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Ornithine Decarboxylase and Tyrosine Kinase Activity in Juvenile Polyps of Childhood

Y. ELITSUR, S. J. G. KOH, J. A. MOSHIER, J. DOSESCU, J. TUREAUD, AND A. P. N. MAJUMDAR

Department of Pediatrics, Pediatric Gastroenterology Division, Marshall University, Huntington, West Virginia 25701-0195 [Y.E., S.J.G.K.], Departments of Internal Medicine and Molecular Biology and Genetics [J.A.M., J.D.], and Division of Gastroenterology, VA Medical Center [J.T., A.P.N.M.], Wayne State University School of Medicine, Detroit, Michigan 48201

ABSTRACT

Juvenile polyps (JP) are the most common colonic tumor in children. Although considered benign, malignant transformation has been reported in JP. Ornithine decarboxylase (ODC) and tyrosine kinase (TyK) enzymes are markers for a rapid cell proliferation index. DNA aneuploidy score and p53 gene expression are late malignant changes seen in patients with colon cancer. In this study, we investigated ODC and TyK activities as well as DNA aneuploidy score and p53 expression in juvenile polyps compared with the adjacent normal colonic mucosa. Results showed that ODC was significantly increased in JP compared with the adjacent normal colonic mucosa. TyK activity was increased in 3/5 polyps and decreased in 2/5 polyps compared with the mucosa. Mean TyK activity was higher in JP compared with normal mucosa but did not reach significance (707 and 632 pmol/mg pmol, respectively). Moreover, changes in phosphorylization of TyK proteins was also observed in JP but not in normal mucosa. JP had a normal DNA aneuploidy score and showed no expression of p53 gene. We conclude that JP do not express p53 gene and aneuploidy but had higher activity of ODC and TyK enzymes, suggesting a higher stage of cell proliferation. (***Pediatr Res* 38: 574–578, 1995**)

Abbreviations

ODC, ornithine decarboxylase
TyK, tyrosine kinase
JP, juvenile polyp
RIPA, radioimmunoprecipitation assay

JP are the most common form of colon tumors in children (1). Malignant transformation of such polyps has been reported in patients with inherited premalignant conditions, *i.e.* familial adenomatous polyposis (2–4), but JP in children without such diseases are considered to be benign. Nevertheless, malignant changes within JP have been anecdotally reported in the domestic and foreign journals (3–10). Moreover, adenocarcinomas of the colon have also been reported in children (11, 12).

ODC is the first limiting enzyme in the polyamine biosynthetic pathway (13). Polyamines and ODC are synthesized by all eukaryotic cells and are thought to play a crucial role in proliferation of normal and neoplastic cells in the human gut epithelium (14–18). Thus, ODC has been used as an early marker for cell proliferation index and for early detection of colon cancer (18, 19).

TyK are a family of proteins which are widely expressed during cell proliferation, differentiation, and transformation (20–22). Increased ODC activity and TyK activity was reported in colon mucosa of patients with adenomatous polyps but not in hyperplastic polyps (23).

Tumorigenesis of human colon is preceded with various intracellular oncogene expression and gene alteration (24). One of the changes occurs on chromosome 17p where the p53 gene is located. p53 gene expression may be used as a late marker for colon cancer development. Thus, the expression of the p53 gene and DNA ploidy analysis of JP may further be used to evaluate the potential of such polyps to transform into malignant polyps.

The aim of this study was to compare the relationship between ODC and TyK in JP and normal adjacent mucosa in children who do not have increased risk factors for colon cancer development. In addition JP are examined for their potential malignant transformation by their p53 expression and aneuploidy score.

METHODS

Children Clinical Data

A total of 10 children participated in the study. All had fresh rectal bleeding and five showed the polyp radiologically. Polyps were located in the sigmoid (five) and in the rectum (five).

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Correspondence and reprint requests: Y. Elitsur, M.D., Department of Pediatrics, Division of Gastroenterology, Marshall University School of Medicine, 1801 Sixth Avenue, Huntington, WV 25701-0195.

Children's ages ranged between 1.5 and 17 y. In three patients, there was a family history of colon cancer, and two other patients had a positive family history for colon polyps. Two patients had a previous history of JP removal and had more than one polyp removed during endoscopy.

