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• *CLINICAL RESEARCH* •

Cyclooxygenase-2 promotes angiogenesis by increasing vascular endothelial growth factor and predicts prognosis in gallbladder carcinoma

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Abstract

AIM: To investigate the relationships between the expression of cyclooxygenase-2 (COX-2), vascular endothelial growth factor (VEGF) and the degree of vascularization, clinicopathologic feature, survival time of patients with gallbladder carcinomas.

METHODS: Sixty-four gallbladder carcinoma specimens were evaluated for COX-2, VEGF expression by immunohistochemical methods. Microvessel counts (MVC) were determined using CD_{34}. The relationships between COX-2, VEGF expression, CD_{34}-stained MVC, clinicopathologic features and survival time were analyzed. The correlations between COX-2 and VEGF expression, CD_{34}-stained MVC were also investigated.

RESULTS: COX-2, VEGF immunoreactivity were observed in 71.9% (46/64) and 54.7% (35/64) specimens, respectively. The average MVC in 64 cases of gallbladder carcinoma was 57±14 per high power vision field. The status of MVC was closely correlated with Nevin staging, tumor differentiation and lymph node metastasis (P<0.01, 0.002, and 0.003, 0.000, respectively). Increased VEGF expression was significantly correlated with tumor differentiation (poorly and moderately>well differentiated, P<0.05, P = 0.016). Clinical stages had no relation with the expression of VEGF (P>0.05, P = 0.612). There was a positive correlation between COX-2 expression and clinical stages. The positive rate of COX-2 was higher in cases of Nevin stages S_4-S_5 (81.8%) than in those of Nevin stages S_1-S_3 (50.0%) with a statistical significance (P<0.01, P = 0.009). The expression of COX-2 did not vary with differentiation (P>0.05, P = 0.067). Statistically significant differences were also observed according to lymph node metastasis, COX-2 expression and VEGF expression (P<0.01, 0.000, and 0.001, respectively). There was no relation between VEGF, COX-2 expression, MVC and the age and sex of patients. MVC and VEGF positive rate in the COX-2 positive gallbladder carcinoma tissue was higher than that in the COX-2 negative tissue (P<0.05, 0.000, and 0.032, respectively). Patients with VEGF, COX-2 positive tumors had a significantly shorter survival time than those with negative tumors (P<0.05, 0.004, 0.01, respectively).

CONCLUSION: Augmented tumor neovascularization induced by VEGF may be one of the several effects of COX-2 responsible for poor prognosis of human gallbladder carcinoma. COX-2 inhibitor, either in combination therapy with other agents, or for chemoprevention, may be effective via suppression of angiogenesis in this fatal disease.

Key words: Gallbladder neoplasms; Neovascularization; Cyclooxygenase; Vascular endothelial growth factor

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INTRODUCTION

Most solid tumors require new blood vessels to provide the nutrients necessary for growth and survival[1]. Neovascularization has been shown to correlate directly with the expression level of several independent genes such as vascular endothelial growth factor (VEGF) that promote angiogenesis. Several proteins and enzymatic pathways also take part in the complex process of angiogenesis. Cyclooxygenase (COX-2) that catalyzes the formation of prostaglandin from arachidonic acid contributes to the regulation of angiogenesis by various genes, including fibroblast growth factor-α, VEGF, platelet-derived growth factor and tumor growth factor-β[2]. COX-2 seems to be involved at various steps in the processes of malignant transformation and tumor progression. Investigations have shown that COX-2 overexpression is associated with increased proliferation, reduced apoptosis, and angiogenesis[3-5]. Chemoprevention based on COX-2 has been achieved in a restricted group

of patients and clinical enthusiasm has been expressed for COX-2-based treatment to be extended as chemoprevention in a wider range of patients or as adjuvant therapy of established diseases[6,7].

Expression of COX-2 is responsible for enhanced tumor growth and angiogenesis in various tumors. However, the role of COX-2 in tumor neovascularization of human gallbladder carcinoma is yet to be delineated.

To analyze the relationships between the expression of COX-2, VEGF, microvessel counts (MVC), clinicopathologic features and survival time of patients in gallbladder carcinomas, 64 specimens were evaluated for COX-2, VEGF expression and CD_{34}-stained MVC by immunohistochemical methods.

