Expression of IL-32 modulates NF-κB and p38 MAP kinase pathways in human esophageal cancer

Key Words: esophageal cancer, IL-32, cytokines, NF-κB, p-p38 MAPK

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**Background**

Esophageal cancer is the seventh leading cause of cancer death in males in USA, and there is a strong link has been demonstrated between inflammation and esophageal cancer, interleukin (IL)-32 is a recently described proinflammatory cytokine characterized by the induction of nuclear factor NF-κB activation, the p38 MAPK also plays an important role in key cellular processes related to inflammation and cancer. we investigated whether the IL-32 expression may be involved in esophageal carcinogenesis through modulates the activity of NF-κB and p- p38 MAPK.

**Method**

Malignant esophageal and blood samples were obtained from 65 operable untreated patients, normal samples was obtained from 35 patients operated for other reasons as control. IL-32 expression visualized by immunohistochemistry, Real time RT-PCR for IL-32 mRNA expression, NF-κB phosphorylation and phosphorylated P38MAPK were analyzed by immunoblotting, ELISA for further detection IL-32 and cytokines (TNF-α, IL-1β, IL-6 and IL-8 ) concentration in the patients sera.

**Results**

IL-32 expression was increased in malignant esophageal samples compared with control (P = 0.01), IL-32 expression detected at the mRNA level (P = 0.007 vs. control), and its correlated with NF-κB activation (P = 0.003), and up-regulation of phospho p38MAPK (P= 0.004), and also increased in the cytokines concentration compared with the control samples p<0.05.

**Conclusions**

Understanding the pathway of IL-32 expression to stimulates the secretion cytokines via the activation of NF-κB and up-regulation of p-p38MAPK might be responsible in the esophageal carcinogenesis and used as tumor marker.
Introduction

Historically, esophageal carcinoma has been well described since the beginning of the 19th century, the first successful resection was performed in 1913 by Frank Torek (1), and according to the statistics adapted from the American Cancer Society's publication 2011, an estimated 16,980 adults (13,450 men and 3,530 women) in the United States will be diagnosed with esophageal cancer. It is estimated that 14,710 deaths (11,910 men and 2,800 women) from this disease will occur in 2011. Esophageal cancer is the seventh most common cause of cancer death among men, and there has been a marked increase in the incidence of esophageal adenocarcinoma over the last 2 decades, where adenocarcinoma has supplanted squamous cell cancer as the dominant pathologic phenotype.

There is a strong link has been demonstrated between inflammation and esophageal cancer, Interleukin-32 (IL-32), previously called natural killer cell transcript 4 (2), has been recognized as a pro-inflammatory cytokine recently, it is mainly expressed in natural killer cells, T cells, epithelial cells and blood monocytes. The gene encoding IL-32, which is organized into eight exons, is located on human chromosome 16p13.3; six splice variants have been described (IL-32a, IL-32b, IL-32g, IL-32d, e and z) of which IL-32g is the full-length isoform without any exonic deletions (3, 4). IL-32 is now recognized as pro-inflammatory mediator through its stimulation of TNF-α, IL-1β, IL-6, and IL-8 production, and its activation of the nuclear factor-κB (NF-κB), and p38 mitogen-activated protein (MAP) kinase pathways (5). IL-32 has been implicated in inflammatory bowel disease (6), also found increased levels of IL-32 are present in the lungs of patients with chronic obstructive pulmonary disease and correlate with airflow decreases that are associated with this condition (7) and expression by gastric, lung and pancreatic cancers has been reported (8,9).

Nuclear factor kappa B (NF-κB) is a ubiquitous transcription factor whose activity is controlled at a subcellular level, comprises a family of inducible transcription factors that serve as important regulators of the host immune and inflammatory response (10). Upon stimulation of NF-κB by a wide variety of stimuli, including cytotoxic agents and irradiation, NF-κB translocates to the nucleus and binds to its specific DNA site and subsequently up-regulates the transcription of a wide variety of genes involved in the inflammatory and immune response. Several reports have shown a role for NF-κB in regulating cell proliferation, tumor development, and cell transformation (11), there is very little clinical or in vivo experimental
data on the effects of NF-κB inhibition on tumor growth and metastasis. However, the role of the NF-κB signaling pathway in the tumorigenesis of human esophageal cancer is not fully understood, although some articles reported that inhibition of NF-κB can increase the drug sensitivity of esophageal cancer cells in vitro (12,13).

