Journal of Surgical Research 174, 211–214 (2012)
doi:10.1016/j.jss.2011.11.008

ASSOCIATION FOR ACADEMIC SURGERY

Germline Mutations in *SMAD4* Disrupt Bone Morphogenetic Protein Signaling

Jennifer C. Carr, M.D., Fadi S. Dahdaleh, M.D., Donghong Wang, M.S., and James R. Howe, M.D.[1]

Department of Surgery, University of Iowa Carver College of Medicine, Iowa City, Iowa

Originally submitted June 28, 2011; accepted for publication November 8, 2011

Introduction. **Juvenile polyposis (JP) is an autosomal dominant disease that predisposes to GI malignancies. Germline mutations in the tumor suppressor gene *SMAD4* account for approximately 20% of JP cases. SMAD4 is the common intracellular mediator of the TGF-β and bone morphogenetic protein (BMP) pathways. Since mutations in BMP receptor 1A also cause JP, we hypothesize that altered BMP signaling is the underlying defect in JP. We therefore set out to investigate the effect of *SMAD4* mutations on BMP signaling.**

Methods. ***SMAD4*** **mutations identified in JP patients were selected for analysis. These were created in *SMAD4* pCMV expression vectors (EV) using a PCR-based, site-directed mutagenesis (SDM) approach. SDM clones were confirmed by direct sequencing, then co-transfected with an IdI-BMP Luciferase Responsive Element (BRE-Luc) vector and *Renilla* control vector into HEK-293T cells. Lysates were then collected after 48 hours, and luciferase activity was quantified using a luminometer. A pCMV empty vector was used as a negative control, and its luciferase activity was considered the baseline for cellular BMP signaling. Results obtained for each SDM clone were compared to those with the wild type (WT) vector. Statistical analysis was performed with the Student's t-test.**

Results. **Eleven distinct mutations from 16 JP patients were analyzed; seven mutations were nonsense, and four were missense. Both type of mutations resulted in reduction of BMP signaling; missense mutations produced an 8–30% reduction in luciferase activity, whereas nonsense mutations led to 30–60% reduction in luciferase activity when compared to the WT clone (Figure 1). All nonsense mutations led to significantly reduced activity relative to WT ($P < 0.05$), while the reduction in signaling seen in missense mutations was not statistically significant.**

Conclusion. ***SMAD4*** **germline mutations as seen in the JP patients appear to negatively impact downstream BMP signaling. Nonsense mutations resulted in significantly reduced luciferase activity when compared to missense mutations. These results support the hypothesis that disruption of the BMP signaling pathway is the likely etiology of JP in patients with *SMAD4* mutations.** © 2012 Elsevier Inc. All rights reserved.

Key Words: **juvenile polyposis; *SMAD4*; bone morphogenetic protein (BMP).**

INTRODUCTION

Juvenile polyposis (JP) is an autosomal dominant disorder with a prevalence of 1 in 100,000 individuals [1]. Affected patients develop hamartomatous polyps in the gastrointestinal tract, most notably in the colorectum and stomach [2]. Although these polyps are typically benign in nature, there is a potential for malignancy, with the risk of developing colon cancer estimated to be up to 50% over a patient's lifetime [3–5].

Two genes have previously been linked to the development of this disease: *SMAD4*, located on chromosome 18q21.1 [6, 7], and *BMPR1A*, located on 10q22-23 [8]. Mutations of these genes are responsible for approximately 40% of reported JP cases [9]. Both of these genes are in the bone morphogenetic protein (BMP) pathway, which is part of the transforming growth factor-β (TGF-β) superfamily. The BMP pathway plays a role in various cellular processes, including differentiation, development, reproduction, and apoptosis [10–12]. Since germline mutations in *SMAD4* and *BMPR1A* cause JP, and these genes are both involved in the bone morphogenetic protein (BMP) signaling pathway,

[1] To whom correspondence and reprint requests should be addressed at Department of Surgery, University of Iowa Hospitals and Clinics, 200 Hawkins Drive, Iowa City, IA, 52242-1086. E-mail: james-howe@uiowa.edu.

0022-4804/$36.00
© 2012 Elsevier Inc. All rights reserved.

aberrant BMP signaling is implicated in the evolution of JP.

Uncovering the effect of specific JP patient mutations on BMP signaling is important to better delineate the mechanism of polyp formation and cancer predisposition. *SMAD4*, the intracellular mediator of BMP signaling, forms hetero-oligomers with SMADs 1,5 and/or 8 after their phosphorylation by ligand-activated BMPR1A. The aim of this study was to recreate mutations found in JP probands in an expression vector, and to evaluate their effect on BMP signaling in an *in vitro* model.