MATERIALS AND METHODS

Clinical materials

Sixty-four surgically resected and paraffin-embedded samples of gallbladder carcinoma in 1985-2001 were collected. The mean age of patients was 57±5.2 years and the ratio of male to female was 28-36 years. None of them received any preoperative radiochemotherapy. According to Nevin grading criteria[8], the clinical grade distribution was grades S_1, S_2, S_3 in 20 cases (31.2%), grades S_4, S_5 in 44 cases (68.8%). Histologically, all of them were adenocarcinomas, 21 were well-differentiated (32.8%), 17 moderately-differentiated (26.6%) and 26 poorly-differentiated (40.6%) adenocarcinomas. In addition, the lymph node status was known in 40 cases (62.5%), 52 cases were followed-up (81.3%).

Reagents and methods

Rabbit polyclonal antibody to VEGF (A-20) and COX-2 (H-62) was purchased from Santa Cruz Inc. Mouse monoclonal antibody against CD_{34} (QBEnd/10) and SP immunohistochemical reagent box UltraSensitive™ S-P kit (kit 9710) were purchased from Maixin-Bio Co., Fuzhou, China. Four-micrometer-thick sections were cut from formalin-fixed, paraffin-embedded surgical specimens from 64 cases of gallbladder carcinoma.

The expression of VEGF, COX-2, and CD_{34} was assessed by SP immunohistochemical method. Briefly, slides were deparaffinized, rehydrated, and treated with 3% H_2O_2 for 10 min to quench endogenous peroxidase activity. Slides were treated with normal rabbit serum for 20 min to block nonspecific bindings. The sections were incubated overnight at 4 ℃ in moisture chambers with a battery of Abs including anti-COX-2 and anti-VEGF and anti-CD_{34}, and those in the control group were dyed with the first antibody substituted by PBS. Positive control slides were always included in each immunoassaying.

Evaluation of staining

The slides were evaluated under a transmission light microscope by two separate investigators in a blind manner. For COX-2 or VEGF assessment, staining intensity was scored as 0 (negative), 1 (weak), 2 (medium), and 3 (strong). Extent of staining was scored as 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%) according to the percentages of positive staining areas in relation to the whole carcinoma area. The sum of intensity and extent scores was used as the final staining score (0-7) for COX-2[9,10]. Tumors having a final staining score >2 were considered to be positive. For MVC assessment, according to Weidner[11], the areas containing a large number of microvessels or "hot spots" were identified at low magnification (×40) under a light microscope. Then counts were made under 200× field in the densest area of microvessels. The average number of microvessels in 10 fields was recorded as the MVC for each tumor.

Statistical analysis

Statistical analysis was performed by χ^2 test and t test using SPSS software (version 10.0). $P<0.05$ or $P<0.01$ was considered statistically significant.

RESULTS

Expression of VEGF, COX-2 and MVC

VEGF and COX-2 were located at brownish yellow stained granules in cytoplasm (Figure 1). The microvessels were heterogeneously distributed in malignant tissues. The positive expression of CD_{34} was mainly presented at brownish yellow or brownish granules in cytoplasm of vascular endothelial cells (Figure 2). The expression rate of VEGF and COX-2 in 64 cases of gallbladder carcinoma was 54.7% (35/64) and 71.9% (46/64) respectively. MVC was 37 to 82 per high power vision field with an average of 57±14.

