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*Original Article*

# The effect of prediagnostic aspirin use on the prognosis of stage III colorectal cancer

Bun Kim[1,2], Soo Jung Park[3], Sung Pil Hong[3], Jae Hee Cheon[3], Won Ho Kim[3], Tae Il Kim[3]

*[1]Department of Medicine, Graduate School, Yonsei University College of Medicine, Seoul, Korea; [2]Center for Cancer Prevention and Detection, National Cancer Center, Goyang, Korea; [3]Department of Internal Medicine, Division of Gastroenterology, Yonsei University College of Medicine, Seoul, Korea*

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**Abstract:** Background: Many studies have suggested that the regular use of non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin, has a protective effect and survival benefit on colorectal cancer (CRC). However, recent data suggest that CRCs have different responses to NSAIDs depending on the timing of NSAID initiation, duration of NSAID use, and molecular characteristics of the tumor. The aim of this study was to evaluate the effect of long-term prediagnostic aspirin use on the prognosis of stage III CRC. Methods: From 2007 to 2009, patients who were diagnosed with stage III CRC were recruited, and their medical records were retrospectively analyzed. Patients were divided into prediagnostic aspirin users (who used aspirin for more than three months continuously before CRC diagnosis) and non-users (who did not use of aspirin and NSAIDs). The two groups were compared in terms of recurrence, cancer-specific mortality, disease-free survival (DFS), and cancer-specific survival. In an experimental study, three CRC cell lines (Caco2, SW480, and DLD-1) were pretreated with aspirin (1 mM) for four days or 28 days to make aspirin-resistant cells, treated with 5-fluorouracil (5-FU; 2 μM), and apoptosis was measured with flow cytometry using Annexin-V and propidium iodide double staining. Results: Compared with the aspirin non-users (N=565), the prediagnostic aspirin users (N=121) were not different in terms of baseline characteristics including tumor characteristics, except for comorbidities and diabetes medication and statin use, which were higher in the prediagnostic aspirin users. Recurrence and cancer-specific mortality in stage III CRC were significantly higher in prediagnostic aspirin users than non-users (46.7% vs. 32.3%, *P*=0.003 and 32.2% vs. 19.8%, *P*=0.003, respectively). Survival analysis using Cox proportional hazards modeling demonstrated that DFS was significantly worse in prediagnostic aspirin users than non-users (HR, 1.525 (1.018-2.286); *P*=0.041). In cell line experiments, long-term aspirin pretreatment induced an increase in 5-FU-induced apoptosis in SW480 cells compared with control treatment without aspirin pretreatment. However, Caco2 cells showed a significant decrease of apoptosis in the same experiments and no change in DLD1 cells. Conclusion: Prediagnostic long-term aspirin use in stage III CRC could be a negative prognostic factor depending on the characteristics of the CRC.

**Keywords:** Aspirin, colorectal cancer, prognosis

## Introduction

Colorectal cancer (CRC) is one of the most common malignancies in both Asian and Western countries [1, 2]. In proportion to the increasing CRC incidence, the health care costs of patients with CRC are also increasingly accelerating due to the development of new treatment regimens. To reduce cancer recurrence and improve survival in patients with CRC, the use of tumor-suppressing substances as adjuncts to conventional chemotherapy has been a focus of investigation. The results suggest that these substances could potentially be used for the prevention of recurrence. Many observational and randomized, controlled studies have suggested a protective effect of regular aspirin use against colorectal neoplasms [3-6]. Favorable outcomes associated with aspirin use after the diagnosis of colorectal cancer suggest that aspirin may be a promising agent for adjuvant therapy [7-10]. Therefore, in this respect, non-steroidal anti-inflammatory drugs (NSAIDs) including aspirin are inexpensive and well-tolerated drugs that may prove to be an effective agent in preventing CRC recurrence, although

this benefit should be estimated relative to side effects including gastrointestinal and hemorrhagic toxicity.

However, in the studies where the effects of NSAIDs or aspirin use on survival after colorectal cancer diagnosis were evaluated, a greater reduction in colorectal cancer mortality was found in patients who used aspirin only after diagnosis [7, 10, 11]. Furthermore, a large prospective cohort study about aspirin use and survival in non-advanced colorectal cancer showed that compared with non-users, participants who regularly used aspirin after diagnosis had a better prognosis [9, 12]. In contrast to aspirin use after diagnosis, long-term use of aspirin or NSAID prior to cancer diagnosis did not show consistent results in cancer-specific or overall survival [7, 11].

