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Observational Study

Clinical implications of interleukin-31, interleukin-32 and interleukin-33 in stomach cancer

Liu QH *et al*. Clinical implications of three interleukins in stomach cancer

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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 IL-31, IL-32 and IL-33 expression and clinicopathological parameters of GC patients.

METHODS

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

RESULTS

We found that IL-31, IL-32 and IL-33 were all lower in GC than that in adjacent non-GC gastric tissue (P for all < 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 for all < 0.05). Higher IL-32 correlated with longer survival in two GC subgroups: T4 invasion depth and TNM I-II stage. Univariate/multivariate analysis revealed that IL-32 is an independent prognostic factor for GC within the T4 stage subgroup. Circulating IL-33 was significantly lower in GC patients at stage 4 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 is further discussed.

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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 IL-31, IL-32 and IL-33 expression and clinicopathological parameters of GC patients. IL-31, IL-32 and IL-33 in GC and non-GC tissues were correlated with 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.

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 recent diagnostic advances, including endoscopic examination, many GC patients are diagnosed with advanced stage disease, resulting in a poor five-year survival rate (< 20%), emphasising the critical need for development of a reliable biomarker(s)[3] with high specificity and sensitivity, to improve 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 subtypes 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 stomach cancers, 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 T-cells

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 IL-32 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 micro-environments.

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 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 ST2-ERK1/2 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 have specifically assessed the relationships among IL-31, IL-32 and IL-33 in GC utilising the same cohort of patients. In our study we aim to identify the expression of IL-31, IL-32 and IL-33 in GC and assess their inter-correlation and clinical significance. Our data may provide useful information for both our 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 in 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 within 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 were: rabbit anti-IL-31 polyclonal antibody (22859-1-AP, Proteintech, China), rabbit anti-IL-32 polyclonal antibody (11079-1-AP, Proteintech, China) and rabbit anti-IL-33 polyclonal antibody (12372-1-AP, Proteintech, China). The dilution for all three antibodies was 1:100. An HRP-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 10 GC patients prior to preoperative chemotherapy in the Affiliated Hospital, Xuzhou Medical University, China. Blood from 10 healthy age and sex matched non-cancer persons presenting for a routine health check-up were collected as controls in our study. Consents were obtained from both GC patients and healthy controls. The circulating cytokine study has also been 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 were 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, followed 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, Nanjing, Jiangsu Province, China). 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's 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 (Table 1). Notably, there were four early GC patients, specifically stage T1 patients, among the 180 GC tissues (Table 1). The management of patients after gastric resection uniformly followed Chinese guidelines for diagnosis and treatment of GC 2018, the National Health Commission of The People's Republic of China[36]. All patients have 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 cancer-related 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 IL-31 (Figure 1C), IL-32 (Figure 2G) and IL-33 (Figure 2K) were 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 for all < 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* for all < 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* for both < 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 IL-31, 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

Correlation between decreased IL-31, IL-32 and IL-33 with overall survival of GC patients. To evaluate whether decreased IL-31, IL-32 and IL-33 correlated 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 and IL-33 166291 AU. Kaplan-Meier survival curves were constructed to compare the survival of high and low expression GC patient groups in relation to IL-31 (Figure 5D), IL-32 (Figure 5E) and IL-33 (Figure 5F) expression. Our data reveal that there were no correlations between IL-31, IL-32 and IL-33 expression with 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 IL-33 (Figure 5I) expression in different subgroups of GC (Figure 5). Figure 4 shows decreased IL-32 staining correlated with significantly worse survival of patients in the TNM I-II tumour stage subgroup (*P* = 0.006) (Figure 5K) and in the tumour invasion depth T4 subgroup (*P* = 0.004). There was no significant differences found 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 found in the combination of IL-31, IL-32 and IL-33 expression for the prognosis of GC patients (Supplementary Figure 6).

Further analysis of the correlation of IL-32 with overall survival in subgroups of GC patients

Univariate and multivariate Cox regression analysis was used to examine whether IL-32 was an independent prognostic marker for subgroups of GC patients, including IL-32 level of expression, age, sex, tumour differentiation, lymph node invasion, tumour size, the 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, respectively. 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 within 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 analysis revealed no significant correlations between decreased IL-32 expression and survival of GC patients within the TNM I-II stage subgroup of GC patients (Table 3).

DISCUSSION

The current study has demonstrated that the levels of expression of IL-31, IL-32 and IL-33 are all decreased within GC tissue compared to adjacent non-cancer gastric tissue and that the extent of these reductions in expression are higher in younger patients below the age of 60 years. Additionally, in the case of IL-32 and IL-33, expression was found to be lower in females compared to males. However, the level 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 within 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 *H. pylori* contributing to > 60% of all stomach cancers[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 IL-31 in the pathogenesis of cancer is unclear. Malignant T-cells 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 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 IL-31 receptor A (IL-31RA) and 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 signalling 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 IL-33 has been found to be of diagnostic and prognostic value[27], but another report shows no significant associations[28] between IL-33 and overall 5 year survival rate[32]. IL-33 promotes GC invasion/migration *via* stimulating MMP-3 and IL-6 *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 IL-33 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 IL-33 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.*, IL-32 has been reported to inhibit colon cancer and leukaemia[22,23], but promotes pancreatic cancers[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 IL-32 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 concerning IL-32 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 IL-32 may mediate host defence against the development of GC. Furthermore, we found that high IL-32 expression correlated with longer survival of GC patients, within the T4 stage and TNM I-II stages subgroups and that IL-32 is an independent prognostic factor for survival within the T4 stage subgroup with GC. 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 undertaken[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 tumour-associated 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 have shown 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 the expression levels of the corresponding cytokines within 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 between 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 benefit 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 among IL-31, 32, 33 and 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. Firstly, 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 larger sample size with a range of different backgrounds will be performed in future.

Secondly, normal healthy stomach 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 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. However, regrettably more than half of the patients were lost to follow-up during the course of the study, consequently only 77 GC patients had complete follow-up (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 IL-31, 32 and 33 during the development of GC. If we had only selected the 77 GC patients with complete follow-up for all aspects of this study, then we would be highly likely to lose some important information and/or statistical power in exploring the correlation of these cytokines and clinical presentations. However, the survival analysis could only be performed on the adequately followed-up sub-cohort of 77 patients. We are currently collecting more samples with a full history and complete follow up, 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 comment on 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 were all decreased, which was correlated with younger age of the GC patients. IL-32 and IL-33 were also correlated with the sex of the GC patients. Decreased IL-32 was correlated with poorer survival of GC patients within the T4 stage and TNM I-II stage subgroups. Downregulation of IL-32 was an independent prognostic factor for survival of T4 GC patients. Finally, low IL-33 in peripheral blood may be considered as an objective prediction marker for the development of GC. However, further studies are required to investigate the mechanism of the role of these ILs in GC.

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