Nuclear factor-κB1 expression levels in human gastric adenocarcinoma

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ABSTRACT

NF-κB pathway is a link between inflammation and cancer and is involved in cellular responses to different stimuli. Gastrointestinal lumen is exposed to many inflammatory agents such as foods, free radicals and bacterial or viral antigens. The aim of the present study was to evaluate the possible role of NF-κB1 in gastric adenocarcinoma. To detect the relative level of NF-κB1 transcript, total RNA was extracted from tissue specimens, a cDNA library was generated, and quantitative RT-PCR was performed for thirty human gastric adenocarcinoma tissue samples and thirty matched normal gastric tissue samples. NF-κB1 expression showed two-sidedness, which means that a group of 11 sample pairs showed up-regulation and a group of 16 sample pairs showed down-regulation. No histopathologic characteristics of samples could justify the observed two-sidedness. The NF-κB1 two-sidedness expression indicates the involvement of NF-κB1 and in a larger scale, NF-κB signaling pathway in gastric carcinogenesis. Our results show the complexity of regulatory mechanisms involved in developing and controlling the process of gastric cancer pathogenesis.

Key words: Gastric cancer; NF-κB1; qRT-PCR; Gene expression

INTRODUCTION

Gastric cancer is the fourth most common cancer and the second leading cause of cancer death in the world [1]. Most cases of gastric cancer are sporadic and more than 95% of gastric tumors are adenocarcinoma [2]. Based on the Lauren classification, adenocarcinoma of the stomach is classified as intestinal and diffuse types. The intestinal type seems to be nutrition- and H. pylori- infection dependent [3]. The transcription factor NF-κB, the nuclear factor kappa-light-chain-enhancer of activated B cells, is recognized as a crucial cellular primarily regulator factor...
of the genes involved in stress, immune and inflammatory responses such as cell death and cancer. NF-κB is detected in various types of cells involved in the expression of growth factors, cell adhesion molecules, cytokines and chemokines. Constitutive activation of NF-κB pathway and increased expression of its components are reported in some cancers and have been connected with multiple aspects of oncogenesis or chemotherapy resistance in carcinoma cells [4-8]. Gastrointestinal lumen is exposed to many environmental carcinogens in the diet and inflammatory agents such as H. pylori, which cause DNA damage and the activation of NF-κB pathway respectively. NF-κB activation leads to additional DNA damage through the production of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2, which enhance the production of reactive oxygen species (ROS). The transformed cells further proliferate through NF-κB activation by the inhibition of apoptosis and increased production of growth factors and cytokines [9-11]. Two distinct NF-κB activation pathways have been described. The most common, NF-κB heterodimers, is NF-κB1 (p50)-RELA, which regulates the expression of immunoregulatory and anti-apoptotic genes through the classical NF-κB pathway [9, 12]. Some NF-κB signaling components involving NF-κB1 are over-expressed in esophageal squamous cell carcinoma [7]. Increased expression of NF-κB1 in melanoma through the production of p50 homodimers, the poorly understood third NF-κB pathway, leads to angiogenesis induction by interleukin-6 (IL-6) transcription enhancement [8]. In the present study, in order to understand the possible molecular mechanism of NF-κB1 pathogenesis of cancer, we evaluated the expression level of this gene in gastric adenocarcinoma samples.

MATERIALS AND METHODS

Clinical samples collection: Thirty pairs of human gastric samples, including thirty human gastric adenocarcinoma tissues and thirty matched normal gastric tissue samples, were obtained from the Iran Tumor Bank, Imam Khomeini Hospital, with the patients’ informed consent. Removed tissues were stored immediately in liquid nitrogen. A record of clinopathological parameters for each patient was received along with each sample (Table 1). The Ethics Committee of Tarbiat Modares University approved the design of the experiment.

<table>
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<th>Characteristics</th>
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<td>Type of adenocarcinoma</td>
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<td>Diffuse type</td>
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<td>Distant metastasis</td>
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<td>10</td>
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<td>No</td>
<td>20</td>
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Table 1: Clinopathological characteristics of samples used for the study
RNA extraction and cDNA synthesis: Total RNA was isolated from the gastric tissue samples, using RNX™-plus solution (Cinnagen, Tehran, Iran) according to the manufacturer’s instructions. To measure the purity (260/280 ect) and integrity of extracted RNA, we used NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, USA) and agarose gel electrophoresis respectively. To obtain RNA free from DNA, treatment of total RNA was performed using RNase free DNase I (Fermentas, Canada) according to the manufacturer’s instructions. A cDNA library was generated through reverse transcription using MMLV Reverse Transcriptase (Fermentas, Canada) with 3 g of the total RNA, oligo dT18 (MWG, Germany) in a total volume of 20 µl, according to the manufacturer’s instructions.