Through various molecular mechanisms such as inhibition of cyclooxygenase and the NF-κB signaling pathway and activation of apoptotic signals [13], NSAID use is known to correlate with prevention of CRC development. However, nothing is known about the behavior of CRC that has developed under long-term NSAID exposure by evading the tumor suppressive effect of NSAIDs.

We hypothesized that CRC development, even in long-term NSAID users, might induce different tumor characteristics such as poor response to adjuvant chemotherapy and more recurrence compared with CRC in NSAID non-users.

Therefore, we suggest the possibility that tumors that initially developed despite long-term exposure to aspirin may be less susceptible to any potential effects of adjuvant chemotherapy on tumor recurrence after surgery due to changed tumor biology, and they may have a different prognosis compared to tumors that have never been exposed to aspirin. However, up to now, there has been little data about prognosis in prediagnostic aspirin users comparing to aspirin non-users, particularly in the context of stage III or IV CRC.

In focusing on these points to maximize the effect on tumor recurrence, we investigated the effect of long-term prediagnostic aspirin-use on tumor recurrence in stage III CRC patients and demonstrated the possible effects of prediagnostic long-term aspirin use in an *in vitro* experimental model.

## Materials and methods

### *Patients*

From January 2007 to December 2009, 925 patients who were diagnosed with stage III CRC at Severance Hospital were recruited. A total of 239 cases were excluded due to: 1) age less than 20 years at diagnosis (N=2), 2) loss to follow-up within one month without any tumor response evaluation at Severance Hospital (N=98), 3) coexistence of other malignancies within five years prior to diagnosis of CRC (N=55), 4) pathology other than adenocarcinoma (N=3), 5) taking NSAIDs only after diagnosis with CRC (N=78), and 6) use of non-selective NSAIDs or COX-2 selective NSAIDs only (N=3). Ultimately, a total of 686 patients were included in the study. They were divided into two groups: prediagnostic aspirin users (N=121) who used aspirin for more than three months continuously prior to CRC diagnosis and non-users (N=565) who did not use aspirin, non-selective NSAIDs, or COX-2 selective NSAIDs.

### *Study design*

A retrospective study was conducted based on medical records. Patient-related factors such as age, sex, body mass index (BMI), smoking history, alcohol history, family history, Eastern Cooperative Oncology Group (ECOG) performance status, and comorbidities were investigated. Also, tumor-related factors such as T stage, N stage, primary site (colon or rectum), initial carcinoembryonic antigen (CEA) level, pathology (adenocarcinoma or mucinous malignancy), pathologic differentiation, and microsatellite instability (MSI) were investigated. In addition, first-course treatment and type of chemotherapy were surveyed. Aspirin-related factors such as type, dose, duration of aspirin usage, and timing in relation to colorectal cancer diagnosis were investigated, and other medications (metformin, thiazolidinediones, insulin, and statins) that may affect colorectal cancer prognosis were also surveyed.

For evaluation of the prognosis of colorectal cancer, we used recurrence rate, cancer-specific mortality rate, disease-free survival (DFS), and cancer-specific survival.

### *In vitro cell line experiments*

*Cell lines and aspirin treatment:* The human CRC cell lines (Caco-2, SW480, and DLD1)

**Table 1.** Comparison of baseline characteristics between aspirin non-users and prediagnostic aspirin users with stage III colorectal cancer