Mitogen-activated protein (MAP kinase phosphatase-1) is one of many phosphatases coded by the mammalian genome (14) and is a member of a subfamily of phosphatases known as the dual-specificity phosphatases, the function of these phosphatases is to dephosphorylate the two types of residues serine/threonine and therefore inactivate the MAP kinases, p38MAPKs, this dephosphorylation activity has been shown to be context-dependent; not all three types of MAP kinases are targeted for dephosphorylation in a given situation, at least in the case of MKP-1 (15), they mainly consist of ERK, JNK/SAPK, and P38. Despite structural similarities, MAPKs play diverse roles in regulating cell function, p38 MAPKs are activated by environmental and genotoxic stresses and have key roles in inflammation, as well as in tissue homeostasis, as they control cell proliferation, differentiation, survival and the migration of specific cell types (16). The functions of p38 MAPKs in cancer development are complex, which is consistent with the wide range of cellular responses that they modulate, however, p38MAPKs may also have oncogenic functions that are mediated by its involvement in key processes of cancer progression, such as invasion, inflammation and angiogenesis and there is much evidence to support a role for p38MAPKs as a tumour suppressor, and this function is mostly mediated by both negative regulation of cell cycle progression and the induction of apoptosis, although the induction of terminal differentiation also contributes to its tumoursuppressive function (17),(18).

Our hypothesis is that, the pathway of NF-κB and P38 MAP kinase modulated by expression of IL-32 protein in malignant esophageal tissue.

**Methods and materials**

**Human tissue and serum**

In this study, blood samples and tissues were obtained from 65 patients with operable untreated patients esophageal cancer and from 35 samples of endoscopically normal esophagus tissue was obtained from patients investigated for other reasons as control, the collected blood centrifuged (in 10000 RPM, for 10 minutes at 4 °C) and each tissue sample was divided to two part one for immunohistochemistry and other part for tissue homogenate for protein assay ethical approvals for this study were obtained by the Ethical review board at Colorado University.
**Reagents**

Recombinant human IL-1, TNF-α and anti-human IL-32 antibodies were purchased from R&D Systems (Minneapolis, MN), and all other reagents were purchased from Sigma.

**Immunohistochemistry (IHC) studies:**

For immunohistochemistry on the formalin-fixed, paraffin-embedded samples, sections were treated as previously reported (19), in briefly goat polyclonal anti-human IL-32 antibodies (R&D Systems) were used as the primary antibodies, after incubation with the primary antibodies (4 μm) were cut from each block sections treated with a biotin-conjugated goat anti-rabbit IgG (Vector, Burlingame, CA), and avidin-biotin-peroxidase complexes (ABC, Vector) were for visualization. for double immunostaining procedures using the anti-IL-32 antibodies plus the anti-cytokeratin antibodies (DAKO, Kyoto, Japan), the mixture of anti-IL-32 antibodies (diluted 1:100) and anti-cytokeratin antibody was applied first and incubated overnight at 4 °C in a humidified chamber. Cy2-labeled anti-goat IgG (diluted 1:100 in phosphate-buffered saline containing 0.1% Tween 20; chemicon, Temecula, CA) plus the Cy3-labeled anti-cytokeratin antibodies (diluted 1:100) were then applied for 60 min at room temperature. The images were obtained with the digital confocal laser-scanning system MRC-600 (Bio-Rad).

**Detection of IL-32 and cytokines in the sera of patients**

The level of IL-32 and cytokines (TNF-α IL-6 IL-1β) in the patient’s sera was detected by enzyme-linked immunosorbent assay methods as described elsewhere (20).

**Western Blot Analyses**

Esophageal tissue was homogenized and treated in PBS containing 0.5% Triton X-100 and a protease inhibitor cocktail, as described before (21), and briefly after protein quantification equal amounts of protein were incubated with the relevant antibodies and protein A-Sepharose beads at 4 °C overnight and resolved in 8%-12% SDS–PAGE depending on the molecular weight of the protein to be detected, after blocking overnight 48C in 5% milk in PBS-0.1% Tween 20, membranes were incubated overnight with antibodies of NF-kB (Santa Cruz Biotechnology, Santa Cruz, CA), following secondary antibody incubation, immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Buckinghamshire, England).
**Real time RT-PCR analyses**

The normal esophagus and malignant samples was assessed by Real time RT-PCR analyses for IL-32 mRNA expression according to the method as previously described (22), briefly Real time PCR was performed using a light cycler 2.0 system (Roche Applied Science), and the data were normalized versus β-actin for human IL-32.

**Statistical Analysis**

Differences between esophageal cancer patients and normal control group were tested using Student’s t-test. Differences were considered significant at p<0.05.

**RESULTS**

**IL-32 Expression strongly positive in esophageal cancer cells**

In control esophagus tissue, immunohistochemical staining showed weak IL-32 expression, while in tumor tissue its appeared strongly positive (Figure 1A), and to further characterize the IL-32 expressing cells in the esophagus we used real-time RT-PCR to determine the level of IL-32 mRNA expression, Real-time RT-PCR data showed a good correlation (p = 0.007) with the immunohistochemical findings (Figure 1B).

**IL32 increased NF-κB activity in esophageal tumor tissues**

The activation of NF-κB plays a critical role in cancer cell survival, and to determine whether IL-32 increased the activation of NF-κB in esophageal cancer cells we used Western Blot analyses for NF-κB binding activity, which showing a strong band of P-NF-κB activation in tumor tissues (P = 0.003) (Figure 2).

