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MATRIX METALLOPROTEINASES IN THE URINE AND TISSUE OF PATIENTS WITH JUVENILE POLYPS: POTENTIAL BIOMARKERS FOR THE PRESENCE OF POLYPS

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Abstract

Background & Aims—Juvenile polyps are the most common type of pediatric gastrointestinal polyp and are typically characterized as hamartomatous overgrowths. Juvenile polyps are highly vascularized tissues and display a markedly increased mucosal microcirculation. Matrix metalloproteinases (MMPs) are a family of zinc-dependent enzymes that play an essential role in: the physiologic degradation of the extracellular matrix in normal development, tumor invasion and metastasis, as well as angiogenesis. We hypothesized that the presence of these enzymes in urine may serve as clinical biomarkers of juvenile polyps.

Patients and Methods—In this preliminary study, we analyzed 32 urine samples collected prospectively from 16 subjects with known or suspected juvenile polyps who presented to the endoscopy unit for colonoscopic evaluation and 16 age and sex-matched controls. Urinary MMPs were analyzed by zymography and their localized tissue expression was assayed via immunohistochemistry of tissue sections.

Results—MMPs were detected in the urine of patients with juvenile polyps with significantly higher frequency when compared to urine of controls subjects. In addition, immunohistochemistry demonstrated that high levels of MMPs were localized in the epithelium and lamina propria of polyp tissue when compared to colonic tissue collected from healthy control subjects.

Conclusions—These data are the first to demonstrate that MMPs are present in the urine and tissue of patients with juvenile polyps, and these enzymes have the potential to serve as surrogate markers for the presence of polyps.

Keywords

Matrix Metalloproteinases; biomarkers; polyps; juvenile polyps; juvenile polyposis syndrome

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Introduction

Juvenile polyps are the most common type of pediatric gastrointestinal polyp and are typically characterized as hamartomatous overgrowths^{1, 2}. Solitary juvenile polyps can occur at any age, but appear most common in preschool children. Children with juvenile polyps typically present with blood in their stool and sometimes anemia. Solitary polyps are generally considered to be a separate entity from juvenile polyposis syndrome (JPS), and it is not known whether the existence of a single juvenile polyp in a child will predispose to the growth of additional polyps or increased risk of colorectal neoplasia in later life^{3, 4}.

JPS is an autosomal dominant condition in which affected individuals develop multiple gastrointestinal juvenile polyps, predominantly in the colon, but may also affect the rest of the gastrointestinal tract^{1, 2, 5}. The rare and often fatal form called juvenile polyposis of infancy is characterized by diarrhea, protein-losing enteropathy, bleeding, and rectal prolapse⁶. The more common form of JPS affecting the colon, stomach and small bowel occurs in the first or second decade with rectal bleeding and anemia^{7–10}. A family history of the condition is found in 20–50 percent of patients with apparently an autosomal dominant inheritance pattern. In JPS, epithelial dysplasia is common and the cumulative risk of colorectal cancer is greater than 50%⁸. Colonic and gastric carcinoma and dysplasia in JPS have prompted reclassification of this entity as a pre-malignant condition. The reported malignant degeneration rate of polyps is 17.6%¹¹. This high risk of gastrointestinal cancer warrants frequent endoscopic screening of both affected and at-risk family members.

JPS has been associated with germline mutations of *SMAD4*, *BMPRI1A*. These two mutations account for approximately 40% of JPS cases¹². *ENG* has recently been shown to be present in 2 patients with JPS¹³; however its role as a pre-disposition gene still requires additional confirmation^{14, 15}. All three genes encode proteins that are involved in the transforming growth factor (TGF- β) signaling pathway. The PTEN gene mutation in patients with juvenile polyposis is a controversial topic. It is generally believed that it likely represent Cowden Syndrome or Bannayan Riley Ruvalcaba patients that have not yet expressed the extraintestinal clinical features of these conditions.

