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Site-specific Hypermethylation of *RUNX3* Predicts Poor Prognosis in Gastric Cancer

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Background and Aims. Methylation status of *RUNX3* remains largely unknown in gastric cancer (GC). The aim of this study was to prognostically evaluate the methylation level of CpG sites within *RUNX3* promoter region in GC.

Methods. Using pyrosequencing, we quantitatively explored the methylation status of 8 CpG sites within *RUNX3* promoter region for 76 gastric cancer and 24 normal gastric tissues. We then analyzed the association between methylation level of each CpG site and clinicopathological characteristics and outcomes in the cohort.

Results. Methylation of *RUNX3* promoter was significantly higher in GC than normal subjects. Overall methylation level was closely associated with tumor invasion and TNM stage. Positive associations were found between hypermethylation of the following concerned sites and variables: site −1392, −1397, −1403, −1415 and tumor invasion, as well as TNM stage; site −1392 and lymph node metastasis along with number of lymph node metastases; site −1415 and cancer recurrence; site −1403, −1415 and cancer-related deaths. In multivariate analysis, tumor invasion was correlated with sites −1392 and −1397. Lymph node metastasis was associated with site −1392. Most importantly, methylation of site −1415 was associated with poor survival by using Cox survival regression.

Conclusion. Analysis of *RUNX3* gene promoter by quantitative pyrosequencing suggested methylation status of *RUNX3* is different in normal and tumor tissues. *RUNX3* methylation level is associated with GC, especially the methylation at site −1415 contributes to the poor prognosis in GC. Thus, *RUNX3* methylation may serve as a valuable diagnostic and prognostic biomarker in GC.

Key Words: Runt-related transcription factor 3 (RUNX3), Gene methylation, Gastric cancer, Prognostic value.

Introduction

Gastric cancer (GC) is the fourth most common human malignant disease and the second major cause of cancer deaths worldwide (1). Although its incidence is declining in some parts of the world, GC is still highly prevalent in Asia, particularly China (2). Moreover, due to lack of available diagnostic and prognostic biomarkers, most GC patients are diagnosed at an advanced stage and often followed by a poor prognosis (3). Thus, establishment of useful biomarkers is critical and hopefully to improve early diagnosis and cure rate of GC. A growing amount of evidence points out that alterations in epigenetic machinery such as DNA methylation are critical causes of cancer initiation and progression (4). Hypermethylation in CpG island regions of tumor-suppressor gene promoters are commonly found early during cancer development (5). A series of methylated tumor suppressor genes such as *CDH1* (6), *DKK3* (7), and *MGMT* (8) have been shown to have predictive value for GC prognosis.

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Runt-related transcription factor 3 (*RUNX3*) is a classic gene in the Runx family. Through recruitment of Smad complexes, RUNX3 protein induces transcription and activates target genes such as *p53*, *p21*, *ATBF1*, *Notch 1*, *p27*, and *caspase3* (9–11) so as to effect cell differentiation, cell cycle regulation, apoptosis and malignant transformation. Previous studies have indicated that loss of RUNX3 expression is involved in numerous instances of tumorigenesis such as colon (12), breast (13), and ovary (14). In GC it has been reported that loss of RUNX3 contributes to hyperplasia and intestinal metaplasia of gastric mucosa epithelial cells in an animal model (15), whereas restoration of RUNX3 expression activates apoptotic pathway in GC (16). It has been classified that promoter methylation majorly accounts for reduced RUNX3 expression in GC (17). Quantification of serum *RUNX3* methylation has shown great potential value for detecting and diagnosing GC in another study (18). However, for postoperative evaluation of GC, the value of *RUNX3* hypermethylation has not yet been substantiated.

In this study we evaluate the methylation level of *RUNX3* in GC and estimate its prognostic significance. To complement the relevant clinical data, the study applied bisulfite pyrosequencing assays to check the methylation status of *RUNX3* gene promoter region in a GC cohort. This method facilitated us to quantitatively analyze the methylation level of *RUNX3* gene in both overall and site-specific terms. Thus, the prognostic value of *RUNX3* hypermethylation for GC should be understood more clearly.

