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Upregulation of the growth arrest-specific-2 in recurrent colorectal cancers, and its susceptibility to chemotherapy in a model cell system

Chi-Jung Huang [a,b], Chia-Long Lee [c,d,g], Shung-Haur Yang [e,f], Chih-Cheng Chien [g,h], Chi-Cheng Huang [g,i,j], Ruey-Neng Yang [k,l], Chun-Chao Chang [m,n,*]

[a] Department of Medical Research, Cathay General Hospital, Taipei, Taiwan
[b] Department of Biochemistry, National Defense Medical Center, Taipei, Taiwan
[c] Division of Gastroenterology and Hepatology, Department of Internal Medicine, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan
[d] Department of Internal Medicine, Cathay General Hospital, Taipei, Taiwan
[e] Department of Surgery, Taipei-Veterans General Hospital, Taipei, Taiwan
[f] School of Medicine, National Yang Ming University, Taipei, Taiwan
[g] School of Medicine, Fu Jen Catholic University, New Taipei, Taiwan
[h] Department of Anesthesiology, Cathay General Hospital, Taipei, Taiwan
[i] Breast Center, Cathay General Hospital, Taipei, Taiwan
[j] School of Medicine, Taipei Medical University, Taipei, Taiwan
[k] Ching Kuo Institute of Management and Health, Keelung, Taiwan
[l] Department of Internal Medicine, Sijhih Cathay General Hospital, New Taipei, Taiwan
[m] Division of Gastroenterology and Hepatology, Department of Internal Medicine, Taipei Medical University Hospital, Taipei, Taiwan
[n] Division of Gastroenterology and Hepatology, Department of Internal Medicine, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan

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ABSTRACT

Colorectal cancer (CRC) is one of the most common life-threatening malignances worldwide. CRC relapse markedly decreases the 5-year survival of patients following surgery. Aberrant expression of genes involved in pathways regulating the cell cycle, cell proliferation, or cell death are frequently reported in CRC tumorigenesis. We hypothesized that genes involved in CRC relapse might serve as prognostic indicators. We first evaluated the significance of gene sequences in the feces of patients with CRC relapse by consulting a public database. Tumorigenesis of target tissues was tested through tumor cell growth, cell cycle regulation, and chemotherapeutic efficacy. We found a highly significant correlation between CRC relapse and growth arrest-specific 2 (GAS2) gene expression. Based on cell models, the overexpressed GAS2 was associated with cellular growth rate, cell cycle regulation, and with chemotherapeutic sensitivity. Cell division was impaired by treating cells with 2-[4-(7-chloro-2-quinoxalinyloxy)phenoxy]-propionic acid (XK469), even when the cells were overexpressing GAS2. Thus, downregulation of GAS2 expression might control CRC relapse after curative resection. GAS2 could serve as a noninvasive marker from the feces of patients with prediagnosed CRC. Our findings suggest that GAS2 could have potential clinical applications for predicting early CRC relapse after radical resection, and that XK469 might impair tumor cell division by reducing GAS2 expression or blocking its cellular translocation. This will help in selecting the best therapeutic option, 5-fluorouracil in combination with XK469, for patients overexpressing GAS2 in CRC cells. Thus, GAS2 might act as a prognostic biomolecule and potential therapeutic target in patients with CRC relapse.

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1. Introduction

Colorectal cancer (CRC), a very heterogeneous disease, is one of the most common life-threatening malignances worldwide [1,2]. It is known that CRC develops through a gradual accumulation of genetic changes that lead to the transformation of normal colonic mucosa to dysplastic tissue, and finally to invasive cancer [3,4]. Aberrant expression levels of genes involved in pathways regulating the cell cycle, cell proliferation, or cell death are frequently reported in CRC tumorigenesis [5–7]. Thus, comprehensive gene expression analysis could be useful for classifying unresectable advanced or recurrent CRCs into subgroups by including or identifying potential biomarkers for treatment [8].

CRC relapse markedly decreases the 5-year survival to 6% and affects almost half of patients following surgery [9–11]. Moreover, surgery for patients with a relapse has a worse prognosis than for those with a

Abbreviations: CRC, colorectal cancer; GAS2, growth arrest-specific-2; XK469, 2-[4-(7-chloro-2-quinoxalinyloxy)phenoxy]-propionic acid; 5-FU, 5-fluorouracil; GEO, Gene Expression Omnibus.