Since genetic testing currently misses a majority of JPS cases, physicians rely on clinical criteria to diagnose patients at high risk for JPS. The clinical criteria include: (1) 3–10 or more polyps detected on colonoscopy (2) polyps located outside of the colon (3) any number of polyps with a family history of juvenile polyps^{16, 17}. Patients who meet one of the three criteria will require surveillance colonoscopies to evaluate for further polyp growth. Setting the total number of polyps to make the diagnosis of JPS at 3 polyps does have the potential for patients to undergo unnecessary colonoscopies due to the misdiagnosis of JPS.

Juvenile polyps are highly vascularized tissues with increased mucosal microcirculation¹⁸. Angiogenesis, or the formation of new blood vessels, plays a critical role in physiologic development, tissue repair, and reproduction. Angiogenesis is also crucial in pathologic conditions, such as tumor growth and metastasis^{19, 20}. Once tumors reach a size of greater than a few millimeters, their growth is completely dependent on angiogenesis²¹. The ability of a tumor to induce angiogenesis involves a dysregulation of multiple angiogenesis growth factors and enzymes.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent enzymes that play an essential role in the degradation and remodeling of extracellular matrix and basement membrane proteins that are required for angiogenesis^{20, 22, 23}. MMPs function during normal physiologic processes of tissue repair and morphogenesis. Previous studies have also implicated MMPs in angiogenesis, tumor invasion and metastasis²⁴.

Our laboratory has previously demonstrated that MMPs can be detected in the urine of patients with a variety of cancers^{25–27}. These urinary MMPs are independent predictors of disease status in these subjects. We have also identified and characterized one of the high molecular weight MMP species with activity at 125 kDa as MMP-9 complexed with neutrophil gelatinase associated lipocalin (NGAL)^{27, 28}. The complexing of NGAL with MMP-9 has the function of protecting MMP-9 from autodegradation, thereby preserving its full degradative activity²⁷.

We hypothesized that, due to the vascular nature of juvenile polyps, these enzymes might serve as potential biomarkers of polyp growth and development. Therefore, the purpose of this pilot study was to determine whether MMPs were present in the urine and tissue of patients with juvenile polyps.

Patient and Methods

Patient Urine Collection

Urine was collected according to protocol approved by the Institutional Review Board at Children's Hospital Boston (Boston, MA). Urine samples were obtained from consecutive patients with known or suspected juvenile polyps that presented to the endoscopy unit for colonoscopic evaluation February 2005 to January 2006. Entry criteria included rectal bleeding, history of prior polyp, or a family history of polyps. Exclusion criteria for the study were known inflammatory bowel disease. Patients consenting to the study provided a voided urine specimen prior to the procedure. Patients in the control group were age and sex matched subjects who met entry criteria but had a normal colonoscopy. Urine and tissue samples collected from 16 patients with juvenile polyps (8 subjects with JPS and 8 with non-syndromic juvenile polyps) were compared to paired samples collected from 16 age-matched controls. For the purposes of analysis, data collected from patients with isolated juvenile polyps were pooled with those from patients with juvenile polyposis syndrome. Patients with JPS were defined as meeting one of the following criteria: 1) any number of polyps with a family history of juvenile polyps; 2) polyps located outside of the colon; 3) five or more polyps detected on colonoscopy. Submitted urine samples were tested for the presence of blood and leukocytes using Multistix 9 Urinalysis Strips (Bayer, Elkhart, IN) as previously described²⁵.

Sample Preparation and Substrate Gel Electrophoresis

Samples were collected, divided into aliquots, and frozen immediately (–20°C) until ready for assay. Substrate gel electrophoresis (zymography) was conducted as previously described^{25, 28}. In brief, proteins were resolved on a 10% SDS PAGE gel containing 0.1% gelatin under non-denaturing conditions. Gels were washed in Triton-X-100 and then incubated overnight at 37 °C in substrate buffer [50 mmol/L Tris (pH 8), 5 mmol/L CaCl₂, and 0.02% NaN₃]. Gels were then stained with 0.1% Coomassie Blue and destained for 1 hour. Gelatinolytic activity was detected as bands of clearance on a blue background.

