IL-32 expression indicates unfavorable prognosis in patients with colon cancer

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Abstract. Recently, interleukin (IL)-32 has been demonstrated to represent a novel biomarker for evaluating the prognosis of patients with gastric and lung cancer; however, its clinical significance in colon cancer remains unknown. In the present study, the IL-32 expression in 60 patients with colon cancer was examined with an immunohistochemistry assay. IL-32 expression was determined in 37 (61.67%) patients with colon cancer. Additionally, IL-32 was associated with tumor size and Dukes’ stage. By using the Kaplan-Meier method, patients with positive IL-32 expression had shorter overall survival time, compared with those with negative IL-32 expression. Multivariate analysis indicated that IL-32 could be an independent prognostic factor in patients with colon cancer; therefore, IL-32 may be a novel prognostic biomarker and therapeutic target for colon cancer.

Introduction

Colon cancer is a common malignancy type of the gastrointestinal tract (1). In 2012, colorectal cancer in China was 16.9 per 100,000 in males and 11.6 per 100,000 in females, and the age-standardized mortality was 9.0 per 100,000 in males and 6.1 per 100,000 in females (1). Notable progress has been achieved in colon cancer therapy; however, metastasis and recurrence of colon cancer still influences the outcome of patients profoundly (2). To improve patient prognosis, it is necessary to determine more effective biomarkers for diagnosis and treatment of colon cancer.

Inflammation is associated with the development of multiple types of cancer, such as esophageal cancer and pancreatic cancer (3,4). Previous studies have determined that the novel pro-inflammatory cytokine interleukin (IL)-32 is associated with the development of numerous types of cancer, such as breast cancer (5) and esophageal cancer (6). Additionally, IL-32 has increased expression levels in gastric cancer (7), pulmonary adenocarcinoma (8), pancreatic adenocarcinoma (9) and esophageal cancer (6).

Paradoxically, a number of researchers consider that overexpression of IL-32 has a tumor-suppressing effect (10-12). In thyroid cancer, cytological experiments have demonstrated that the number of BC-PAP and FTC133 apoptotic thyroid cancer cells decreases following suppression of IL-32 expression, while overexpression of IL-32 promotes apoptosis in cancer cells (10). The apoptosis-inducing effect of IL-32 has also been observed in HeLa cells, which originated from a cervical cancer sample (11). In a previous study, B16 melanoma cells were subcutaneously injected into transgenic and non-transgenic mice overexpressing IL-32. The volume and weight of the resulting tumor samples were measured on day 26, and those in transgenic mice had significantly reduced size and weight, compared with the non-transgenic mice (12).

In the present study, the IL-32 expression in colon cancer was assessed and its association with clinicopathological parameters was investigated. To the best of our knowledge, the present study provides the first evidence of the role of IL-32 in the prognosis of patients with colon cancer.

Patients and methods

Patients and samples. In the present study, 60 colon cancer specimens obtained from the Department of Pathology, The First Affiliated Hospital of Henan University of Science and Technology (Luoyang, China) between January 2006 and January 2007 were examined. The patients included 35 males and 25 females with a mean age of 56.2 years (range, 42-71 years). Pathological data were studied prospectively following surgery. The survival time was calculated from the date of surgery to the follow-up deadline (August 2016) or date of mortality. Two experienced pathologists from the Department of Pathology, the First Affiliated Hospital of Henan University of Science and Technology, were blinded to the results of the present study. Clinicopathological parameters of these patients are displayed in Table I. Tumor differentiation and Dukes’ stage were classified in accordance with the report by Fukushima et al (13). Written informed consent was

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obtained from each patient upon collection of the samples. The study protocol was approved by the Human Ethics Review Committees of Henan University of Science and Technology (approval no. 2013-PJ152).