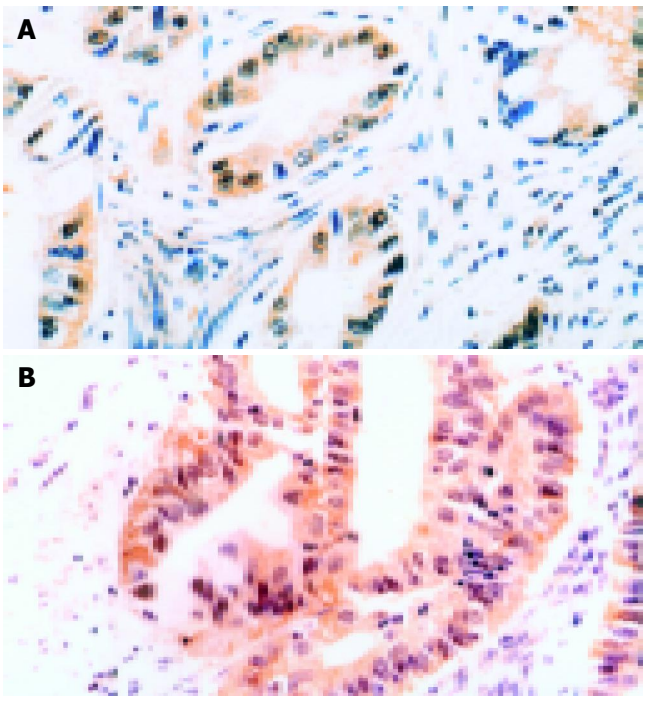

Figure 1 Expressed VEGF (**A**) and COX-2 (**B**) in gallbladder carcinoma (S-P ×400).

Relationship between VEGF and COX-2 expression, MVC and clinicopathological characteristics of gallbladder carcinoma

The status of VEGF, COX-2 and MVC is summarized in

Table 1. The status of MVC was closely correlated with Nevin staging, tumor differentiation and lymph node metastasis. The patients with lymph node metastasis and poorer differentiation or in Nevin stages S_4-S_5 had higher MVC ($P<0.01$, 0.000, 0.003, and 0.002, respectively). Increased VEGF expression was significantly correlated with tumor differentiation (poorly and moderately>well differentiated, $P<0.05$, $P = 0.016$). Clinical stages had no relation with the expression of VEGF ($P>0.05$, $P = 0.612$). A statistically significant correlation was observed between COX-2 expression and clinical stages. The positive rate of COX-2 was higher in cases of Nevin stages S_4-S_5 (81.8%) than in those of Nevin stages S_1-S_3 (50.0%) with a statistical significance ($P<0.01$, $P = 0.009$). The expression of COX-2 did not vary with differentiation ($P>0.05$, $P = 0.067$). Both COX-2 and VEGF levels showed a positive correlation with lymph node metastasis. COX-2 and VEGF levels were significant in cases having lymph node metastasis higher than in those having no lymph node metastasis ($P<0.01$, 0.000, and 0.001, respectively). The VEGF, COX-2 levels and MVC did not correlate with the age and sex of patients ($P>0.05$).

Table 1 COX-2, VEGF, and MVC status in relation to clinicopathological characteristics of patients

Pathological characteristics	*n*	Positive VEGF (%)	Positive COX-2 (%)	MVC
Sex				
Male	28	17 (60.7)	21 (75.0)	56±12
Female	36	18 (50.0)[a]	25 (69.4)[a]	58±16[a]
Age (yr)				
⩾60	30	16 (53.3)	22 (73.3)	54±13
<60	34	19 (55.9)[a]	24 (70.6)[a]	60±15[a]
Clinical stage				
S_1, S_2, S_3	20	10 (50.0)	10 (50.0)	49±15
S_4, S_5	44	25 (56.8)[a]	36 (81.8)[b]	64±12[b]
Degree of differentiation				
Well differentiated	21	7 (33.3)	12 (57.1)	50±14
Moderately and poorly differentiated	43	28 (65.1)[c]	34 (79.1)[a]	61±13[b]
Lymph node metastasis				
Positive	40	28 (70.0)	35 (87.5)	65±12
Negative	24	7 (29.2)[b]	11 (45.8)[b]	45±7[b]

[a]$P<0.05$, [b]$P<0.01$, [c]$P<0.05$ *vs* control.

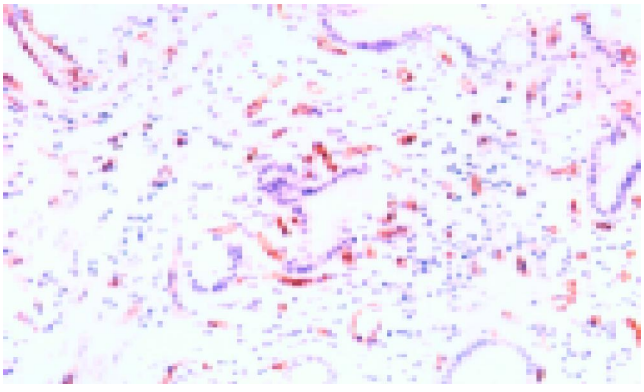

Figure 2 Immunohistochemical staining of CD_{34} in gallbladder carcinoma tissue (S-P ×200).