**IL-32 modulate P38MAPK activity in tumor tissues**

The levels of phosphorylated p38 MAPK increased in malignant esophageal tissue compared with control due to the rapid up-regulation of p-p38 activity by IL-32 (P= 0.004) (Figure 3).

**Increased of IL-32 protein levels up-regulated the cytokines in esophageal cancer patients**

To investigate the potential of IL-32 as a serological marker for esophageal cancers, we analyzed sera the average IL-32 level in the sera of the esophageal cancer patients was 207 pg/ml and this value was higher than that of the control (p<0.05), the average IL-32 level in the sera of control was 95 pg/ml. We analyzed cytokine levels in plasma by ELISA , the levels of TNF-α,
IL-6 and IL-1β, were significantly increased in plasma of esophageal cancer patients compare to the control, (Figure 4).

**DISCUSSION**

To our knowledge, this is the first study that demonstrates the link between expression of IL-32 protein levels in the malignant esophagus and the pathway of NF-κB and P38MAPK, in the recent studies have demonstrated that IL-32 is expressed by cells of epithelial origin, such as colon, gastric, and lung cancer cells (23), and the up-regulation of IL-32 is present in synovial tissue of patients with rheumatoid arthritis (24), where it is correlated with the expression of proinflammatory cytokines, such as IL-1b, IL-18, and TNF-α, with markers of clinical severity. Moreover, epithelial expression of IL-32 is enhanced in the inflamed mucosa of patients with Crohn’s disease (25), also IL-32 seems to be involved in myelodysplastic syndrome and chronic myelomonocytic leukaemia, HIV infection (26), also in a mouse model of sepsis, IL-32 was shown to potentiate peritonitis-induced elevations in serum levels of TNF-α and IL-1β, and over-expression of the IL-32β isoform led to reduced time to death (27).

In the present study, we demonstrates that NF-κB is activated in esophageal tumor by over expression of IL32, and down regulation of the P38MAPK, and here raises the possibility that P38 kinase is the common central component relaying multiple cell stress pathways to the cell death program. The Dejardin et al (28) have shown a high constitutive NF-κB activity in a number of adenocarcinoma cells and this activation of NF-κB in tumor tissues could be possibly due to a direct consequence of the degradation of IκB-α or the presence of a mutant form of IκB-α. Mohamed et al (29) have shown a thirty-one percent of the NF-κB negative esophageal adenocarcinoma were stage I or IIa compared with 12% of the NF-κB positive esophageal adenocarcinoma, whereas 87.5% NF-κB positive tumors were stage IIb or III compared with 69.5% of the NF-κB negative tumors.

Here, we showed that the expression levels of IL-6, IL-1B, and TNF-α are strongly positively in malignant esophageal tumor correlated with those of IL-32 highly expression, many studies report the 5-year survival analyses of patients with lung cancer expressed IL-32, frequently paralleled by those of IL-6, IL-1B, and VEGF, was related to a significantly shorter overall survival or with a trend toward a worse prognosis for the patients with lung cancer (30).
The study was done by Qian-Xian et al (31) show that cisplatin treatment strongly activates P38 kinase in esophageal cancer cells. Moreover, inhibition of P38 activity by the specific inhibitor SB203580 almost completely blocked cisplatin-induced apoptosis, also others data (32,33) explained that P38 activation is a critical step in cisplatin-induced apoptosis.

In conclusion, we demonstrated that IL-32 is expressed in the human esophageal cancer, and may play an important role in inflammatory responses and cancer growth. We still have limited data on IL-32 functions, but If this is proven to be true, then IL-32 could be considered not solely as a therapeutic target in esophageal cancer, but also as a valid biomarker for disease progression.

Reference

Fig. 1
A. Representative immunohistochemical staining of IL-32 in esophageal cancers, sections from the paraffin blocks were examined for IL-32 expression using mAb raised against IL-32 show its strongly positive in the nuclei and cytoplasms of esophageal cancer cells, while the control section show negative isotype with mouse IgG1 antibody shows negative staining. (Left side pictures H&E staining, right side pictures immunohistochemical staining).
B. The Real-time RT-PCR data showed a good correlation with the immunohistochemical findings, the relative expression level of IL-32 mRNA was low in control, and highly expression in tumor.
The Western Blot analyses showed that increased P-NF-κB in tumor compared with control and no difference in the total NF-κB (left side gel). The IL-32 up-regulated the P-pNF-κB in the esophageal tumor compared with control (right side gel).
To investigate regulatory mechanisms of P-p38MAPK underlying IL-32 expression in tumor using western blots showing an increase in P-p38MAPK activation, but no change in total p38 MAPK in malignant esophagus and control.
Fig. 4
A: IL-32 protein levels in the plasma of esophageal cancer patients and control. Plasma samples evaluated for reactivity with IL-32 antibody using an ELISA. The p value between patients and control patients <0.05 (p=0.01).

B: increased the cytokines levels in the plasma of esophageal cancer patients compared with control using an ELISA, p value <0.05. Normal value of cytokines in plasma: (TNF-α = 0, IL-6 = 0-149, IL-1β = 0).