MATERIALS AND METHODS

Reporter Plasmids

Germline *SMAD4* mutations found in JP patients [9] were recreated in a *SMAD4* pCMV6-XL5 expression vectors (Origene, Rockville, MD). A PCR-based, site-directed mutagenesis (SDM) approach was used to generate the individual mutations. Primers containing specific patient mutations were designed using the QC Primer Design Software (Stratagene). PfuUltra (Stratagene) was then used to amplify mutant constructs under the following conditions: 95° for 30 s, 65° for 1 min, and then 7 min at 68° for 18 cycles. *E. coli* bacterial cells were then transformed with the mutant expression vectors. For each mutation, 10 colonies of the transformed *E. coli* cells were selected at random, and were allowed to propagate 12 h in an orbital shaker at 37° in 5 mL of Luria Burtani media supplemented with 100 ug/mL of ampicillin. Plasmid DNA was then extracted using the PureLink Quick Plasmid Miniprep Kit (Invitrogen, Carlsbad, CA). The plasmid DNA was then sequenced to verify successful creation of each mutation.

Cell Culture and Transfection

Human embryonic kidney cells (HEK-293T), obtained from American Type Culture Collection (Manassas, VA), were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin G-streptomycin sulfate (PS). After confluence of 90% was achieved, the cells were cotransfected with 1 ug of the BRE-Luc vector (a plasmid reporter vector with a BMP responsive element promoter cloned upstream from a luciferase gene), 1 ug of the *SMAD4* expression vector (wild-type or mutants), and 200 ng of Renilla (internal control vector for transfection efficiency), using 6 uL Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). Each transfection was performed in triplicate. After 4 h, fresh media was added.

Luciferase Assays

Forty-eight hours post-transfection, 500 uL of lysis buffer was added to the cells, which were incubated at room temperature for 15 min. Twenty uL of lysate was transferred to a reading tube that contained 100 uL of the Luciferase Assay Reagent II (LARII) solution, and luciferase activity was measured at 562 nm for 10 s using a TD 20/20 luminometer (Turner BioSystems, Sunnyvale, CA). Once the initial readings were performed, 100 uL of Stop and Glo reagent (Promega, Madison, WI) was added, and Renilla luciferase activity measured at 480 nm for 10 s. The final amount of luciferase activity for each construct was determined by subtracting the background luciferase activity from the control pGL3 basic vector without construct, and then normalizing to the Renilla luciferase activity for each individual reaction. A Student's t-test was then performed to assess the statistical significance of differences between triplicate results obtained for each mutant construct relative to the wild-type.

RESULTS

In our juvenile polyposis database of 119 probands, 22 patients with *SMAD4* mutations were identified. Six of these patients shared one mutation (Exon 9: Del AGAC 1244-47, D415EfsX20), while two others shared substitutions of nucleotide 1081. After accounting for the duplication of these mutations, 14 distinct mutations were identified. One mutation was a splice site variant, and was not further evaluated. Eleven of these mutations were re-created by SDM in the *SMAD4* expression vector. Seven of these mutations were non-sense: 608delC (P203HfsX38, Exon 4); 1037delC (P346LfsX38, Exon 8); 1162C > T (E388X, Exon 9); 1193G > A (W398X, Exon 9); 1244_7delACAG (D415EfsX20, Exon 9); 1343_65del22 (Q448QfsX37, Exon 10); and 1588delC (R530TfsX7, Exon 11). Four mutations were missense: 989A > G (E330G, Exon 8); 1081C > A (R361S, Exon 8); 1393G > A (V465M, Exon 10); 1525T > A (W509R, Exon 11).

Luciferase activity from the different constructs is shown in Fig. 1. The light units produced by the wild-type (WT) expression vector (standardized to the Renilla control) were set at 100%. The negative control, which consisted of a pCMV vector without an insert, produced 31% of the wild-type light units, indicating the baseline level of BMP signaling mediated through endogenous cellular SMAD4. As a group, the non-sense mutations led to a 30%–60% reduction in luciferase activity compared with the wild-type vector. More specifically, the 608delC mutation led to a 59% reduction in luciferase activity, 1037delC was reduced 30%, 1162 C > T 52%, 1193 G > A 48%, 1244_7delACAG 33%, 1343_65del22 29%, and 1588delC 61% ($P < 0.05$ for all constructs).

The missense mutations led to lesser reductions in BMP signaling, ranging from 8%–30%. The 989 A > G (E330G) substitution led to an 8% reduction, 1081

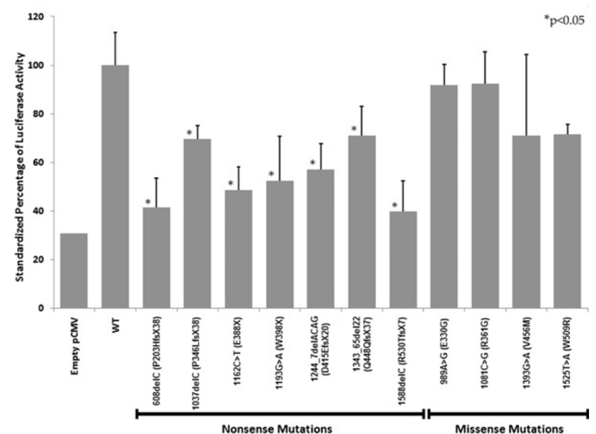

FIG. 1. The effect of *SMAD4* mutations on BRE-Luc luciferase activity. Asterisks denote values that were statistically significantly different from wild-type ($P < 0.05$).

Download English Version:

https://daneshyari.com/en/article/6254644

Download Persian Version:

https://daneshyari.com/article/6254644

Daneshyari.com