Correlation between MVC, VEGF and COX-2 expression rate

As is shown in Table 2, MVC (62±13) in COX-2 positive gallbladder carcinoma tissue was higher than that (45±10) in COX-2 negative tissue ($t = 5.424$, $P<0.01$, $P = 0.000$). VEGF positive rate (45.3%, 29/46) in COX-2 positive gallbladder carcinoma tissue was higher than that (33.3%, 6/18) in COX-2 negative tissue ($\chi^2 = 4.608$, $P<0.05$, $P = 0.032$), suggesting that the expression of COX-2 was related to increased VEGF and tumor angiogenesis.

Table 2 Correlation between MVC, VEGF and COX-2 expression rate

Characteristics	VEGF expression		MVC
	Positive	Negative	
COX-2 expression			
Positive	29	17	62±13
Negative	6	12[a]	45±10[b]

[a]$P<0.05$, [b]$P<0.01$ *vs* control.

Relationship between expressions of COX-2 and VEGF and prognosis of gallbladder carcinoma

Statistically significant differences in survival were observed according to the expressions of COX-2 and VEGF. As is shown in Table 3, the 3-year survival rate of patients with COX-2 or VEGF positive tumors was significantly less than that of patients with COX-2 or VEGF negative tumors ($\chi^2 = 6.656$ and 8.276, $P = 0.01$, $P = 0.004$, respectively).

Table 3 Relationship between expressions of COX-2 and VEGF and prognosis of gallbladder carcinoma

Characteristics	*n*	Survival time (yr)		Rate of 3-yr survival (%)
		<3	>3	
VEGF expression				
Positive	35	25	10	28.6
Negative	17	5	12	70.6[b]
COX-2 expression				
Positive	38	26	12	31.6
Negative	14	4	10	71.4[a]

[a]$P<0.05$, [b]$P<0.01$ *vs* control.

DISCUSSION

In 1971, Folkman *et al*[12], initiated a new field of research about tumor angiogenesis and found that several factors take part in the process of angiogenesis. The extent of angiogenesis appears to be an important prognostic factor for many solid tumors, and MVC correlates with stage and survival of many cancers[13]. Our study showed that the status of MVC was closely correlated with Nevin staging, tumor differentiation and lymph node metastasis. The patients with lymph node metastasis, poorer differentiation or in later stage had higher level of MVC in gallbladder carcinomas, indicating that MVC might be one of the most important parameters in predicting the prognosis of gallbladder carcinoma.

COX-2 is a key enzyme in prostaglandin biosynthesis. Recent evidence indicates that COX-2 modulates angiogenesis not only by increasing production of prostaglandins but also by augmenting the release of angiogenic peptides such as VEGF, basic fibroblast growth factor (bFGF), and nitric oxide from tumor cells[14]. COX-2 is expressed in human tumor neovasculature and neoplastic cells[15]. A large body of evidence suggests that COX-2 is up-regulated in carcinoma tissues and plays roles in promoting neovascularization, cell proliferation, growth and metastasis of carcinoma cells[16]. We found that some pathological variables such as Nevin staging and metastasis had a significant correlation with high COX-2 expression. Our study demonstrated that the intensity of COX-2 expression correlated with metastasis lymph nodes. Tsujii *et al*[17], found that induction of COX-2 expression in colon cancer cells activates membrane-type metalloproteinases, which may explain the increased invasiveness and greater metastatic potential of COX-2-expressing tumors. We also found that COX-2 expression had a significant impact on patient survival. Patients with positive COX-2 tumors survived a shorter time than those with negative COX-2 tumors. A similar survival time of patients with negative COX-2 tumors has been reported by Sheehan *et al*[18]. Interestingly, overexpression of COX-2 was more common in moderately and poorly differentiated gallbladder carcinoma as compared with well-differentiated carcinoma, but without statistical significance. Results from our research suggest that tumor invasiveness and metastasis are responsible for the worse prognosis of patients bearing COX-2 positive tumors.

The development, invasion, metastasis, staging of human neoplasm have been shown to correlate directly with the expression level of several angiogenesis regulating factors such as VEGF and bFGF[19]. Studies have also shown that VEGF is significant in the angiogenic process as a prognostic factor[20]. The data of our studies indicate that VEGF expression is strongly associated with poorer differentiation, positive lymph node metastasis and worse prognosis of gallbladder carcinoma, suggesting that VEGF plays a significant role in tumor invasion and metastasis.

