INTERNAL MEDICINE

□ CASE REPORT □

Epigenetic Inactivation of Tumor Suppressor SFRP2 and Point Mutation in KRAS Proto-Oncogene in Fistula - Associated Mucinous Type Anal Adenocarcinoma: Report of Two Cases

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Abstract

The secreted frizzled-related proteins (SFRPs) genes are unmethylated in normal colorectal mucosa tissue but abberant methylation profiles can be detected in colorectal cancer (CRC), adenomas, and in aberrant crypt foci. The aim of the current study was to clarify whether SFRP2 methylation and K-ras structural mutation in fecal DNA can be found in stool and tumoral tissues of individuals with fistula-associated mucinous type anal adenocarcinomas (MTAA).Two man patients (68 and 56 years old) were treated for anorectal fistula in the surgical department. Patients were evaluated for clinical findings, tumoural tissue samples were examined histopathologically and DNA from fecal and tumoral tissue samples were isolated. K-ras mutation and promoter hypermethylation of SFRP2 gene in tumoral tissues were assessed by methylation-specific PCR based stripAssay hybridisation technique (Me-PCR) and compared to the healthy controls. Fecal and tumoural tissue samples from both patients were found to be fully hypermethylated profiles for SFRP2 gene and combined point mutations were detected in codon 12 and 13 of K-ras proto-oncogene. The current results showed that the combined effects of somatic mutations in K-ras and epigenetic alterations in SFRP2 genes may play an active role in the development of mucinous type anal adenocarcinoma.

Key words: mucinous anal adenocarcinoma, K-ras mutation, SFRP2, epigenetic alterations

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Introduction

Anal carcinoma represents approximately 1 percent of all tumors of the gastrointestinal tract. Epidermoid (squamous cell) carcinoma is the most common histological variant and represents about 80% of patients with anal carcinomas (1). Perianal mucinous adenocarcinoma is a rare cancer constituting 3 to 11 per cent of all anal carcinomas (2). Silencing by promoter hypermethylation in tumour supressor (TS) genes and activating by hypomethylation of CpG islands in oncogenes are frequently reported in human cancer. The different methylation status in the secreted frizzled-related proteins (SFRPs) genes plays a crucial role in the colorectal carcinomas (CRC). Recently, some study groups demonstrated that SFRP2 methylation is the most sensitive single DNA-based marker in fecal samples for identification of CRC (3, 4). The promoter hypermethylated SFRP2 gene profile rate in fecal and tumoural tissue samples for the same case were reported to be nearly equal (87.0%, 91.3% respectively) in CRC (3, 5). Some recent reports showed that SFRP2 gene is a further Wnt inhibitor and its expression is downregulated in various malignancies such as cervix (6-8), CRC (3-5), breast (9) and lung (10) tissues. Single base pair-substitutions in codons 12, 13 or 61 are common mutations that occur in K-ras gene. Cigarette smoking

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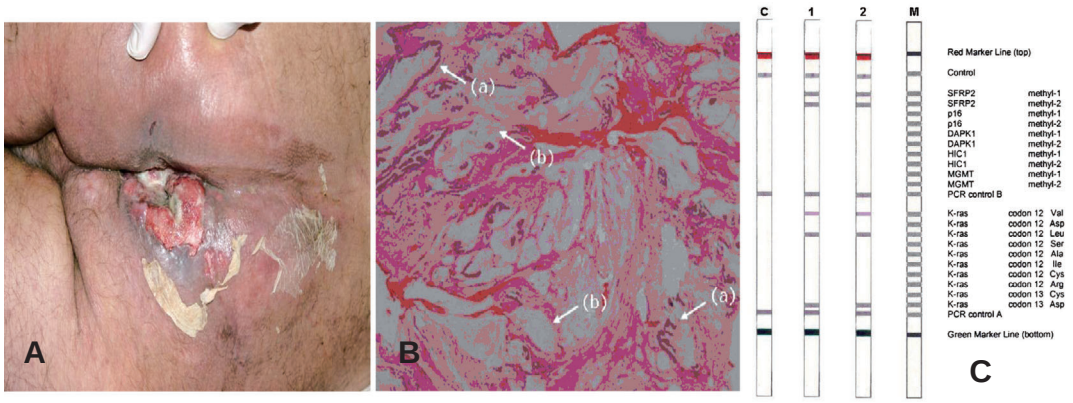

Figure 1. **Phenotype, histopathologic and molecular findings of current case 1 with mucinous type anal adenocarcinoma. A. Fistula-associated anal adenocarcinoma. B. Histopathological overview of adenocarcinoma in tumoural tissue (arrows), (Hematoxylin and Eosin staining, ×100). C. Structural mutation and epigenetic profiles of target genes. The molecular findings of a healthy control and two different biological samples (fecal and solid tumour) from case 1 are shown. Three different point mutations in codon 12 and 13 of K-ras proto-oncogene and fully promoter hypermethylation (both alleles were inactive) of the tumor supressor SFRP2 gene were detected in both fecal and tumoural tissue samples.**