**Immunohistochemistry.** Staining for IL-32 was performed using 10% formalin-fixed, paraffin-embedded serial sections at 37˚C. Sections (4-µm thick) were cut from the selected paraffin blocks and deparaffinized in dimethylbenzene. The slides were microwaved in sodium citrate-hydrochloric acid buffer solution for 4 min for antigen retrieval at 95°C and rehydration with phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, and 2 mM KH2PO4). IL-32 was detected with a mouse polyclonal antibody (cat. no: sc-517408, 1:150 dilution, Santa Cruz Biotechnology, Dallas, TX, USA). Ki-67 (cat. no: GT209401) were purchased from Gene Biotechnology (http://www.genetech.com.cn, Shanghai, China). The antibody reaction was conducted at 37˚C for 3 h. Labeling was detected by adding biotinylated secondary antibodies (http://www.maxim.com.cn/sitecn/myzhjcxthsjh/981.html, KIT-9710, Rabbit, 37˚C for 0.5 h), avidin-biotin complex and diaminobenzidine (all from Maxim-Bio, Fuzhou, China, http://www.maxim.com.cn/sitecn/myzhjcxthsjh/981.html). Sections were then counterstained with 10% hematoxylin at 37˚C for 0.5 h. Expression of IL-32 was scored according to the positive percentage and staining intensity of the stained tumor cells. The percent positivity was scored as ‘0’ (0-25%), ‘1’ (26-50%), ‘2’ (51-75%) and ‘3’ (>75%). The staining intensity was scored as ‘0’ (no staining), ‘1’ (weakly stained), ‘2’ (moderately stained) and ‘3’ (strongly stained). If the product of multiplication between staining intensity and the percentage of positive cells is ≥2, it was considered as immunoreaction positive (+). Ki-67 was scored according the positive number of 100 cancer cells at high magnification by a light microscope (Olympus Corporation, Tokyo, Japan; magnification, x400).

**Western blot analysis.** A total of 5 paired colon cancer and non-cancerous colonic epithelium tissues were lysed (RIPA Buffer Beyotime Institute of Biotechnology) and lysates were obtained by centrifugation at 4°C (35,000 x g, 30 min). BCA Protein assay kit was used to measure protein concentration. Following applying 30 µg total protein to 10% SDS-PAGE, the total protein was transferred to a polyvinylidene fluoride membrane. Following blocking (5% skimmed milk, 37˚C for 3 h), the membranes were incubated with IL-32 antibody at 4°C for 12 h (1:200 dilution; cat. no. sc-50001; Santa Cruz Biotechnology), followed by horseradish peroxidase-conjugated secondary antibody (Rabbit anti-goat IgG, ZB 2306, 1:5,000 dilution, incubation at 37˚C for 2 h). Subsequently, IL-32 expression was detected by enhanced chemiluminescent substrate (cat. no. 34580; Thermo Scientific, Inc.). GAPDH was served as the internal control (1:150 dilution; cat. no. sc-0411; Santa Cruz Biotechnology).

**Statistical analysis.** Statistical analyses were performed using SPSS 13.0 for Windows (SPSS, Inc., Chicago, IL, US). The association between IL-32 and clinicopathological parameters was assessed using Fisher’s exact test.
Results are presented as mean ± SD. Survival curves were calculated using the Kaplan-Meier method and compared by log-rank test. The Cox proportional hazards regression model was performed for multivariate survival analysis. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**IL-32 expression in non-cancerous colonic epithelium and colon cancer tissues.** To investigate the expression of IL-32 in colon cancer, IL-32 staining in 60 colon cancer specimens was assessed using immunohistochemistry. Immunohistochemical results indicated weak immunoreactivity of IL-32 in non-cancerous colonic epithelium (Fig. 1A and B); however, IL-32 staining was distributed in the cytoplasm of cancer cells (Fig. 1C and D). Of the 60 cases, 37 (61.67%) samples exhibited positive staining of IL-32 in the cytoplasm.

To further verify the results obtained from immunohistochemistry, western blot analysis was performed to detect the IL-32 expression in 5 paired colon cancer and non-cancerous colonic epithelium tissues. The data demonstrated that there was notably increased IL-32 expression in colon cancer tissues, compared with complementary non-cancerous tissues (Fig. 2), which was consistent with the immunohistochemical results.

**Association between IL-32 expression and proliferation index (PI).** To investigate the role of IL-32 on proliferation of colon cancer, the association between IL-32 and PI was evaluated. As depicted in Fig. 3, there was significantly increased PI in the group positive for IL-32 expression (64.5±9.2), compared with the IL-32 negative group (33.5±5.8). These data demonstrated that IL-32 may be associated with the proliferation of colon cancer cells.

**Association between IL-32 expression and clinicopathological factors of patients with colon cancer.** As depicted in Table I, there were 26 IL-32 positive samples with Dukes’ stage C+D, compared with only 6 for IL-32 negative samples. Furthermore, positive IL-32 expression was significantly associated with tumor size ≤5 cm, compared with negative IL-32 expression (P=0.015) and this result confirmed that IL-32 may be associated with the proliferation of colon cancer. No significant association was observed between IL-32 expression and other variables, including sex, age, tumor differentiation and lymph node metastasis (P>0.05).