| | Aspirin non-users N=565 | Prediagnostic aspirin users N=121 | *P* value |
|---|---|---|---|
| Age (mean ± SD, years) | 57.55 ± 11.75 | 66.30 ± 9.26 | <0.001 |
| Sex, N (%) | | | |
| M/F | 263 (40.8) | 0.070 | 0.070 |
| BMI (≥25 kg/m$^2$) | 134 (23.7) | 32 (26.4) | 0.525 |
| Smoker, N (%) | | | |
| Never/former/current | 347 (61.2)/107 (18.9)/112 (19.8) | 69 (57.0)/31 (25.6)/21 (17.4) | 0.244 |
| Alcohol, N (%) | | | |
| Never/<1 bottle/day/>1 bottle/day | 307 (54.3)/213 (37.7)/45 (8.0) | 62 (51.2)/50 (41.3)/9 (7.4) | 0.758 |
| Colorectal ca. familial Hx, N (%) | 42 (7.4) | 6 (5.0) | 0.333 |
| ECOG, N (%) | | | |
| 0-1/2 | 118 (97.6)/3 (2.5) | 118 (97.6)/3 (2.5) | 0.206 |
| Comorbidity, N (%) | | | |
| HTN | 142 (25.1) | 103 (85.1) | <0.001 |
| DM | 79 (13.8) | 45 (37.2) | <0.001 |
| CAD | 1 (0.2) | 23 (19.0) | <0.001 |
| CVA | 9 (1.6) | 20 (16.5) | <0.001 |
| Renal failure | 10 (1.8) | 7 (5.8) | 0.019 |
| Hepatitis | 29 (5.1) | 5 (4.1) | 0.643 |
| Hx of TBc | 42 (7.4) | 10 (8.3) | 0.754 |
| Aspirin use duration (mean ± SD, months) | - | 83.26 ± 56.24 | |
| Prediagnostic duration (mean ± SD, months) | - | 61.66 ± 48.12 | |
| Post-diagnostic duration (mean ± SD, months) | - | 21.60 ± 26.18 | |
| Medication, N (%) | | | |
| Metformin | 43(7.6) | 24 (19.8) | <0.001 |
| TZD | 4 (0.7) | 6 (5.0) | 0.003 |
| Insulin | 4 (0.7) | 7 (5.8) | 0.001 |
| Statin | 28 (5.0) | 27 (22.3) | <0.001 |

Variables are expressed as mean ± SD or n (%). Abbreviations: NSAIDs, non-steroidal anti-inflammatory drugs; BMI, body mass index; SD, standard deviation; Hx, medical history; ECOG, eastern cooperative oncology group; HTN, hypertension; DM, diabetes mellitus; CAD, coronary artery disease; CVA, cerebrovascular accident; TBc, tuberculosis; TZD, thiazolidinediones.

were purchased from American Type Culture Collection (ATCC; Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (Hyclone, Logan, UT), 100 units/ml penicillin, 100 mg/ml streptomycin (Invitrogen, Carlsbad, CA), and 2 mM L-glutamine (Life Technologies, Carlsbad, CA). All cells were maintained in a 5% $CO_2$ incubator at 37°C. The media were changed every two days, and the cells were separated via trypsinization using trypsin/EDTA when they reached subconfluence.

Aspirin was purchased from Sigma (St. Louis, MO), dissolved in dimethyl sulfoxide (DMSO) as a 1 M stock solution, and stored at 4°C in the dark. The stock solution was diluted to the appropriate concentrations with medium immediately before use. The concentration of aspirin (1 mM) was chosen based on the intracellular concentrations achieved after oral administration [13]. Each cell line was exposed to 1 mM of aspirin for 48 h, and the medium with 1 mM aspirin was changed regularly for 8 days or 28 days.

*Measurement of 5-FU-induced apoptosis using flow cytometric analysis:* Three CRC cell lines (Caco2, SW480 and DLD-1) were pretreated with aspirin (1 mM) for 8 days or 28 days, and then treated with 5-FU (2 μM) for 24 hours. Apoptosis was measured by flow cytometry using Annexin-V and propidium iodide double staining. Annexin V staining was done using an Annexin V-FITC Apoptosis kit (BD Pharmingen, Franklin Lakes, NJ, USA), according to the manufacturer's protocol. Annexin V- and propidium iodide-positive cells were measured at a fluo-