* Corresponding author at: Division of Gastroenterology and Hepatology, Department of Internal Medicine, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan, No. 250, Wu Hsing St., Taipei 11031, Taiwan.
E-mail address: ccchangcmri@gmail.com (C.-C. Chang).

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primary CRC [12]. However, some biomarkers from patients with stage II CRCs can also be used as prognostic indicators [13]. In such cases, appropriate adjuvant chemotherapy can be administered to these high-risk subgroups, such as those suffering CRC relapse [14]. On the other hand, current therapeutic regimens often lack efficacy in treating such relapses and can add toxic side effects for most patients with no recurrence, who are inefficiently discriminated from the patients with CRC relapse [15–17]. For example, 5-fluorouracil (5-FU) can induce apoptosis in human CRC cells but does not prevent CRC relapse [18,19]. Therefore, identification of biological markers of CRC relapse is critical for targeting treatments effectively to those patients at risk of relapse [20]. This is why appropriate chemoprevention could improve the clinical outcomes for such patients [21,22]. However, few studies have investigated the molecular aspects of CRC relapse. Identifying CRC-related molecules might help in developing neoadjuvant chemotherapy [4,23,24].

We previously screened for differentially expressed genes in the feces of patients with CRC and have now cross-matched them with data from Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) according to CRC relapse [25,26]. The development of biomolecules with prognostic or predictive properties for CRC, including CRC relapse, is imperative for advancing treatment strategies [27]. Here we aimed to investigate the growth arrest-specific 2 (GAS2) gene, found in feces from patients with CRC relapse. Characterization of significant biomolecules was performed at molecular and cellular levels. We showed that the levels of GAS2 mediated the expression levels of G2/M cyclins. Furthermore, we found that cell division was impaired by treating cells with a selective topoisomerase II β inhibitor, even when the cells were overexpressing GAS2. These results imply that suppression of GAS2 expression might control CRC relapse after curative resection, and that GAS2 could be a noninvasive marker in the feces of patients previously diagnosed with CRC.

2. Material and methods

2.1. Patients and CRC cell lines

Two archived specimens were derived from previously formalin-fixed/paraffin-embedded biopsies, which were confirmed as CRC (AJCC stages II and III) by the Department of Pathology at Cathay General Hospital, Taipei, Taiwan. Informed consent was obtained from participants and study guidelines were approved by the institutional review boards. Six CRC cell lines (SW480, ATCC CCL-228; SW620, ATCC CCL-227; HCT 116, ATCC CCL-247; LS123, ATCC CCL-255; LoVo, ATCC CCL-229; and Caco-2, ATCC HTB-37) were cultured according to the recommendations from American Type Culture Collection (ATCC, Manassas, VA, USA). Briefly, all parental SW480 and SW620 cells and their derived cells were maintained in a CO_2-free incubator. To induce cell differentiation, Caco-2 cells were seeded at 3.9×10^4 cells/cm^2 and cultured to confluence for 21 days with changes of medium every 1–2 days [28,29]. The clinical characteristics of these cell lines were obtained from the ATCC Web site (www.atcc.org).

