patients without local recurrences for immunohistochemistry to determine the expression of IL-31, IL-32, and IL-33. Additionally, circulating levels of IL-31, 32, 33 were determined using ELISA. The Mann-Whitney *U* test or the Kruskal-Wallis test was used for statistical analysis.

Research results

IL-31, IL-32, and IL-33 expression was all lower in GC than in adjacent non-cancer gastric tissues ($P < 0.05$). IL-33 level in peripheral blood of GC patients was significantly lower than that of healthy

individuals (1.50 ± 1.11 *vs* 9.61 ± 8.00 ng/mL, ($P < 0.05$). Decreased IL-31, IL-32, and IL-33 expression in GC was observed in younger patients (< 60 years), and IL-32 and IL-33 expression was lower in female patients ($P < 0.05$). Higher IL-32 expression correlated with a longer survival in two GC subgroups: T4 invasion depth and TNM stage I-II. Univariate/multivariate analysis revealed that IL-32 was an independent prognostic factor for GC in the T4 stage subgroup. Circulating IL-33 was significantly lower in GC patients at TNM stage IV than in healthy people ($P < 0.05$).

Research conclusions

IL-31, IL-32, and IL-33 expression in GC is all decreased, which correlates with younger age of the GC patients. IL-32 and IL-33 expression also correlates with the sex of the GC patients. Decreased IL-32 correlates with a poorer survival of GC patients in the T4 stage and TNM stage I-II subgroups. Down-regulation of IL-32 is an independent prognostic factor for survival of T4 GC patients. Finally, low IL-33 in peripheral blood may be considered as an objective predictive marker for the development of GC.

Research perspectives

Further studies are required to investigate the mechanism of action of these ILs in GC.

ACKNOWLEDGEMENTS

We acknowledge the staff from the Department of Pathology, Xuzhou Medical University for their support.

FOOTNOTES

Author contributions: Liu QH collected the samples, performed the histopathological and immunohistochemical examinations, analysed the data, and wrote the paper; Zhang JW collected the samples, analysed the data, and wrote the paper; Liu QH and Zhang JW contributed equally to the study; Xia L performed ELISA and data analysis; Wise SG provided intellectual input; Hambly BD and Tao K revised the manuscript and provided intellectual input; Bao SS designed the experiment and revised the manuscript.

Supported by the National Natural Science Foundation of China, No. 81502030.

Institutional review board statement: This study was approved by the Human Ethics Committee, the Institutional Review Boards of Affiliated Hospital of Xuzhou Medical University and conducted in accordance with the Declaration of Helsinki.

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

Conflict-of-interest statement: The authors declare that no financial or other conflict of interest exists in relation to the content of the article.

Data sharing statement: The data can be available upon request.

STROBE statement: The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.

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S-Editor: Yan JP
L-Editor: Wang TQ
P-Editor: Li X

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