Real-time polymerase chain reaction analysis: To detect the relative level of NF-κB1 transcript, real-time RT-PCR was performed. Real-time PCR of NF-κB1 and GAPDH (as internal control) was performed on an ABI 7500 real-time PCR system (Applied Biosystems, USA) using SYBR® Premix Ex Taq™ (TAKARA, Japan) according to the manufacturer’s instructions. All experiments were conducted at least in duplicate. PCR primers were: NF-κB1 sense: 5’-TACTCTGGCGCAGAATTAGGTC-3’; NF-κB1 antisense: 5’-CTGTCTCGGAGTCTGATTTG-3’; GAPDH sense: 5’-GTGAACCATTGAGAAGTATGACAA-3’; and GAPDH antisense: 5’-CAGCTGCTTTCCACGATAC-3’.

Statistical analysis: Data are expressed as means and standard deviations (SD), and P ≤ 0.05 is considered to be the statistical significance level of the t-tests. Statistical analysis of the data was performed using SPSS software version 18 (Chicago, IL, USA).

RESULTS

The expression of NF-κB1 was not different in tumor and non-tumor samples: To determine the expression level of NF-κB1 in human gastric adenocarcinoma and adjacent normal tissues, real-time PCR was performed for thirty pairs of gastric tissue samples. NF-κB1 was expressed in all normal and adenocarcinoma samples. However, its expression did not show any meaningfully differential expressions in all tumor samples compared to all non-tumor samples (P=0.14) (Fig. 1).

Figure 1: Histogram comparing the mean of NF-κB1 expression between all tumor and non-tumor samples using obtained data by real-time PCR (GAPDH as internal control). NF-κB1 expression does not show meaningfully differential expressions in all tumor samples compared to all non-tumor samples (P=0.14)
NF-κB1 expression shows two-sidedness: As clearly shown in Figure 2, NF-κB1 expression shows two-sidedness, which means that apparently, 14 sample pairs showed up-regulation while 16 sample pairs showed down-regulation. To find out whether the apparent up- and down-regulations were statistically significant the researchers analyzed these two groups, revealing that in a group of 16 samples, NF-κB1 was down-regulated with a fold change (FC) of 4.3 ($P=0.001$) (Fig. 3A) and in a group of 11 samples (three samples in this group were omitted to signify analysis), NF-κB1 was up-regulated with an FC of 3($P=0.001$) (Fig. 3B). Due to this two-sidedness, NF-κB1 expression did not show meaningfully differential expressions in all tumor samples compared to all non-tumor samples.

**Figure 2:** Fold change analysis of NF-κB1 expression in each sample pair. NF-κB1 is apparently up-regulated in 14 sample pairs with fold change $>1$ and is down-regulated in 16 sample pairs with fold change $<1$.

**Figure 3:** Histograms show observed two-sidedness of NF-κB1 expression. (A) In a group of 16 samples NF-κB1 is down-regulated with fold change (FC) of 4.3 ($P=0.001$). (B) In a group of 11 samples NF-κB1 is up-regulated with FC of 3($P=0.001$).
Histopathologic characteristics of the samples cannot justify observed two-sidedness:

We carried out subtle analysis using histopathologic characteristics of the samples. Samples were classified into two groups based on each histopathologic characteristic. No histopathologic characteristic of the samples could justify the observed two-sidedness. NF-κB1 expression did not show a meaningfully different expression in high grade tumors versus adjacent non-tumor tissues (P=0.66) (Fig. 4A1), low grade tumors versus adjacent non-tumor tissues (P=0.2) (Fig. 4A2), high stage tumors versus adjacent non-tumor tissues (P=0.056) (Fig. 4B1), low stage tumors versus adjacent non-tumor tissues (0.86) (Fig. 4B2), tumors with regional lymph node metastasis versus adjacent non-tumor tissues (0.16) (Fig. 4C1), tumors without regional lymph node metastasis versus adjacent non-tumor tissues (0.75) (Fig. 4C2), tumors with distant metastasis versus adjacent non-tumor tissues (0.29) (Fig. 4D1), and tumors without distant metastasis versus adjacent non-tumor tissues (0.46) (Fig. 4D2).