**Table 2.** Comparison of tumor characteristics and treatment modality between aspirin non-users and prediagnostic aspirin users with stage III colorectal cancer

| | Aspirin non-users N=565 | Prediagnostic aspirin users N=121 | *P* value |
|---|---|---|---|
| Initial stage, N (%) | | | 0.727 |
| Stage IIIA | 52 (9.2) | 11 (8.9) | |
| Stage IIIB | 376 (66.4) | 86 (69.9) | |
| Stage IIIC | 138 (24.4) | 26 (21.1) | |
| T stage, N (%) | | | 0.862 |
| T1/T2/T3/T4a/T4b | 16 (2.8)/42 (7.4)/386 (68.3)/119 (21.1)/2 (0.4) | 5 (4.1)/9 (7.4)/82 (67.8) /25 (20.7)/0 (0.0) | |
| N stage, N (%) | | | 0.903 |
| N1a/N1b/N1c/N2a/N2b | 224 (39.6)/139 (24.6)/1 (0.2)/127 (22.5)/74 (13.1) | 39 (32.2)/46 (38.0)/0 (0.0)/17 (14.0)/19 (15.7) | |
| Site, N (%) | | | 0.350 |
| Proximal colon/Distal colon/Rectum | 113 (20.0)/194 (34.3)/258 (45.7) | 31 (25.6)/41 (33.9)/49 (40.5) | |
| Initial CEA (mean ± SD, g/) | 12.76 ± 69.70 | 7.14 ± 13.26 | 0.383 |
| Pathology, N (%) | | | |
| Adenocarcinoma/mucous | 540 (95.6)/25 (4.4) | 117 (96.7)/4 (3.3) | 0.579 |
| Histologic differentiation, N (%) (N=612, N=118) | | | 0.294 |
| WD/MD/PD | 49 (9.1)/458 (85.4)/29 (5.4) | 16 (13.8)/93 (80.2)/7 (6.0) | |
| MSI, N (%) (N=338, N=14) | | | 0.106 |
| MSS/MSI-L/MSI-H | 266 (89.9)/20 (6.8)/10 (3.4) | 64 (92.8)/1 (1.4)/4 (5.8) | 0.322 |
| Surgical therapy, N (%) | 564 (99.8) | 120 (99.2) | |
| RTx, N (%) | | | 0.253 |
| None/neoCCRTx/adjuCCRTx/RTx | 379 (67.1)/86 (15.2)/99 (17.5)/1 (0.2) | 90 (74.4)/13 (10.7)/17 (14.0)/1(0.8) | |
| CTx, N (%) | | | 0.044 |
| None/adju/neo | 20 (3.5)/471 (83.4)/74 (13.1) | 9 (7.4)/103 (85.1)/9 (7.4) | |
| Type of CTx, N (%) | | | 0.293 |
| FOLFOX/FL/Xeloda/TS-1/other | 297 (54.5)/189 (34.7)/43 (7.9)/7 (1.3)/9 (1.7) | 65 (58.0)/31 (27.2)/13 (11.6)/1 (0.9)/2 (1.8) | |

Variables are expressed as mean ± SD or n (%). Abbreviations: NSAIDs, non-steroidal anti-inflammatory drugs; CEA, carcinoembryonic antigen; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; MSI, microsatellite instability; MSS, microsatellite stable; MSI-H, microsatellite instability-high; MSI-L, microsatellite instability-low; RTx, radiotherapy; CCRTx, concurrent chemoradiation therapy; CTx, chemotherapy; FOLFOX, fluorouracil, leucovorin, and oxaliplatin; FL, fluorouracil and leucovorin; TS-1, oral fluoropyrimidine.