Materials and Methods

Ethics Statement

This study was conducted in accordance with the Institutional Review Board and Human Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University School of Medicine, Shaanxi Province, China. Written informed consent was obtained from all related participants for sample collection and data analysis.

Patients and Clinicopathological Parameters

A total of 76 paraffin-embedded GC tissues were collected from the First Affiliated Hospital of Xi'an Jiaotong University. None of these patients underwent chemotherapy or radiotherapy prior to GC surgery. In addition, 24 samples from patients with chronic gastritis who underwent endoscopic biopsy were obtained for normal controls. All of the samples were histopathologically classified by at least two senior pathologists at the Department of Pathology of the Hospital based on the World Health Organization (WHO) criteria. Clinicopathological features were obtained from the patients' files. Follow-up included recurrence and overall survival and were acquired by telephone interview with the patients or their relatives. Time of overall survival was measured in months from the date of diagnosis to the time of cancer-related death or the end time of follow-up. Detailed clinicopathological characteristics are summarized in Table S1 (Supplementary material).

DNA Isolation and Bisulfite Treatment

DNA was extracted from paraffin-embedded GC tissues using standard phenol-chloroform extraction and ethanol precipitation protocol. Detailed information refers to previous description (19). Briefly, after 12 h treatment at room temperature with xylene to remove paraffin, tissues were digested with 1% sodium dodecyl sulfate (SDS) and 0.5 mg/mL proteinase K at 48°C for 48 h, with addition of several spiking aliquots of concentrated proteinase K to facilitate digestion. Genomic DNA was then isolated from the digested tissues using standard phenol/chloroform protocol, dissolved in distilled water, and stored at −80°C until use. For bisulfite treatment, genomic DNA was denatured in a total volume of 20 μL containing 4 μg genomic DNA, 10 μg salmon sperm DNA, and 0.3 mol NaOH for 20 min at 50°C. Denatured DNA was then diluted in 500 μL of a freshly prepared solution containing 3 mol sodium bisulfite (Sigma, St. Louis, MO) and 10 mmol hydroquinone (Sigma) and incubated for 2–3 h at 70°C. After incubation, DNA sample was subsequently desalted with a Wizard DNA Clean-Up System (Promega Corp., Madison, WI) according to the manufacturer's recommendations followed by ethanol precipitation and dried. DNA was then resuspended in 100 μL of deionized water. After bisulfite treatment, all unmethylated cytosine was converted to uracil while leaving methylated cytosine unchanged. Bisulfite-treated genomic DNA samples were stored at −80°C until use.

Primer Design and DNA Methylation Analysis Using Pyrosequencing

Polymerase chain reaction (PCR) assays were designed to amplify a part of the CpG islands in *RUNX3*. The pyrosequencing primers were designed using PyroMark Assay Design software v2.0 (Qiagen, Valencia, CA). The forward primer was biotin labeled. The primer sequences are as follows: forward: 5′-biotin-AAGGGGTGATTTGTAGTG AAGTTTA-3′; reverse: 5′-CTCTACCAATCCAACCCC ACTTCTTCT-3′; sequencing primer: CCCACTTCTTCT TAAACC. PCR reaction mixture was prepared in a total volumn of 30 μL containing ~120 ng of bisulfite-treated DNA, 1× PCR buffer, 3 mmol $MgCl_2$, 300 μmol each of deoxynucleotide triphosphate mixture (dATP, dCTP, dGTP, and dTTP), 300 nmol forward and reverse primers, and 0.75 U of platinum Taq DNA polymerase (Invitrogen Technologies, Carlsbad, CA). The amplification was run in a Thermocycler (Bio-Rad Laboratories, Hercules, CA) as follows: denaturation at 95°C for 5 min; 45 cycles of