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Aberrant CpG Island Shore Region Methylation of *CAV1* Is Associated with Tumor Progression and Poor Prognosis in Gastric Cardia Adenocarcinoma

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Background and Aims. Caveolin-1 (CAV1) is a multifunctional scaffolding protein and plays an important role in tumorigenesis. However, the epigenetic changes of *CAV1* in gastric cardia adenocarcinoma (GCA) have not been investigated so far. The purpose of this study was to clarify the contribution of critical CpG sites in *CAV1* to progression/prognosis of GCA and to further elucidate the effect of critical CpG sites on the ectopic expression of β-catenin in GCA.

Methods. Methylation-specific polymerase chain reaction (MSP) and bisulfite genomic sequencing (BGS) methods were, respectively, applied to examine the methylation status of *CAV1*. RT-PCR and immunohistochemistry methods were used to determine the mRNA and protein expression of *CAV1* and β-catenin.

Results. Decreased mRNA and protein expression of *CAV1* were observed in GCA tumor tissues and were associated with hypermethylation of CpG island shore and transcription start site (TSS) regions in *CAV1*. Hypermethylation of the other two regions within CpG islands in *CAV1* was observed both in tumor and corresponding adjacent tissues but was not related to the transcriptional inhibition of *CAV1*. The methylation status of CpG island shore region in *CAV1* was associated with the ectopic expression of β-catenin and was independently associated with survival in GCA patients.

Conclusions. Hypermethylation of CpG island shore and TSS regions is cancer specific and is closely associated with reduced expression of *CAV1*. The CpG island shore methylation of *CAV1* may play an important role in progression of GCA and may serve as a prognostic methylation biomarker for GCA patients. © 2016 IMSS. Published by Elsevier Inc.

Key Words: Caveolin-1 gene, Methylation, Gastric cardia adenocarcinoma, β-catenin.

Introduction

Gastric cardia adenocarcinoma (GCA), which was formerly registered as esophageal cancer or gastric cancer, is diagnosed independently in very recent years due to the improvement in early endoscopic screening and pathologic diagnosis. GCA was defined by the World Health Organization (WHO) as esophagogastric junction (EGJ) adenocarcinoma with its epicenter located between 1 cm proximal and 2 cm distal of the EGJ (1). GCA is a common upper gastrointestinal cancer (UGIC) in China and has shown increased incidence rate in recent years (2). Based on two national mortality surveys conducted in the 1970s and 1990s, there is an obvious clustering of geographical distribution of GCA in China, with the high mortality being mostly located in rural areas, especially in Taihang mountain range areas

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of North China (3–5). The incidence of UGIC in this area was >100/100,000. Epidemiological studies indicated that there was a strong tendency toward familial aggregation of GCA in high-risk areas of UGIC, suggesting that multiple genetic and epigenetic events may contribute to the occurrence and progression of GCA. However, the precise molecular mechanisms of the development and progression of GCA remain unknown. Therefore, additional elucidation of the molecular mechanisms involved in GCA and the discovery of early detection biomarkers are urgently needed for more effective chemoprevention or treatment.

Caveolin-1 (*CAV1*), an essential component of caveolae (6), maps to human chromosome 7q31.2 and encodes a cytoplasmic 22-kDa scaffold protein. *CAV1* has been implicated as a regulator of a large number of intracellular signalling pathways; however, the effect of *CAV1* in tumorigenesis was controversial with either tumor-promoting or inhibiting activity based on different tumor types and/or tumor stages. In recent years, several studies have reported upregulation of *CAV1* in several malignancies including renal cell carcinoma, glioblastoma, and meningioma (7,8). Conversely, *CAV1* was downregulated in a variety of human cancers such as ovarian cancer (9), breast cancer (10–13), prostate cancer (14), oral squamous cell cancer (15), soft tissue sarcomas (16,17) and lung cancer (18). In addition, locus D7S522 of human chromosome 7q31.2, which is the location of the *CAV1* gene, is frequently deleted in human cancers, further implicating the tumor suppressor role of *CAV1* (19). However, the expression and the inactivation mechanism of *CAV1* in GCA have not been reported so far.

Epigenetic alterations, in particular DNA methylation of promoter CpG islands, play an important role in regulating gene transcription (20,21). Loss of *CAV1* expression with aberrant promoter methylation was observed in several human cancers (14,16,22–25). However, it was reported that the methylation frequency of *CAV1* and the effect of aberrant methylation on gene transcription were different in various tumor types. In recent years, some studies have shown that methylation frequency of each CpG site may be different and some CpG sites, which were closely related to the inhibition of gene transcription, called the critical CpG sites. The critical CpG sites may reside within CpG islands or non-CpG islands. In the present study, we attempted to clarify critical CpG sites of *CAV1* gene in GCA samples and elucidate its effect of their methylation on progression/prognosis of GCA patients, in order to gain more information on the role of hypermethylation of *CAV1* critical CpG sites with regard to the pathogenesis and prognosis of GCA.

Materials and Methods

Patients and Specimens

One hundred and seventy-two paired tumor tissue samples of primary gastric cardia carcinomas and corresponding adjacent non-cancerous tissues were collected from the Fourth Affiliated Hospital, Hebei Medical University between the years 2006 and 2009. All study subjects were ethnically homogeneous Han nationality and were from the same areas, which were the high-risk area of UGIC in Hebei province. The incidence of UGIC in this area was >100/100,000 (26,27). The study was approved by the local ethics committee and informed consent was obtained from all patients. Complete clinicopathologic characteristics and UGIC family history of these cases were available before operation and during follow-up. Individuals with at least one first-degree relative or at least two second-degree relatives having esophageal/cardia/gastric cancer were defined as having a family history of UGIC. Distribution of clinicopathological data in the study cohort is shown in Table 1. Tumor stages were assigned according to a modified American Joint Committee on Cancer (AJCC) and Union for International Cancer Control (UICC) standard (v.7) and tumor grade was classified according to WHO criteria. For this study, all subjects were re-examined and confirmed by professional pathologists for their histopathological diagnosis. All gastric cardia carcinomas were adenocarcinomas and the tumor epicenters located between 1 cm proximal and 2 cm distal of the EGJ (1). The adjacent non-cancerous tissues (normal tissues or hyperplasia tissues) were ~5 cm from tumor tissues and confirmed by microscope examination. Tumor and corresponding adjacent tissues were divided into two parallel parts, one part was frozen at −80°C and extracted DNA and RNA, and the other part was formalin-fixed and paraffin-embedded. Survival data were ascertained through the Tumor Registry and Hospital chart review. Sixteen patients were lost to follow-up.

CAV1 *mRNA Level Quantified by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)*

Messenger RNA (mRNA) expression level of the *CAV1* was determined by RT-PCR. Total RNA was isolated by Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and quantified by UV absorbance at 260–280 nm. Two μg RNA was used to synthesize first-strand cDNA using the advantage RT-for-PCR kit (Clontech, Palo Alto, CA) with random priming as recommended in the protocol provided. Following first-strand synthesis, the reaction mixture was diluted to 20 times and 2.5 μl of diluted cDNA mixture was used for PCR amplification in a final 25 μl reaction volume. All primers and reaction conditions are listed in Supplementary Table S1. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control. PCR products were separated in 2% agarose gel in electrophoresis and visualized with ethidium bromide staining, and quantified using an image analysis system (Gel work-2ID). Optical density (OD) value of the mRNA expression was calculated. The reaction