MMP levels were determined using zymography. Urine samples were run on commercial 10% gelatin zymogram kit (Invitrogen). Gels were stained and photographed. Both proenzyme and activated proteinases appeared as zones of substrate clearing. Different MMPs were distinguished from one another on the basis of their molecular weights. High molecular weight MMP, MMP-9/neutrophil gelatinase-associated lipocalin (NGAL), MMP-9, MMP-2, were determined using this methodology. Identity of MMP species was confirmed by immunoblot analysis as previously reported by us²⁵. Investigators were blinded to the clinical status of the individual from whom the urine sample was obtained. Urine samples were scored on the presence or absence of MMP activity.

Immunohistochemical analysis

Immunohistochemistry of polyps and colonic tissue was conducted using formalin fixed and paraffin-embedded tissue sections. Slides were covered with Peroxidase block (DAKO USA, Carpinteria, CA) for 5 minutes to quench endogenous peroxidase activity followed by a 1:5 dilution of goat serum in 50 mmol/L Tris-HCL (pH 7.4) for 20 minutes to block nonspecific binding sites. Primary murine anti-human MMP-9 (1:500 dilution; Chemicon International, Inc., Temecula, CA), murine anti-human MMP-2 (1:250 dilution; Chemicon International), rat anti-human NGAL (1:100 dilution; R & D Systems, Inc. Minneapolis, Mn), or CD34 (Immunotech, France) was applied in 50 mmol/L Tris-HCL (pH 7.4) with 3% goat serum at room temperature for one hour. Slides were then washed in 50 mmol/L Tris-HCL (pH 7.4) and antigen-antibody binding was detected using horseradish peroxidase-conjugated secondary antibodies (Envision detection kit, DAKO USA).

A pathologist specializing in gastroenterology (J.G.) who was blinded to the clinical history of the subjects scored the staining for each marker on a scale of 0 to 3. A score of zero equaled no expression, 1= one to two foci of expression, 2 = multiple foci of expression (>2 separate foci, <50% of tissue), and 3 = extensive expression (diffuse or >50% of tissue). The relative strength and cellular distribution of the staining was also noted.

MMP-2 ELISA

Quantitative measurement of urinary MMP-2 was obtained by ELISA. Urine samples were normalized to protein to allow for comparison. Protein concentration of the urine was determined by the Bradford method using bovine serum albumin as the standard. MMP-2 levels were determined using a Human MMP-2 ELISA kit (R & D Systems, Inc.) and was performed according to the manufacturer's instructions. This is a quantitative sandwich enzyme immunoassay technique using a polyclonal antibody specific for MMP-2 bound to the plate, and an enzyme-linked polyclonal antibody specific for MMP-2 that is added to the wells.

Statistical Analysis

Positive expression for each urinary MMP biomarker was compared between patients with juvenile polyps and controls using Fisher's exact test for binomial proportions. Protein tissue expression based on immunohistochemistry was evaluated nonparametrically using the Kruskal-Wallis test followed by the Mann-Whitney U-test for pairwise comparisons between normal colonic tissue, solitary polyps, and JPS groups with data presented as medians and ranges for each of three MMP variables (MMP-2, MMP-9, and MMP-9/NGAL)²⁹. Power analysis indicated that for comparing binary proportions of urinary MMPs, sample sizes of 16 patients and controls would provide 80% power ($\alpha = 0.05$, $\beta = 0.20$) to detect differences of 50% in the rate of positive expression using a two-tailed Fisher's exact test (version 6.0, nQuery Advisor, Statistical Solutions, Saugus, MA). Statistical analysis was conducted with the SPSS statistical package (version 14.0, SPSS Inc., Chicago, IL). All two-tailed values of $p < 0.05$ were considered statistically significant.

Results

Elevated MMP Levels are Found in the Urine of Subjects with Juvenile Polyps

Representative zymograms of urine analyzed from patients with juvenile polyps and healthy controls are illustrated in Figure 1. The bands noted on the gel represent zones of clearance where the MMPs have degraded the gelatin substrate. The percentage of positive MMP expression (MMP-2, MMP-9, MMP-9/NGAL and HMW > 150 KD) was significantly higher in subjects with juvenile polyps compared to controls ($P < 0.001$) (Figure 2). There

was no statistical difference in MMP expression between subgroups (solitary and syndromic polyps).