Experimental researches on COX-2 have shown that COX-2 stimulates angiogenesis in colon cancer cell lines by inducing angiogenic factors such as VEGF and bFGF[21]. Jones *et al*[22], also demonstrated that COX-2 inhibitors suppress angiogenesis by affecting three endothelial cell lines and that COX-2 is important for the regulation of angiogenesis in endothelial cells. Based on these findings, we hypothesize that tumor-derived VEGF promotes angiogenesis by inducing the production of COX-2. This hypothesis is supported by our study finding that COX-2 expression was associated with VEGF expression in gallbladder carcinoma and had a direct correlation with tumor MVC. These results suggest that production of VEGF induced by COX-2 is essential to neovascularization. Increased tumor vascularity induced by COX-2 might be a significant cause for the growth, invasion, metastasis and worse prognosis of gallbladder carcinoma. These new findings suggest that COX-2 expression is associated with augmentation of neovascularization. Carcinoma of the gallbladder is the most common and highly malignant tumor of the biliary tract with a poor 5-year survival rate. Whether a similar correlation exists between COX-2 expression and angiogenesis in gallbladder carcinoma is not clear.

Selective COX-2 inhibitors such as nonsteroidal anti-inflammatory drugs (NSAIDs) have drawn much attention. Clinical trials indicate that NSAIDs exhibit a significant antitumor effect on animal models[23,24] and colorectal carcinoma in humans[25,26].

Though the exact mechanism underlying neovascularization is not clear, COX-2 overexpression causes increased VEGF and angiogenesis, which may be the mechanisms underlying the contribution of COX-2 to the angiogenesis of gallbladder carcinoma. In addition, COX-2 inhibition in the presence of growth factor VEGF should be a very significant therapy for the fatal disease.

REFERENCES

1 **Holmgren L.** Antiangiogenis restricted tumor dormancy. *Cancer Metastasis Rev* 1996; **15**: 241–245
2 **Bakhle YS.** COX-2 and cancer: a new approach to an old problem. *Br J Pharmacol* 2001; **134**: 1137–1150
3 Chan G, Boyle JO, Yang EK, Zhang F, Sacks PG, Shah JP, Edelstein D, Soslow RA, Koki AT, Woerner BM, Masferrer JL, Dannenberg AJ. Cyclooxygenase-2 expression is up-regulated in squamous cell carcinoma of the head and neck. *Cancer Res* 1999; **59**: 991-994
4 **Shen ZX,** Cao G, Sun J. Expression of COX-2 mRNA in colorectal carcinomas. *Shijie Huaren Xiaohua Zazhi* 2001; **9**: 1082-1084
5 **Wu QM,** Li SB, Wang Q, Wang DH, Li XB, Liu CZ. The expression of COX-2 in esophageal carcinoma and its relation to clinicoptahologic characteristics. *Shijie Huaren Xiaohua Zazhi* 2001; **9**: 11-14
6 **Steinbach G,** Lynch PM, Phillips RK, Wallace MH, Hawk E, Gordon GB, Wakabayashi N, Saunders B, Shen Y, Fujimura T, Su LK, Levin B, Godio L, Patterson S, Rodriguez-Bigas MA, Jester SL, King KL, Schumacher M, Abbruzzese J, DuBois RN, Hittelman WN, Zimmerman S, Sherman JW, Kelloff G. The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. *N Engl J Med* 2000; **342**: 1946–1952
7 **Vainio H,** Morgan G. Cyclo-oxygenase 2 and breast cancer prevention. Non-steroidal anti-inflammatory agents are worth testing in breast cancer. *BMJ* 1998; **317**: 828
8 **Nevin JE,** Moran TJ, Kay S, King R. Carcinoma of the gallbladder, staging, treatment and prognosis. *Cancer* 1976; **37**: 141-148
9 **Zimmermann KC,** Sarbia M, Weber AA, Borchard F, Gabbert HE, Schrör K. Cyclooxygenase-2 expression in human esophageal carcinoma. *Cancer Res* 1999; **59**: 198-204
10 **Folkman J.** What is the evidence that tumors are angiogenesis dependent? *J Natl Cancer Inst* 1990; **82**: 4-6
11 **Weidner N,** Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis-correlation in invasive breast carcinoma. *N Engl J Med* 1991; **324**: 1-8
12 **Folkman J,** Klagsbrun M. Angiogenic factors. *Science* 1987; **235**: 442-447
13 **Jia L,** Chen TX, Sun JW, Na ZM, Zhang HH. Relationship between microvessel density and proliferating cell nuclear antigen and prognosis in colorectal cancer. *Shijie Huaren Xiaohua Zazhi* 2000; **8**: 74-76
14 **Eberhart CE,** Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN. Up-regulation of cyclooxygenase-2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology* 1994; **107**: 1183-1188
15 **Sano H,** Kawahito Y, Wilder RL, Hashiramoto A, Mukai S, Asai K, Kimura S, Kato H, Kondo M, Hla T. Expression of cyclooxygenase-1 and -2 in human colorectal cancer. *Cancer*