2.2. Lentivirus-mediated RNA interference (RNAi) and overexpression of target genes

The lentiviruses used for GAS2 knockdown or for producing tetracycline-inducible GAS2 were produced by the National RNAi Core Facility located at the Institute of Molecular Biology/Genomic Research Center, Academia Sinica, Taiwan. Lentiviral infection of the target cells was performed as described [30]. In brief, the amount of lentiviral particles was designed to infect 1.5×10^5 cells grown in a 6-well plate for 18 h at a multiplicity of infection of 3. Following 24 h culture, media were then replaced with those containing puromycin (2 μg/mL) to select for infected cells. For GAS2 knockdown in SW620 cells, the short hairpin (sh) RNA for GAS2 (TRCN0000420792) targeting the hairpin sequence (shGAS2; 5′-ATGATGCAGTGAAACGAATTT-3′) was used and shRNA (TRCN0000231719) targeting luciferase (shLuc; 5′-GCGGTTGCCAAGAG GTTCCAT-3′) served as a negative control. To overexpress GAS2 in cells, its cDNA was amplified from a human placenta library (Sigma-Aldrich, St Louis, MO. USA) (see Table 1 in Ref [31]) and the polymerase chain reaction (PCR) bands for GAS2 were sequenced to confirm gene identity. SW480 cells expressing GAS2 were generated either by lentiviral transduction using the all-in-one tetracycline-inducible plasmid (pAS4.1w.Ppuro-aOn) (Academia Sinica, Taipei, Taiwan) or by stable transfection using a plasmid encoding green fluorescent protein (pEGFP C3) (Takara Bio, Shiga, Japan). Conditional GAS2 expression in infected cells was induced by the addition of up to 2 μg/mL doxycycline for up to 72 h. In addition, the stable transfection of GAS2 into SW480 cells (5×10^4 cells/cm^2) was performed using jetPRIME (Polyplus-transfection, Illkirch, France) according to the manufacturer's instructions. The transfected cells were treated with 3 mg/mL Geneticin 24 h post-transfection. Expression of the target gene was routinely examined by quantitative real-time reverse transcription (RT)-PCR or western blotting.

2.3. Cytoplasmic RNA and quantitative real-time PCR

Cytoplasmic RNA was prepared using RNAzol reverse transcriptase (Molecular Research Center, Cincinnati, OH, USA) and quantitative real-time RT-PCR for each sample was performed using the High-Capacity cDNA Reverse Transcription Kits (Life Technologies, Carlsbad, CA, USA), by Oligo(dT) 12–18 priming at 37 °C for 2 h, using 1 μg of RNA per sample, following the manufacturers' instructions. After synthesis of the cDNA, quantitative real-time PCR was performed using a LightCycler Nano (Roche Diagnostics GmbH, Mannheim, Germany), using the FastStart Taqman Probe Master (Roche Diagnostics), and with sets of primers and Universal ProbeLibrary probes (Roche Diagnostics) designed online with ProbeFinder (v. 2.50; Roche Diagnostics). The mRNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, NM_002046 from http://www.ncbi.nlm.nih.gov) was used as a loading and internal control. LightCycler Nano software (v. 1.1.0; Roche Diagnostics) was used to analyze the real-time PCR kinetics.

2.4. Immunodetection of GAS2 protein in colonic cell lines and tissues

Cells were trypsinized and cellular lysates were harvested using PRO-PREP Protein Extraction Solution (iNtRON Biotechnology, Sungnam, Korea) in the presence or absence of 1 × Phosphatase Inhibitor Cocktail (Hycell, Taipei, Taiwan), if the phosphorylated protein was immunodetected by western blotting. For each sample, 20–30 μg of total protein in 1 × NuPAGE LDS sample buffer (Life Technologies) was denatured for 10 min at 95 °C, separated onto a precast polyacrylamide gel (NuPAGE Novex 12% Bis-Tris Gel; Life Technologies), and transferred to a polyvinylidene difluoride membrane (Merck Millipore, Darmstadt, Germany). Various target proteins were probed with the following antibodies at the indicated titers: anti-GAS2 (1:1000; ab55026; Abcam, Cambridge, MA, USA), anti-cyclin B1 (1:1000; ab32053; Abcam), anti-cyclin B2 (1:1000; ab185622; Abcam), anti-histone H3 (1:1000; ab1791; Abcam), anti-phospho-histone H3 on Ser10 (1:200; sc-8656-R; Santa Cruz Biotechnology, Dallas, TX, USA), and anti-GAPDH (1:5000; AM4300; Life Technologies), following standard procedures. Blots were then incubated with anti-mouse or -rabbit secondary antibodies (0.2 μg/mL) conjugated to horseradish peroxidase. Western blots were developed using either Western Lighting Ultra Extreme Sensitivity chemiluminescence kits (NEL113001EA; PerkinElmer, Boston, MA, USA) or VECTASTAIN ABC-AmP DuoLuX chemiluminescent/fluorescent substrate kits for alkaline phosphatase (SK-6005; Vector Laboratories, Burlingame, CA, USA), according to the manufacturers' instructions.

A routine immunohistochemical staining was performed used the biotin–streptavidin–peroxidase method with a VECTASTAIN® Elite ABC kit (Vector Laboratories) with minor modifications [32]. Briefly, the slides with tissue sections were immersed in citrate buffer

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