Approximately 35% of the urine collected from healthy control subjects in this study contained detectable MMP-2 activity. Previous studies have similarly reported that MMP-2 can be detected in the urine of healthy young children, and it is thought that this may reflect physiologic bone remodeling. However MMP activity is not normally present in the urine of children beyond puberty³⁰. Therefore, we employed a quantitative ELISA assay to compare urinary MMP-2 levels collected from patients with intestinal polyps with those collected from healthy control subjects. Our data demonstrate that while MMP-2 is indeed present in the urine of healthy control subjects, it is present at significantly lower levels than those observed in the urine of subjects with intestinal polyps (Figure 3).

MP Expression is Elevated in Juvenile Polyp Tissue

We next sought to determine whether MMPs were over- expressed in juvenile polyp tissue compared to normal colonic epithelium. As such, tissue section collected from patients with non-syndromic polyps and juvenile polyposis syndrome, as well as tissue sections collected from control patients, was analyzed for the presence and localization of MMP-2, MMP-9, and NGAL. MMP-2 expression was significantly increased in both the lamina propria and epithelium of polyp tissue compared to normal colonic tissue (Figure 4). Expression of MMP-9 was similarly expressed at significantly higher levels in the lamina propria of polyp tissue (Figure 4). In addition, MMP-9 was expressed in the endothelium of blood vessels. NGAL expression was significantly increased in both the lamina propria and epithelium of polyp tissue compared to normal colonic tissue (Figure 4). Histological scoring to compare expression of MMP-2, MMP-9 and NGAL in both the lamina propria and epithelium in juvenile polyps, and normal colonic tissue was conducted and the results have been summarized in Table 1.

Increased Microvessel Density in Juvenile Polyps

In order to determine the extent of angiogenesis in polyp and control tissues, we used the endothelial marker CD34 to stain the microvasculature. CD34 immunostaining revealed increased microvessel density in the lamina propria of juvenile polyp tissue compared to the lamina propria of normal colonic tissue (Figure 4). In juvenile polyp tissue, the mean vessel count was 28.1 +/- 7.3 microvessels per 400X field compared to 10.3 +/- 1.5 microvessels per 400x field in normal colonic epithelium. ($p < .001$ by Wilcoxon rank sum test).

Discussion

These data are the first to demonstrate elevated MMP levels in polyp tissue collected from patients with solitary and juvenile polyposis syndrome. We were similarly able to detect elevated MMP levels in the urine of patients with these intestinal polyps. In our pilot study, we found no statistical difference between MMP levels in the urine and tissue of solitary juvenile polyps and JPS polyps. A larger prospective study has been initiated to determine whether a urinary MMP fingerprint might be present and useful in distinguishing patients with non-syndromic polyps from those with JPS. We hypothesized that due to the vascular nature of juvenile polyps, and the extracellular matrix remodeling accompanying their growth, MMPs would be upregulated in juvenile polyps regardless of whether they are syndromic or non-syndromic. The potential clinical utility of this test lies in its potential to non-invasively monitor pediatric patients with a history of juvenile polyps for additional polyp growth.

MMP-2, MMP-9, MMP-9/NGAL, and the HMW MMP species were present with high frequency in the urine of our polyp subjects compared to healthy controls. The presence of MMPs in the urine of the patients studied correlates with an over-expression of MMP-2, MMP-9, and NGAL in the polyp tissue itself. CD34 immunostaining also demonstrated an increased microvessel density, thereby further supporting the conclusion that these polyps have increased neovascularization. MMP-2 and MMP-9 were detected in the endothelium of polyp tissue by immunostaining, and their increased presence in these tissues is consistent with their previously outlined role in angiogenesis^{20, 22}.

The finding that MMP9/NGAL is upregulated in both the urine and tissues of individuals with juvenile polyps is an interesting one, and the role of NGAL in the pathogenesis of juvenile polyps merits further investigation. It is possible that NGAL may serve to protect MMP-9 from autodegradation²⁷, thereby facilitating the increased extracellular matrix remodeling activity required for both polyp formation and angiogenesis.