**Figure 4:** Association between NF-κB1 expression and clinopathological characteristics of samples. NF-κB1 expression is not meaningfully different in (A1) low grade tumor samples versus adjacent non-tumor samples, (A2) low grade tumor samples versus adjacent non-tumor samples, (B1) high stage tumor samples versus adjacent non-tumor samples, (B2) low stage tumor samples versus adjacent non-tumor samples, (C1) N+ (with regional lymph node metastasis) tumors versus adjacent non-tumor samples, (C2) N- (without regional lymph node metastasis) tumors versus adjacent non-tumor samples, (D1) DM+ (with distant metastasis) tumors versus adjacent non-tumor samples, and (D2) DM- (without distant metastasis) tumors versus adjacent non-tumor samples.
DISCUSSION

NF-κB signaling pathway serves as a link between cancer and inflammation [9]. The surface of the stomach is exposed to inflammatory agents. Induced inflammation by *H. pylori* infection has direct correlation with incidence of the intestinal type of gastric adenocarcinoma [3, 4]. NF-κB pathway components’ up-regulation and constitutive activation of the pathway have been connected to multiple aspects of carcinogenesis such as induction of cell proliferation, inhibition of apoptosis, angiogenesis, and tissue invasion [12].

NF-κB1 (p50)-RELA heterodimer is the major dimeric form of NF-κB components [9]. Recently, Arora et al. reported that miR-9 and let-7g enhance the sensitivity to ionizing radiation by NF-κB1 suppression in a lung cancer cell line [13]. To determine the expression level of NF-κB1, real-time PCR was performed on thirty pairs of gastric adenocarcinomas (28 of intestinal type and 2 of diffuse type) and adjacent normal gastric tissues from thirty patients. NF-κB1 expression showed two-sidedness. The obtained data revealed that NF-κB1 was down-regulated in a group of 16 samples but up-regulated in a group of 11 samples. This observed two-sidedness could not be justified by any histopathological characteristics of samples. Previously, reduced expression of NF-κB1 was reported in metastatic prostate cancer, which was parallel to the reduced expression of IκBα (Inhibitor of κB). The down-regulation of IκBα may result in the constitutive activation of NF-κB pathway despite the reduced level of NF-κB components. However, the mechanism underlying NF-κB1 down-regulation remains to be elucidated in prostate cancer [14]. Therefore, the expression level of the other components of NF-κB pathway intensely affects the activity of the pathway and can, therefore, be the cause of incapability to classify observed two-sidedness with histopathological characteristics. This means that probably, in some samples with reduced NF-κB1 expression, the additional activity of the pathway is due to the variable expression of other components such as the absence of IκBα component, resulting in an unexpected higher grade or stage. On the other hand, in our study, the majority of samples (28 of 30) were of an intestinal type and induced inflammation by *H. pylori* infection, which is the major activator of NF-κB pathway, had direct correlation with the incidence of the intestinal type of gastric adenocarcinoma. Consequently, the presence or absence of NF-κB pathway activator such as *H. pylori* infection can be the other cause of the mentioned incapability. Expression level of miRNAs, which may regulate NF-κB1 expression, can result in the reduced level of NF-κB1 transcript as Bazzoni et al.’s report also revealed that miR-9 controls NF-κB1 transcript level through a regulatory loop [15, 16]. In a group of 11 samples there was up-regulation, which was in accordance with the reported upregulation of 9 gastric adenocarcinoma samples by Ying et al. They revealed that NF-κB1 was down-regulated by miR-9 up-regulation in human gastric adenocarcinoma. Similarly, they did not report any histopathologic characteristics for their nine samples. Our study was focused on NF-κB1 expression, which is a key component of the NF-κB signaling pathway. Therefore, the necessity of simultaneous study of more components is felt to obtain more exact judgments about the activity level of the pathway. On the other hand, further investigation of two group samples, with and without *H. pylori* infection, could be helpful.

In accordance with one previous study, we showed that NF-κB1 was up-regulated in a subset of gastric adenocarcinoma samples relative to non tumor samples. In addition, our study revealed that NF-κB1 was down-regulated in the other subset. The observed two-sidedness indicate the involvement of NF-κB1 and in larger scale NF-κB signaling pathway in gastric carcinogenesis and show the complexity of regulatory mechanisms involved in controlling NF-κB1 expression in the process of gastric carcinogenesis.
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Conflict of Interest: There is no conflict of interest in this study.

REFERENCE