Colon Tissues

Colonic biopsies and JP were obtained from 10 children who underwent colonoscopy for rectal bleeding. After polypectomy, small pieces of the colonic polyps and adjacent colonic biopsies were quickly frozen and stored at −70°C until analyzed. The polyps and their adjacent colonic biopsies were cleared of any histologically malignant changes. Due to ethical constrains, there was a shortage of control biopsies to complete all studies in each pair. Accordingly, JP and adjacent control pair were used for ODC activity, TyK activity, and TyK-phosphorylated protein studies upon samples availability. The study was approved by the local Human Investigational Committee. In other experiments, a total of eight pathologic blocks of JP were examined for p53 expression and DNA ploidy analysis. These specimens had no malignant features on histologic examination.

Biochemical Assays

ODC assay. ODC activity was determined according to the micro method of Beaven *et al.* (25), as established in our laboratory (17). Briefly, the cell pellet was homogenized in a buffer containing 50 mM HEPES (pH 7.4), 0.1 mM EDTA, 0.04% Brij 35, 4 mM DTT, and 0.5 mM pyridoxal 5′-phosphate. Homogenates were centrifuged at 47 000 × *g* for 30 min at 4°C, and the supernatants were assayed for ODC activity. The incubation mixture contained 10 μl of cell homogenate and 10 μl of incubation buffer containing 50 mM HEPES (pH 7.4), 0.1 mM EDTA, and 0.144 μCi of L-[1-^{14}C]ornithine hydrochloride (58 mCi/mmol). Protein concentration was determined by the method of Bradford using a kit from Bio-Rad according to the manufacturer's instructions.

TyK activity. This was measured according to the method of Dangott *et al.* (26) as previously described by Majumdar *et al.* (27). Briefly, tissues were solubilized in RIPA buffer (20 mM sodium phosphate, pH 7.4, containing 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxychloate, 150 mM NaCl, 5 mM EDTA, 5 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 10 μg/ml leupeptin, and 5 mM sodium pyrophosphate and diluted with an equal volume of homogenizing buffer (25). The diluted material was used as a source for the enzyme. The reaction mixture in a final volume of 50 μl contained 2.5 μmol of Tris-HCl, 2/5 μmol of $MgCl_2$, 0.5 nmol of orthovanadate, 0.2% Triton X-100, 3 μmol of ATP, 0.4 μCi of [γ-^{32}P]ATP, and 100 μg of Glu-Tyr polymer. The reaction at 24°C for 10 min was initiated with a mucosal membrane fraction (10–15 μg of protein) and was terminated by applying 20 μl of the reaction mixture onto 3-cm^2 Whatman No. 3 MM filter paper. The filters were washed extensively in 10% trichloroacetic acid containing 10 mM sodium pyrophosphate, rinsed with 95% ethanol, dried, and counted in 5 ml of scintillation mixture. The results were expressed as picomoles of ^{32}P incorporated per mg of protein.

Autophosphorylation and identification of phosphorylated proteins. This was carried our according to the method of Majumdar *et al.* (28, 29). Briefly, the reaction mixture contained, in a final volume of 50 μl, the following: 2.5 μmol of HEPES (pH 7.8), 2.5 μmol of $MgCl_2$. 0.5 nmol of orthovanadate, 0.5 nmol of [γ-^{32}P]ATP (4 × 10^6 disintegration/min), and 0.02% Triton X-100. The reaction at 0–2°C for 30 min was initiated with 75 μg of protein and was terminated by adding an equal volume of RIPA lysis buffer (20 mM sodium phosphate, pH 7.4, containing 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 150 mM NaCl, 5 mM EDTA, 5 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 5 mM sodium pyrophosphate, and 3 μg of antiphosphotyrosine antibody (MAb, Boehringer Mannheim, Indianapolis, IN). After incubation for 3 h at 4°C, the immune complex was precipitated with pansorbin (a) and recovered by centrifugation at 10 000 × *g* for 30 min. The immunoprecipitates were dissolved by incubating at 100°C for 7 min in a small volume of stopping buffer (62.5 mM Tris-HCl, pH 6.8, 6% SDS, 20% glycerol, and 10% 2-mercaptoethanol)/RIPA lysis buffer (1:1) and centrifuged again. The supernatant was then subjected to SDS-PAGE. After electrophoresis, the gel was fixed, washed, and finally exposed to X-Omat AR film as described previously (28, 30). The molecular mass of the labeled bands was calculated from marker proteins run concurrently.