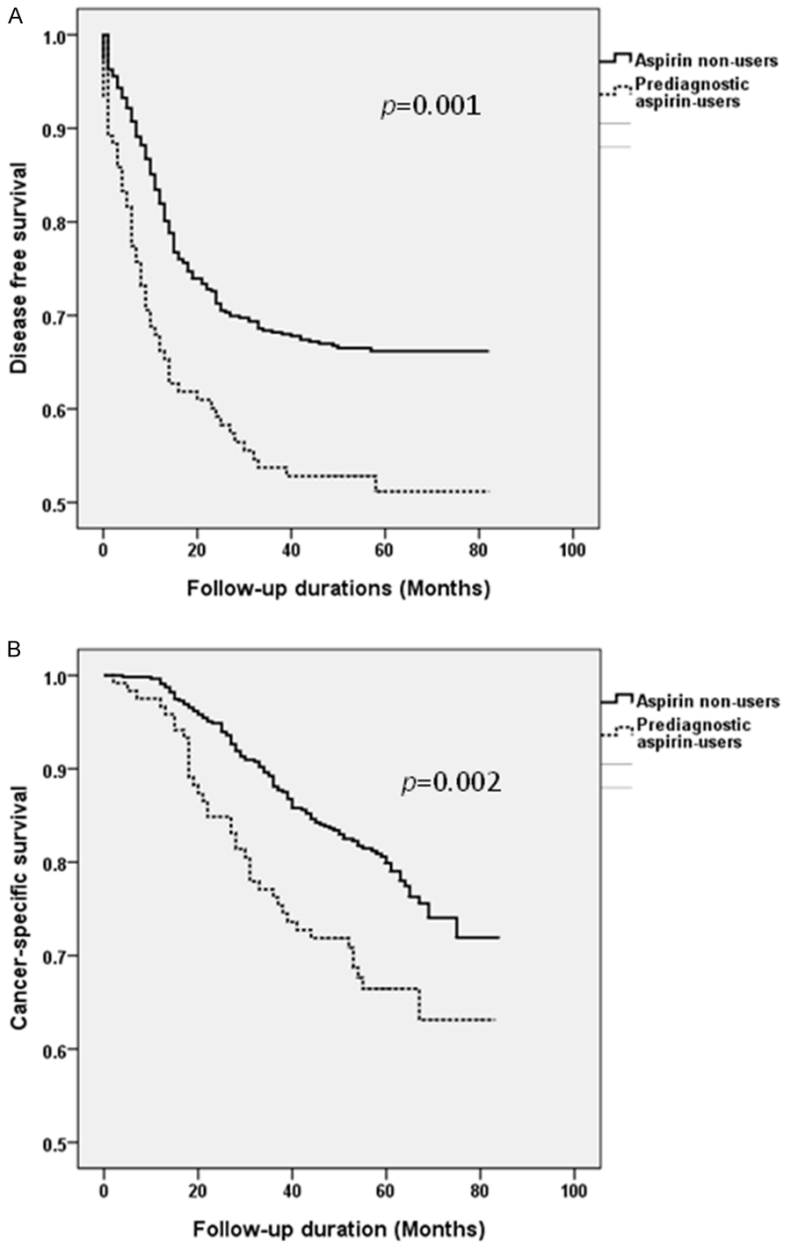


**Figure 1.** Disease-free survival (DFS) and cancer-specific survival of prediagnostic aspirin users and non-users among patients with stage III colorectal cancer. A. DFS was significantly shorter in prediagnostic aspirin users than in non-users (*P*=0.002). B. Cancer-specific survival was also decreased in the prediagnostic aspirin user group compared with the non-user group (*P*=0.003). Kaplan-Meier curves were used to stratify prediagnostic aspirin use.

rescence intensity of $1\times10^4$ cells using the FACSCalibur system (Becton & Dickinson, San Jose, CA, USA). For evaluation of apoptosis, FITC Annexin V-positive and PI-negative (early apoptosis) cells and FITC Annexin V- and PI-positive (end stage apoptosis and death) cells were calculated together.

*Statistical analysis*

Continuous variables were expressed as mean ± standard deviation (SD). Baseline characteristics of the aspirin non-users and prediagnostic aspirin users were compared by Student's t-test for continuous variables and Chi-square test or Fisher's exact test for categorical variables. The logistic regression method was used for multivariate analysis. The Kaplan-Meier method was used to estimate the distribution of the time from diagnosis to DFS and cancer-specific survival according to whether or not aspirin was used. Cox proportional hazards modeling was used to control for multiple risk factors that have been shown to influence colorectal cancer prognosis to compute 95% confidence intervals (CIs). Results were considered to be statistically significant with $P<0.05$. All statistical analyses were performed using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA).

