

Analysis of SMAD4 gene promoter methylation in pancreatic and endometrial cancers

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ABSTRACT

Background. Promoter hypermethylation of the SMAD4 gene has been registered in some cancer types, but in general doesn't appear to be a frequent event in carcinogenesis. However, only a few published studies deal with this topic and not many cancer types have been analyzed. The aim of this study was to establish SMAD4 gene promoter methylation status in pancreatic and endometrial cancers. **Methods.** Patients included in the study (62 subjects) were diagnosed and surgically treated at the University of Belgrade, Clinical Center of Serbia. Patients with pancreatic carcinoma (17 subjects) underwent surgical removal of the pancreatic adenocarcinoma at the First Surgical Clinic, while the patients with endometrial carcinoma (45 subjects) underwent hysterectomy with adnexectomy at the Institute for Gynecology and Obstetrics. Extraction of DNA from fresh tissue samples was performed and the methylation status of the SMAD4 gene promoter was studied by a previously designed PCR-based HpaII and MspI restriction enzyme assay. The resulting PCR products were analyzed by electrophoresis in 2% agarose gels. **Results.** Neither of the analyzed samples was found to be hypermethylated. **Conclusion.** This is the first report on SMAD4 methylation status in pancreatic and endometrial tumor specimens, and supports the viewpoint that SMAD4 hypermethylation is not a common event in malignant tumors. Nevertheless, promoter hypermethylation remains a candidate mechanism for SMAD4 inactivation in malignant tissue as a potential cause of decreased or lost SMAD4 expression in certain tumor types, and should be further investigated in different tumor types and larger cohorts of patients.

Key words: endometrial cancer; pancreatic cancer; Methylation

INTRODUCTION

As a multistep process, carcinogenesis is promoted by a series of genetic changes that involve activation of oncogenes and inactivation of tumor suppressor genes. Apart from genetic changes affecting the coding regions, genetic and epigenetic alterations in regulatory regions represent a common mechanism of inactivating tumor suppressor genes (1). Aberrant methylation of cytosine in the gene promoters is common event in cancers and promoter hypermethylation leading to decreased gene transcription frequently affects tumor suppressor genes in malignant tissue.

SMAD family member 4 (SMAD4) is a pivotal intracellular mediator of the transforming growth factor beta (TGFβ) signaling pathway. This protein is composed of 552 amino acids and is encoded by the gene located in the region 21.1 of the chromosome 18. It is essential for maintenance of tissue homeostasis and cell cycle regulation both during the development and in adult tissues. Although SMAD4 is known to be frequently inactivated in cancers, the mechanisms of its inactivation other than coding region mutations remain underinvestigated (2, 3, 4).

Only a few studies have dealt with the investigation of the 5' regulatory region of the SMAD4 gene and little is known about its transcriptional regulation (5, 6, 7). Existing data are limited to the fact that SMAD4 transcription is regulated by a complex region spanning over 80kb upstream from the transcription start site. This region contains alternative promoters that regulate SMAD4 transcription in a time-specific manner, but their exact roles and pattern of activation remain unknown (8). Various alterations in the SMAD4 gene coding region were found in different human malignancies, but altered transcriptional regulation due to mutations or aberrant

methylation in the SMAD4 gene regulatory regions as potential molecular mechanisms of carcinogenesis remain severely understudied (4, 10).

Although the role of specific segments within SMAD4 5' regulatory region and their contribution to the SMAD4 gene regulation in health and disease remain to be investigated, some mutations within these regions have already been found in tumors and confirmed to be functionally relevant, which may indicate their significance for malignant process in the cell. Two somatic mutations that disturb transcription were found in endometrial carcinoma (11). Another somatic alteration in this region was found in tumor tissues of patients with pancreatic, colorectal and endometrial cancer and it was shown to significantly reduce promoter activity of this segment (6, 7, 12). Relatively small number of studies analyzed methylation status of the SMAD4 5' regulatory region, mostly in gastrointestinal cancers, and it was not found to be a major mechanism of SMAD4 inactivation (9, 13, 14, 15).

Promoter hypermethylation of the SMAD4 gene has been registered in some cancer types, but in general doesn't appear to be a frequent event in carcinogenesis. However, only a few published studies deal with this topic and not many cancer types have been analyzed. The aim of this study was to establish SMAD4 gene promoter methylation status in pancreatic and endometrial cancers.

MATERIAL AND METHODS

Subjects

Patients included in the study (62 subjects) were diagnosed and surgically treated at the University of Belgrade, Clinical Center of Serbia. Patients

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with pancreatic carcinoma (17 subjects) underwent surgical removal of the pancreatic adenocarcinoma at the First Surgical Clinic, while the patients with endometrial carcinoma (45 subjects) underwent hysterectomy with adnexectomy at the Institute for Gynecology and Obstetrics. The study was approved by the Ethics Committee of the Clinical Center of Serbia and all patients gave a written informed consent. The procedures applied were in accordance with the international ethical standards. In all patients, standard histopathological analysis was performed and the stage of the cancer was determined using TNM Classification of Malignant Tumours based on criteria proposed by the American Joint Committee on Cancer (AJCC) (16).