Immunohistologic Study of p53 Suppressor Oncogene Product

Expression of the p53 suppressor oncogene product was demonstrated by the modified technique of immunoperoxidase methods previously reported (31–33). Commercially available anti-p53 suppressor oncogene product (BP53–12-1) (Biogenix, San Ramon, CA) was used as a marker. Briefly described, the deglycerated tissue sections were treated with 1:20 dilution of anti-p53 protein and 1:40 dilution of horseradish peroxidase-conjugated Ig (Cappal Laboratories, Inc., Cochranville, PA) for 60 and 30 min, respectively. For color reaction, 0.05%, 3,3-diaminobenzidine tetrachloride (Sigma Chemical Co., St. Louis, MO) was used. Adenocarcinoma from an adult colon was used as a positive control.

DNA Analysis

The Feulgen-stained slides were analyzed using the CAS 200, a video-based interactive image cytometer (Cell Analysis Systems, Elmhurst, IL) that has been previously described (34). Briefly, after proper calibration, microscopic fields were selected, and the chosen images were digitalized. The integrated optical density of Feulgen-stained nuclei was proved to be proportional to the amount of DNA present in the nuclei. The nuclei of JP were analyzed using the Quantitative Ploidy Analysis soft-ware package (Cell Analysis Systems). The nuclei analyzed were chosen to reflect the typical histopathologic patterns of JP.

In the DNA histograms produced by the cell image cytometer, JP were classified as diploid if they had a single G_0/G_1 peak. DNA aneuploid would be present if an additional distinct peak was demonstrated different from the diploid peak. The DNA index was calculated as the ratio of the observed peak to the standard peak of 7.18 pg of DNA.

Statistics

Nonparametrial statistic method, Mann-Whitney statistical analysis, was used in this study. $p < 0.05$ was considered as the level of significance.

RESULTS

ODC activity. A total of seven pairs of JP and histologically normal mucosa were available for ODC determinations. In 6/7 cases, ODC activity was higher in polyp tissue compared with control mucosa (Fig. 1). The mean ODC activities were 356 (range 22–887) and 1145 (range 187–2079) pmol of CO_2/mg protein/h in normal mucosa and polyp, respectively ($p < 0.03$). For each sample ODC measurement was done in triplicate, and variability was <10%.

TyK activity and phosphorylation. A total of five pairs of tissue samples were available for determination of TyK activity assay. Although in 3/5 samples TyK activity was higher in the polyp compared to control, no significant difference was achieved. The mean TyK activity was 632 (range 430–1010) and 707 (range 565–821) for normal and polyp tissues, respectively (Fig. 2). Two of the polyp samples with increased TyK activity were also assayed for tyrosine-specific phosphorylation of proteins. Results revealed that, in addition to two prominent 80- and 70-KD bands, two other bands with molecular masses of 170 and 120 kD were phosphorylated. Extent of tyrosine-specific phosphorylation of these proteins was found to be 2–4-fold in samples from JP when compared with the controls (Fig. 3).

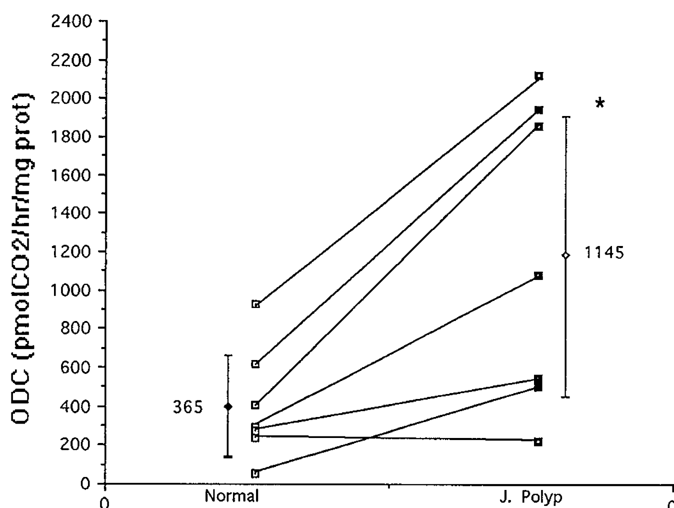


Figure 1. ODC activity in JP and normal mucosa. ODC activity was measured in JP and adjacent normal mucosa as described in Methods. The mean ODC activity was higher in JP compared with adjacent normal mucosa (1145 and 365 pmol of CO_2/h/mg of protein, respectively). A total of seven pairs of tissue samples were investigated, each done in triplicate determinations, and variability was <10%. *$p < 0.05$.