## Results

*Baseline characteristics*

The baseline characteristics of the aspirin non-users and the prediagnostic aspirin users are summarized in **Table 1**. The mean age was significantly higher in prediagnostic aspirin users than non-users (66.34 ± 9.20 vs. 57.95 ± 11.62, $P<0.001$). Comparing the two groups, other patient-related factors (sex, BMI, smoking history, alcohol history, family history of CRC, ECOG performance status, and comorbidities) also were not significantly different. However, comorbidity

**Table 3.** Cox regression analysis of factors related with recurrence and cancer-specific mortality

| | | Disease-free survival | | Cancer-specific survival | |
|---|---|---|---|---|---|
| | | Hazard ratio (95% CI) | *P* value | Hazard ratio (95% CI) | *P* value |
| Age (mean ± SD, years) | | - | 0.591 | - | 0.073 |
| Sex | | - | 0.541 | - | 0.590 |
| Comorbidity | HTN | - | 0.302 | - | 0.213 |
| | DM | - | 0.106 | - | 0.436 |
| | CAD | - | 0.366 | - | 0.784 |
| | CVA | - | 0.561 | - | 0.251 |
| | Renal failure | - | 0.765 | - | 0.574 |
| Pre-diagnosis aspirin use (vs. non-use) | | 1.525 (1.018-2.286) | 0.041 | - | 0.170 |
| Metformin | | - | 0.781 | - | 0.478 |
| TZD | | - | 0.326 | - | 0.875 |
| Insulin | | - | 0.722 | - | 0.067 |
| Statin (vs. non-user) | | 0.488 (0.281-0.848) | 0.011 | 0.480 (0.239-0.966) | 0.040 |
| Initial stage | | | | | |
| IIIB (vs. IIIA) | | 3.248 (1.498-7.040) | 0.003 | - | 0.112 |
| IIIC (vs. IIIA) | | 6.372 (2.890-14.051) | <0.001 | 6.875 (2.460-19.216) | <0.001 |
| Pathologic differentiation | | | | | |
| MD (vs. WD) | | - | 0.114 | - | 0.273 |
| PD (vs. WD) | | 3.574 (1.758-7.265) | <0.001 | 6.006 (2.469-14.607) | <0.001 |
| Cancer primary site | | | | | |
| Rectum (vs. colon) | | 1.482 (1.122-1.958) | 0.006 | - | 0.616 |
| CTx (vs. non-CTx) | | 0.462 (0.232-0.921) | 0.028 | - | 0.064 |

Abbreviations: CI, confidence interval; HTN, hypertension; DM, diabetes mellitus; CAD, coronary artery disease; CVA, cerebrovascular accident; TZD, thiazolidinediones; WD, well differentiated; PD, poorly differentiated; CTx, chemotherapy. Cox proportional hazard model adjusted for age, sex, comorbidity, pre-diagnosis aspirin use, medication, cancer site, initial stage and pathological differentiation.

prevalence, including hypertension, diabetes mellitus, coronary artery disease, cerebrovascular accident, and renal failure was higher in prediagnostic aspirin users than in non-users (*P*<0.001, *P*<0.001, *P*<0.001, *P*<0.001, and *P*=0.020, respectively). The baseline medications, including metformin, TZD, insulin, and statins, were used more frequently in prediagnostic aspirin users due to preexisting underlying diseases (*P*<0.001, *P*=0.003, *P*=0.001, and *P*<0.001, respectively). The average duration of aspirin use in the prediagnostic aspirin users was 82.74 ± 56.02 months.

The tumor characteristics between the two groups were not significantly different, including initial stages, which were determined based on AJCC (American Joint Committee on Cancer) 7$^{th}$ edition, primary site, pathology, pathologic differentiation, CEA at diagnosis, and MSI status. The treatment modality among the three groups was also not significantly different in terms of surgery, radiation therapy, and chemotherapy (**Table 2**).

*Prognostic differences between prediagnostic aspirin users and non-users with stage III colorectal cancer*

Long-term prediagnostic aspirin use affected the prognosis of stage III colorectal cancer in both the univariate analysis and multivariate analysis. Between the two groups, the prediagnostic aspirin user group showed a higher rate of recurrence and cancer-specific mortality than non-users (recurrence: 46.7% vs. 32.3%, *P*=0.003 and cancer-specific mortality: 32.2% vs. 19.8%, *P*=0.003). The prediagnostic aspirin user group had a shorter DFS in the univariate analysis than the non-user group (33.32 ± 25.54 months vs. 40.46 ± 23.90 months, *P*=0.006). Also, the prediagnostic aspirin user group had a shorter overall survival than the non-user group, although it was not statistically