Lanes

Lane C - Healthy control case. The gene profiles of the blood tissue from a healthy control show fully hypomethylated-active gene profiles of the SFRP2 and all other tumor suppressor genes that studied. The structural K-ras proto-oncogene also showed a normal appearance and no point mutation was detected. Lanes 1 and 2 − The promoter methylation status of target tumour supressor genes such as; SFRP2, p16, DAPK1, HIC1 and MGMT in fecal (lane 1) and tumoural samples (lane 2) from case 1. Show promoter hypermethylation in SFRP2 gene in both samples. No signals were detected in p16, DAPK1, HIC1 and MGMT genes. Two point mutations in codon 12 (Val and Leu) and one in codon 13 (Asp) of K-ras proto-oncogene were detected in fecal, tumoural samples. M − Standart size marker (Vienna Lab, StripAssay).

is a frequent cause of K-ras mutations which have been widely hypothesized to be related to direct tobacco exposure (11).

Therapies that are based on epidermal growth factor receptor (EGFR) inhibitor have emerged as effective treatments in a subset of patients with metastatic CRC. On the other hand such therapies are ineffective in tumors with mutations of codons 12 and 13 of the K-ras proto-oncogene. The epigenetic inactivation of tumour supressor DAPK1, SFRP2, p16, MGMT, p53 is widely reported in CRC in humans. The purpose of this study was to clarify the possible role of mutated K-ras proto-oncogene and epigenetically inactivated TS genes in fistula - associated mucinous type anal adenocarcinoma.

Case Report

Case 1

A 68-year-old man with a chronic history of complex fistulas (for 6 years) and abscesses presented to our hospital with a horseshoe fistula and anal abscess (Fig. 1, A). Multiple biopsies from the abscess crater, fistulous tract and the perianal skin opening were identified as mucinous adenocarcinoma of the anal canal in the histological examination (Fig. 1, B). Genomic DNA from fecal and tumoral samples showed three point mutations (Val and Leu in codon 12 and Asp in codon 13) in K-ras and full promoter hypermethylation of SFRP2 gene (Fig. 1, C-Lanes 1,2). There was no evidence of inguinal adenopathy. No intestinal lesion was seen at colonoscopic examination. He was advised to have an abdominal perineal resection (APR), but he refused this procedure. The patient was re-admitted with complaints of bleeding, discharge, and anal discomfort after 2 years. He was advised to have a chemoradiation therapy. FOLFOX-4 regimen and pelvic radiotherapy (30 Gy)-capesitabine therapy were performed. The patient died 14 months later.

Case 2

A 56-year-old man had a symptomatic history of fistula prior to mucinous adenocarcinoma diagnosis, for 5 years. He had multiple minor surgical interventions, under different specialists, over months or years prior to a diagnosis of mucinous adenocarcinoma. Colonoscopic examination of the patient was normal. Magnetic resonance imaging (MRI) had shown changes that were suspicious, but not diagnostic. No inguinal lymph node was detected. Pathologic examination of biopsies from the abscess crater, fistulous tract and the

perianal skin openings were revealed as mucinous adenocarcinoma of the anal canal. He was advised to have a neoadjuvant chemoradiation therapy. FOLFOX-4 regimen and pelvic radiotherapy (30 Gy) -capesitabine therapy were performed. After neoadjuvant chemoradiation therapy, this patient was also advised to have an abdominal perineal resection (APR). The patient died 28 months later.

Genotyping

Both patients were clinically examined and multiple tissue biopsies were taken for molecular and histopathological examinations. For the tissue specific correlation of promoter hypermethylation profiles in target TS genes, three different tissues were analyzed histopathologically, and epigenetically. Fecal and tumoral tissue specimens from both current MTAA patients were used for total genomic DNA isolation and epigenetic analysis. Peripheral blood sample from a healthy person who has no familial history of CRC and/or such a gastrointestinal problem was used as a negative control. Direct in vitro amplification of the proto-oncogene K-ras and tumor supressor genes of SFRP2, P16, DAPK1, P53 and MGMT were performed by modified and amplified DNA fragments detected by methylation-specific PCR based stripAssay hybridisation technique (Me-PCR) (10). Me-PCR has a high-sensitive performance to discriminately amplify and detect methylated CpG dinucleotides by using methylation site-specific primers on bisulfite-converted target genes. Primers only anneal to amplify target genes containing methylcytosines that are unmethylated while the 5-methylcytosine (5-mC, epicytosine) resists this bisulfite action.

Fecal and tumoural samples from case 2 also showed fully hypermethylated profiles for SFRP2 gene and combined point mutations (Val in codon 12 and Asp in codon 13) for K-ras proto-oncogene. Other target tumour supressor genes had hypomethylated active profile in all samples that were studied in both cases.