JPS is a pre-malignant condition that requires close follow up with repeat colonoscopy 7–11, 31–33. One of the difficulties in differentiating patients with solitary or nonsyndromic polyp patients from those with JPS is the lack of consensus clinical criteria to classify JPS and current clinical classification may potentially over diagnose JPS 16, 17, 34, 35. Presently available genetic testing is not routinely performed at many academic centers. Further, existing data suggest that a majority of JPS subjects will not be detected using current technology^{12, 36, 37}. As such, making a diagnosis of JPS can be difficult and children may require repeat colonoscopic evaluations to survey for new polyp growth. Our data suggest that urinary MMPs may be markers for growth of juvenile polyp tissue in patients with both JPS and non-syndromic polyps. The clinical utility of this test may lie in its ability to non-invasively follow children with a history of juvenile polyps to serve as a surrogate marker for the presence of new polyps. This would provide clinicians with objective data to assist in determining when a patient would most benefit from repeat colonoscopic evaluation. This is the focus of our future studies.

Taken together, these data suggest that MMPs may be crucial for polyp formation and thus may serve as a biomarker to detect these highly vascular lesions. Future studies will be focused on determining the role of MMPs in polyp development as well as initiating longitudinal studies to determine whether these non-invasive urinary proteins might be useful additions to surveillance guidelines for these diseases.

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Abbreviations

BMPR1A	bone morphogenic protein receptor 1A
FAP	Familial Adenomatous Polyposis
HMW	high molecular weight
IHC	immunohistochemistry
JPS	Juvenile Polyposis Syndrome
MMPs	matrix metalloproteinases

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Figure 1.

Substrate gel electrophoresis (zymography) analysis was performed on the urine from subjects with polyps and healthy age and sex matched controls. Urine was electrophoresed in gelatin-embedded polyacrylamide gels. Gelatin is the substrate for MMP-2 and MMP-9. Neutrophil Gelatinase Associated Lipocalin (NGAL) is a protein that has been found in the urine of polyp patients and migrates in complex with MMP-9 at approximately 125 kDa.

Figure 2.

Comparison of MMP expression between juvenile polyp (JP) patients and controls. A significant elevation in each MMP biomarker ($P < 0.001$) was detected.

Figure 3.

Quantitative analysis of MMP-2 by ELISA showed significantly elevated levels of MMP-2 in the urine of polyp subjects as compared with the urine of age-and sex matched controls (medians: 0.046 vs. 0 ng MMP-2/ug total protein, $p < 0.001$, Mann-Whitney U -test).

Figure 4.

Immunohistochemistry was performed on polyp tissue and normal colonic tissue. Antibodies specific for CD34, MMP-2, MMP-9, and NGAL were used. CD34 staining showed increased microvessel density in polyp tissue. MMP-2 expression was increased in both the lamina propria and the epithelium of polyp tissue compared to normal colonic tissue. MMP-9 expression was higher in the lamina propria and endothelium of polyp tissue. NGAL expression was increased in both the lamina propria and epithelium of polyp tissue compared to normal colonic tissue.

Table 1

Protein Tissue Expression as Determined by polyp Immunohistochemistry

	Epithelium			Lamina Propria		
	Normal colonic tissue (n=6)	Juvenile Polyp tissue (n=20)	P value	Normal colonic tissue (n=6)	Juvenile Polyp tissue (n=20)	P value
Biomarker						
MMP-2	0 (0–0)	1 (0–2)	<0.001*	0 (0–0)	1 (0–3)	<0.001*
MMP-9	1 (1–1)	1 (0–2)	0.98	0 (0–0)	2 (0–2)	0.009*
NGAL	0 (0–0)	1 (0–3)	0.004*	1 (1–1)	3 (1–3)	0.004*

0= absent, 1= 1–2 foci only; 2 =more than 2 Separate Foci, <50% of tissue; 3 = extensive staining, >50% of tissue.

Data represent median Values with ranges in parentheses. MMP = matrix metalloproteinase, NGAL = neutrophil gelatinase-associated lipocalin.