**Association between IL-32 expression and outcome of patients with colon cancer.** In the present study, the survival of 60 patients with colon cancer, divided into IL-32-positive and -negative groups, was investigated. Patients positive for IL-32 expression had less favorable prognoses, compared with patients negative for IL-32 expression, which indicates that IL-32 may be an independent prognostic factor for colon cancer (IL-32-positive group vs. IL-32-negative group: P<0.05; Fig. 4).

Multivariate analyses were performed to confirm that IL-32 is an independent prognostic predictor for patients with colon cancer. Cox proportional regression models together with other pathological features, including sex, age, tumor size, Dukes’ stage, tumor differentiation and lymph node metastasis, were used. As presented in Table II, multivariate analysis indicated that IL-32 expression, Dukes’ stage, tumor differentiation and lymph node metastasis could be independent prognostic factors in patients with colon cancer. Notably, lymph node metastasis had the most significant parameter (P=0.014), followed by IL-32 expression (P=0.026), Dukes’ stage (P=0.035) and tumor differentiation (P=0.042).

**Discussion**

Inflammation promotes the development, invasion, and metastasis of a number of cancer types, such as colorectal cancer and nasopharyngeal carcinoma (14,15); the novel pro-inflammatory cytokine IL-32 has been demonstrated to have tumor-promoting and tumor-suppressing effects under certain conditions (5,16-22).
Tsai et al (23) determined that overexpression of IL-32 can cause gastric cancer cells to transition to spindle cells and epithelial cells to transition to mesenchymal cells, enhancing the ability of tumor cells to move and inducing the expression levels of IL-8, vascular endothelial growth factor, metalloproteinase (MMP)2 and MMP9 through the p-Akt/β-catenin pathway and the p-Akt/hypoxia inducible factor-α pathway. Subsequently, this enhances the ability of the cancer cells to invade other tissues and metastasize (23). Immunohistochemical staining of 120 patients with gastric cancer indicated that the strength of IL-32 staining in the tumor cytoplasm was notably increased, compared with the surrounding non-neoplastic mucosal cells (23). Ishigami et al (7) investigated 181 patients with...
gastric cancer and concluded consistent results to the aforementioned study (23). Additionally, IL-32 expression determined to be associated with lymph node metastasis and the progression of gastric cancer (7). Multiple-factor analysis demonstrated that IL-32 is an independent prognostic factor for patients with pulmonary adenocarcinoma (24). In vitro investigation of pulmonary adenocarcinoma A549 cells indicated that IL-32 has the ability to activate nuclear factor (NF)-κB and induce the generation of MMP2 and MMP9, consequently promoting the ability of cancer cells to invade other tissues (8).

However, Heinhuis et al (10) determined that IL-32β and IL-32γ promote apoptosis in thyroid cancer cells by activating the caspase-3/caspase8 pathway, and IL-32γ exhibited a more pronounced ability to induce apoptosis due to it downregulating C-X-C motif chemokine receptor 1 expression, blocking the survival signaling pathway of IL-8 (10). Furthermore, IL-32γ overexpression in the colon cancer cell line SW620 causes suppression of cell proliferation along with reduced activity of NF-κB and signal transducer and activator of transcription (STAT3), which indicates that IL-32γ can regulate tumor cell apoptosis and tumor development by suppressing NF-κB and STAT3 signals (22).

In the present study, it was determined that IL-32 expression is increased in colon cancer tissues, compared with non-tumoral colorectal tissues. Additionally, it was determined that IL-32 expression is significantly associated with an earlier Dukes' stage and a tumor size ≤5 cm. Therefore, IL-32 may participate in the tumorigenesis of colon cancer. Using multivariate Cox proportional hazards regression analysis, the role of IL-32 in the prognosis of patients with colon cancer was investigated. Consistent with data reported by Ishigami et al (7) in gastric cancer, the present results also indicated IL-32 staining to be associated with shorter overall survival time by multiple-factor analysis, and it could be considered as an independent prognostic factor for patients with colon cancer.

To conclude, the present data demonstrated that IL-32 expression participated in the progression of colon cancer. IL-32 staining is also associated with unfavorable prognosis in colon cancer; therefore, IL-32 could be considered as a novel biomarker for predicting the prognosis of colon cancer, but further confirmation is required in a larger cohort to provide novel cancer treatments.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JMZ and YHA designed the study. JMZ, WW and YGF analyzed the data. GLY collected and analyzed clinical samples, and also was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent from all patients and ethical approval was obtained from the Human Ethics Review Committees of Henan University of Science and Technology (approval no. 2013-PJ152).

Patient consent for publication

Patients have provided written informed consent for the publication of any associated data and accompanying images.

Competing interests

The authors declare that they have no competing interests.

References