Discussion

Although the pathogenesis of anal canal adenocarcinoma remains unclear, many factors have been reported. The aim of this article was to report two rare cases of mucinous type anal adenocarcinoma who were treated in the Hospital of Cumhuriyet University Faculty of Medicine. The histopathological examination of tumoural tissues showed poorly differentiated, production of an abundant amount of intra and/or extracellular mucinous fluids with large cavities of adenocarcinoma in both cases. Mucinous type anal adenocarcinomas have a higher pathologic stage at the time of diagnosis (12), and a greater tendency for metastasis and poor prognosis (13). Local inflammation, chronic anorectal fistulas, Crohn's disease, anal intercourse and human papillomavirus have been implicated as etiological factors (1, 2). A chronic, destructive, and inflammatory pathology often predisposes to malignant transformation and progression. Long-standing fistulas of the gastrointestinal tract have been the most widely affected by malignant transformation (14). Gaertner et al reported 14 patients with histologically proven fistula-associated anal adenocarcinoma (11 patients had preexisting chronic anal fistulas, 10 had Crohn's disease, and 1 had previously received pelvic radiation therapy) (15). Most anal adenocarcinomas are currently believed to originate from the epithelial elements of the anal glands (16). But, there has been some debate as to whether the fistula is the source of the tumor, or whether the fistula is the presenting feature of a slow-growing, indolent carcinoma. Chronic perianal fistula evolution into adenocarcinoma is rare (17). Pollastri et al concluded that any chronic inflammatory disease must be treated early and adequately to avoid neoplastic changes (18). A high index of suspicion and biopsy of fistulous tracts and abscesses are the keys to early diagnosis and treatment. Rectal ultrasound and dynamic contrast-enhanced MRI can facilitate the diagnosis.

The current management of anal mucinous adenocarcinoma remains controversial. Some authors believe Abdomino-Perineal Resection (APR) with permanent colostomy, should be considered as the standard treatment. Others propose that combined chemoradiation should be adopted as a possible treatment in certain patients. Belkacemi et al concluded that chemoradiation appeared to be the preferred primary modality of treatment for early-stage anal adenocarcinoma, because it controls the tumor while maintaining anorectal function (19). APR was recommended to be reserved for persistent or recurrent disease. Li and co-workers reported that APR with adjuvant chemoradiation was the preferred principal treatment at adenocarcinoma of the anal canal (20). Beal et al reported that the combination of APR and combined modality therapy (whether it is neoadjuvant or adjuvant) is a reasonable approach for the treatment of this rare tumor (21). Though there is currently no standard protocol for the treatment of primary anal adenocarcinoma, recent studies have shown that locally advanced anal adenocarcinomas could benefit from pre or postoperative chemoradiation therapy. However, accurate and complete removal of the tumor, which usually entails abdominoperineal resection, is often necessary.

Aberrant methylation of the CpG islands, which are concentrated within the gene promoter regions, prevents gene transcription and causes inactivation of the tumor suppressor gene which is currently believed to play a major role in human carcinogenesis. These aberrations include hypomethylation leading to oncogene activation and chromosomal instability, hypermethylation and tumor suppressor gene silencing and chromatin modification acting directly and cooperatively with methylation changes to modify gene expression. Adenocarcinomas are the phenotypic consequence of an accumulation of epigenetic changes in TS genes and structural mutation in distinct proto-oncogenes that result in unrestrained cellular proliferation. The structural point mutations of the oncogene K-ras were reported in 15 to 30% of adenocarcinomas, especially in smokers (22, 23). SFRP2 gene is

claimed to be a tumor suppressor gene that is inactivated by epigenetic CpG hypermethylation especially in CRC and MTAA (3, 6, 8, 24, 25). The promoter hypermethylation seems to be the predominant mechanism of SFRP2 gene silencing. Here, we report the combined effect of fully inactive SFRP2 and structural mutated K-ras oncogene in both current two cases with MTAA. The current results showed that genomic DNA from fecal and tumoral samples from case 1 have three point mutations (Val and Leu in codon 12 and Asp in codon 13) in K-ras and full promoter hypermethylation profile for SFRP2 gene (Fig. 1, C-Lanes 1,2). The same epigenetic profile for SFRP2 gene was also detected in both studied samples from case 2. While other TS genes such as p16, HIC1, DAPK1 and MGMT were in fully hypomethylated—in the active profile in both of the current cases, only SFRP2 gene was hypermethylated.

In conclusion, the current findings strongly support the hypothesis that the combined effect of epigenetic inactivation of TS SFRP2 and K-ras oncogene plays an important role in the development of anal mucinous type anal adenocarcinoma and it could be used for molecular screening in the future. Despite new therapy protocols, the prognosis of mucinous adenocarcinoma is poor. The present fecal and solid tumoural tissue DNA methylation assay provides a possible meaning to non-invasive screen for MTAA in humans to reduce the mortality as claimed by Veeck et al (9).

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