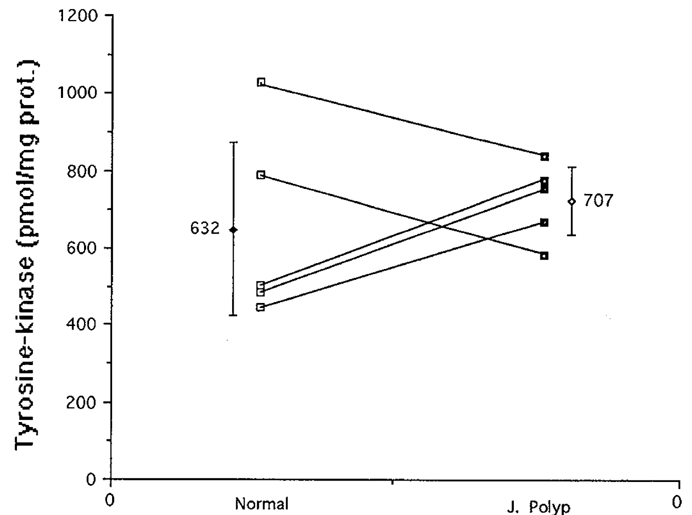


Figure 2. TyK activity in JP and normal mucosa. TyK activity was measured in JP and adjacent normal mucosa as described in Methods. TyK activity in JP tissue was higher than in adjacent normal mucosa in three out of five sample pairs. The mean TyK activity was higher in JP compared with adjacent normal mucosa (707 and 632 pmol/mg of protein, respectively). A total of five pairs of tissue samples were investigated, each done in triplicate determinations, and variability was <10%.

p53 and DNA analysis. p53 expression was determined in a total of eight juvenile polyps. No histologic changes of malignant or premalignant features were found in any polyps. p53 was not detected in any of these polyps but was strongly detected in the adenocarcinoma from an adult colon used as a positive control (data not shown). In addition, DNA ploidy showed no abnormal aneuploid peaks (Fig. 4).

DISCUSSION

Although JP are considered to be benign colonic tumors in children, several reports have suggested that malignant transformation may occur within such polyps (8, 12). This histologic transformation should be a concern to the pediatricians and to the pediatric gastroenterologists who are treating these patients. It is estimated that 3–5% of children with a solitary polyp may develop an adenomatous polyp (35). In a retrospective study Giardiello *et al.* (8) reported an association of JP (solitary/multiple) and the development of colorectal neoplasia in young age. In a recent report (36), a long-term outcome of patients with solitary JP was investigated. In this survey the authors concluded that the relative risk of dying for patients who have previously had a solitary juvenile polyp in comparison with the general population was similar and, thus, these patients do not require future follow-up.

Unlike ODC activity, TyK activity was similar in JP and normal mucosa. Although TyK and ODC activities are interdependent, they may not share the same intracellular regulatory mechanisms. Our data support this hypothesis. It is clear that, until we dissect the influence of ODC on the signal transduction pathways, we cannot assume that TyK must be activated whenever ODC is increased. It may also be argued that the TyK activity in our study represents the sum activity of many families of enzymes, thus will fail to document a specific

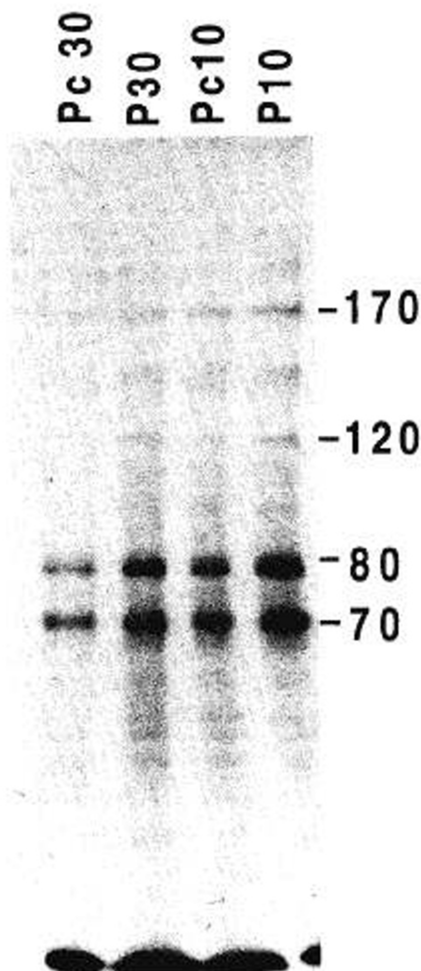
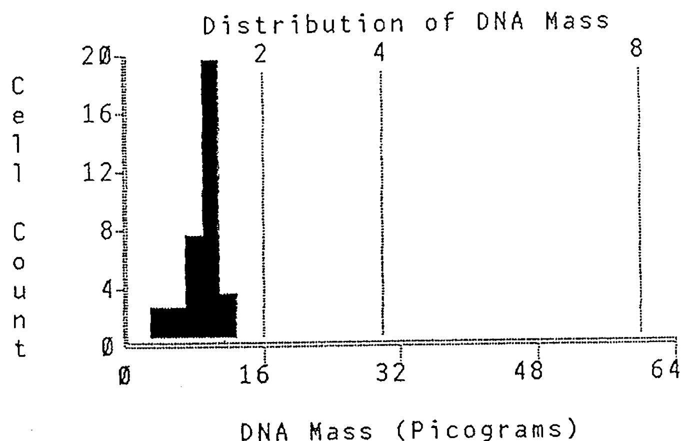