Methylation analysis

The fragment selected for this analysis was a CG-rich region that encompasses CpG islands upstream from the non-coding exon 1 of the SMAD4 gene. Extraction of DNA from fresh tissue samples was performed by QIAamp DNA Blood Mini Kit (Qiagen). The methylation status of the SMAD4 gene promoter was studied by a previously designed PCR-based *HpaII* and *MspI* restriction enzyme assay (5). The method was tested on a set of ten DNA samples extracted from blood samples of patients with pancreatic cancer, and then applied for the analysis of 62 samples extracted from tumor tissue. The assay is based on the ability of the *HpaII* restriction enzyme to distinguish CpG sites that are methylated versus those that are nonmethylated. Approximately 300 ng of tumor DNA was digested in a total volume of 15 μ L for 16 h at 37 °C. The reactions contained either no enzyme (control), 5 units of *HpaII* or 5 units of *MspI*. All 15 μ L of each digest were analyzed by PCR in 25 μ L reactions containing: 1x Reaction buffer B (Solis BioDyne), 1.5 mM MgCl₂, 0.2 mM of each dNTP, 10% of DMSO, 5 pmol of each primer and 1U of FIREPol DNA Polymerase (Solis BioDyne). Primers used to amplify 408 bp long fragment of the SMAD4 gene promoter containing six *HpaII*/*MspI* restriction sites were: 5'-CAAGTTGGCAGCAACAACAC-3' and 5'-ACATGGCGCGTTACCT-3'. The amplification was performed for one cycle of 95 °C for 10 min followed by 40 cycles of 95 °C for 30 sec, 60 °C for 45 sec, and 72 °C for 45 sec, followed by one cycle of 72 °C for 10 min. The product of PCR amplification should be detected only when the original target DNA contains methylated *HpaII* restriction sites. The resulting PCR products were analyzed by electrophoresis in 2% agarose gels.

RESULTS AND DISCUSSION

This is the first report on SMAD4 methylation status in pancreatic and endometrial tumor specimens. As expression of SMAD4 appears to be decreased or lost in most pancreatic and colorectal cancers, as well as in some endometrial carcinoma tissues, promoter hypermethylation remains a candidate mechanism for SMAD4 inactivation in malignant tissue and is worth investigating in carcinomas. The CG-rich segment of the SMAD4 5' regulatory region is located 18 kb upstream from the transcription start site (5, 9). Its methylation status was investigated in different malignant tumors.

The first report of SMAD4 promoter methylation in any malignancy was the study in which 70% of esophageal carcinoma cases were found to be hypermethylated at SMAD4 promoter (13). Hypermethylation of the

SMAD4 promoter was detected in 45% of advanced prostate cancer cases, while lower frequency was observed in cases of gastric carcinoma (5%) (9, 14). Hypermethylation of the SMAD4 promoter was also observed in 25% of tumors from patients with lung cancer and idiopathic pulmonary fibrosis (15). Hypermethylation was not found in colorectal malignancies and small intestinal neuroendocrine tumors (5, 17, 18). Such high variability in SMAD4 methylation status in different tumor types could be due to the size of study groups, as well as applied methodology. In our study, neither of the analyzed samples was found to be hypermethylated. These results are consistent with previous studies, which indicate that promoter hypermethylation is not common mechanism of SMAD4 inactivation in malignant tissue. Considering that other applied methods are more sensitive than restriction enzyme assay, a possibility remains that SMAD4 is methylated to a minor extent, which remained undetected in our study due to insensitivity of the restriction enzyme assay. In two studies that analyzed colorectal cancer no SMAD4 methylation was detected (5, 17). These studies also employed restriction enzyme assay for this purpose, hence possibility remains that the methylation was present to a certain extent not detectable by this technique. Most of the other studies relied on methylation-specific PCR combined with direct DNA sequencing, which is more sensitive approach than use of methylation-specific enzymes (9). Pyrosequencing, as even more sensitive technique than methylation-specific PCR, was previously employed to analyze SMAD4 methylation in small intestinal neuroendocrine tumors, but only low levels of SMAD4 methylation were registered in the tumor tissue (18). The studies which detected SMAD4 hypermethylation with a high frequency have included relatively small number of patients (less than 30), so the relevance of these findings is questionable (9, 13, 15). In studies conducted in larger cohorts of patients, SMAD4 hypermethylation was either detected with a low frequency or not detected at all, indicating that it is not common in cancer cells (5, 14, 17). Our study supports the viewpoint that SMAD4 hypermethylation is not a common event in malignant tumors. Promoter hypermethylation remains a candidate mechanism for SMAD4 inactivation in malignant tissue as a potential cause of decreased or lost SMAD4 expression in certain tumor types, and should be further investigated in different tumor types and larger cohorts of patients.

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Conflict of Interest

Authors declare no conflicts of interest

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