Res 1995; **55**: 3785-3789

16 **Seki S,** Kitada T, Sakaguchi H, Hirohashi K, Kinoshita H. Cyclooxygenase-2 expression in the adenoma-carcinoma sequence of human gallbladder. *Am J Gastroenterol* 2002; **97**: 2146-2147

17 **Tsujii M,** Kawano S, DuBois RN. Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. *Proc Natl Acad Sci USA* 1997; **94**: 3336-3340

18 **Sheehan KM,** Sheahan K, O'Donoghue DP, MacSweeney F, Conroy RM, Fitzgerald DJ, Murray FE. The relationship between cyclooxygenase-2 expression and colorectal cancer. *JAMA* 1999; **282**: 1254-1257

19 **Li Z,** Shimada Y, Uchida S, Maeda M, Kawabe A, Mori A, Itami A, Kano M, Watanabe G, Imamura M. TGF-alpha as well as VEGF, PD-ECGF and bFGF contribute to angiogenesis of esophageal squamous cell carcinoma. *Int J Oncol* 2000; **17**: 453-460

20 **Giatromanolaki A,** Koukourakis MI, Kakolyris S, Turley H, O'Byrne K, Scott PA, Pezzella F, Georgoulias V, Harris AL, Gatter KC. Vascular endothelial growth factor, wild-type p53, and angiogenesis in early operable non-small cell lung cancer. *Clin Cancer Res* 1998; **4**: 3017-3024

21 **Tsujii M,** Kawano S, Tsuji S, Sawaoka H, Hori M, DuBois RN. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. *Cell* 1998; **93**: 705-716

22 **Jones MK,** Wang H, Peskar BM, Levin E, Itani RM, Sarfeh IJ, Tarnawski AS. Inhibition of angiogenesis by nonsteroidal anti-inflammatory drugs: insight into mechanisms and implications for cancer growth and ulcer healing. *Nat Med* 1999; **5**: 1418-1423

23 **Piazza GA,** Alberts DS, Hixson LJ, Paranka NS, Li H, Finn T, Bogert C, Guillen JM, Brendel K, Gross PH, Sperl G, Ritchie J, Burt RW, Ellsworth L, Ahnen DJ, Pamukcu R. Sulindac sulfone inhibits azoxymethane-induced colon carcinogenesis in rats without reducing prostaglandin levels. *Cancer Res* 1997; **57**: 2909-2915

24 **Boolbol SK,** Dannenberg AJ, Chadburn A, Martucci C, Guo XJ, Ramonetti JT, Abreu-Goris M, Newmark HL, Lipkin ML, DeCosse JJ, Bertagnolli MM. Cyclooxygenase-2 overexpression and tumor formation are blocked by sulindac in a murine model of familial adenomatous polyposis. *Cancer Res* 1996; **56**: 2556-2560

25 **Giardiello FM,** Hamilton SR, Krush AJ, Piantadosi S, Hylind LM, Celano P, Booker SV, Robinson CR, Offerhaus GJ. Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. *N Engl J Med* 1993; **328**: 1313-1316

26 **Matsuhashi N,** Nakajima A, Fukushima Y, Yazaki Y, Oka T. Effects of sulindac on sporadic colorectal adenomatous polyps. *Gut* 1997; **40**: 344-349

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