Figure 3. TyK phosphorylation in JP and adjacent normal mucosa. TyK-phosphorylated proteins were measured in 2/3 JP samples which expressed an increased level of TyK activity. Four different bands were identified (70, 80, 120, and 170 kD). These bands showed 2–4-fold higher expression compared with control (P10 *vs* Pc10 and P30 *vs* Pc30, respectively). P, JP mucosa; Pc, normal mucosa.



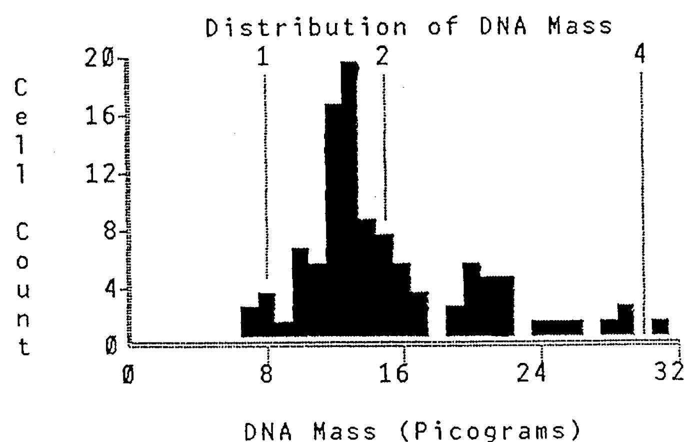


Figure 4. DNA ploidy analysis. DNA ploidy analysis was performed on eight JP histologic blocks as described in Methods. A representative JP sample, which previously expressed higher ODC activity, and TyK activity showed a single DNA peak (*a*) compared with multiple DNA peaks in colon cancer specimen (*b*, positive control).

change in a single enzyme. Increased phosphorylation of specific protein seen in JP may support such a hypothesis. Future determination of a specific TyK enzyme is warranted to resolve this enigma.

Colorectal tumorigenesis is a multistep phenomenon which develops through various intracellular chromosomal alterations. The genetic model for this process suggests that several genetic alterations should occur before cancer will be fully expressed (24, 37). One of these late changes occurs in the p53 gene, located on chromosome 17. ODC and TyK expression are secondary markers for a cellular proliferation index and tumor transformations. In this study, we found that the ODC level but not the TyK level is increased in JP compared with adjacent colon mucosa, but these changes were not associated with increased expression of p53 or changes in aneuploidy score. Moreover, we also did not find any histologic markers of premalignant changes in these polyps, *i.e.* adenomatous changes and metaplasia. We suggest that the increase of enzymatic marker (ODC) in JP reflects its higher stage of proliferation compared with the normal mucosa. Because p53 is a late genetic event in the malignant transformation cascade (24), we speculate that earlier genetic alterations toward malignancy do not occur in the JP. The recent epidemiologic study by Nugent *et al.* (36) supports this hypothesis.

In summary, in this study, we investigated the potential tumorigenicity of solitary/multiple JP in children by measuring ODC and TyK activity, the late malignant transformation markers, p53 expression, and DNA ploidy score. We report a higher activity of ODC but not TyK in JP compared with normal control, probably reflecting their higher stage of proliferation. There was no evidence of either p53 gene expression or aneuploidy in JP. Further studies, looking at earlier changes in the adenoma-carcinoma sequence to rule out premalignant potential, are warranted.

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