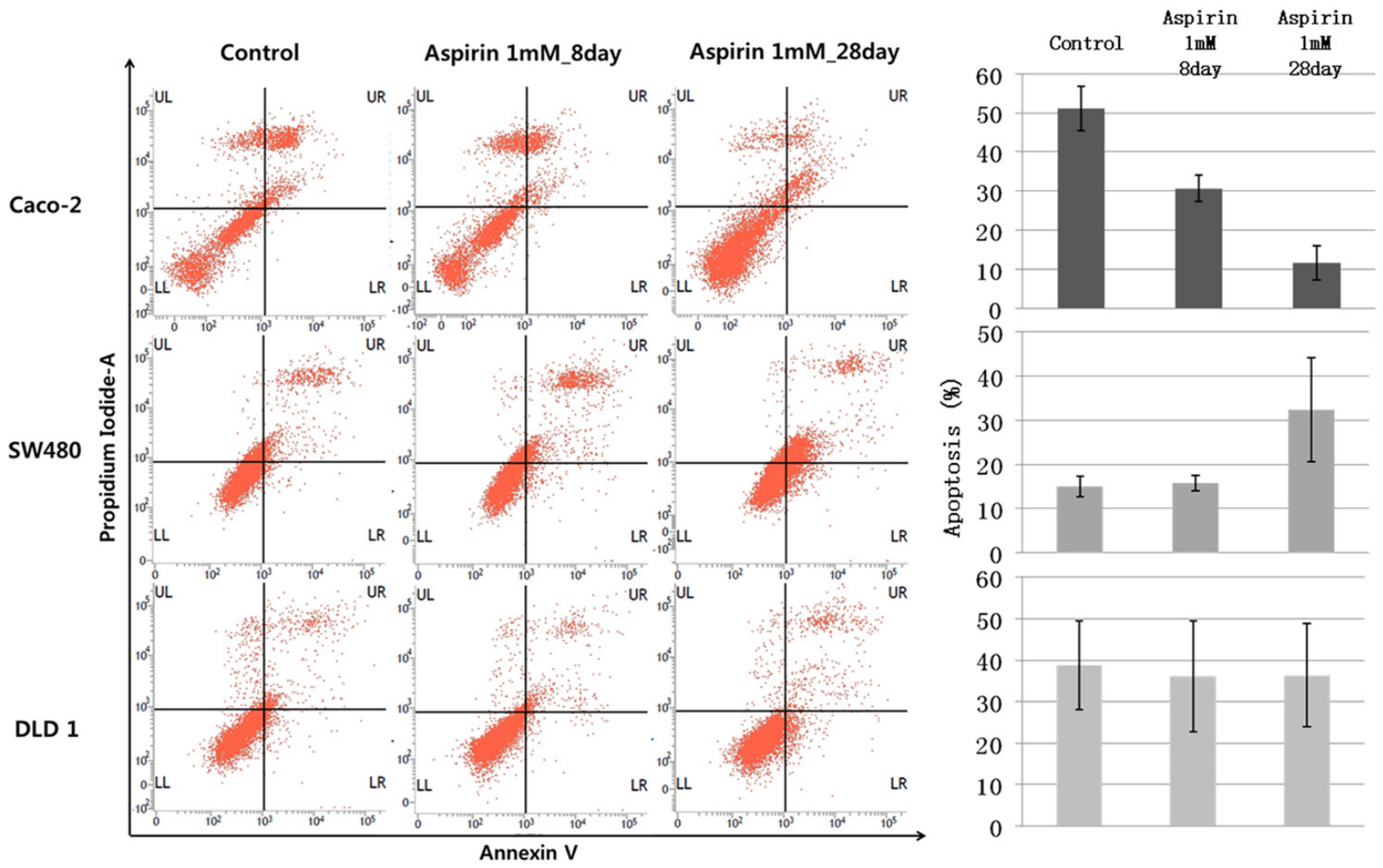


**Figure 2.** 5-FU-induced apoptosis after long-term pretreatment of aspirin in colon cancer cell lines. Aspirin (1 mM) was pretreated for 8 or 28 days in three CRC cell lines (Caco2, SW480, and DLD-1). Then, after 24 hr of treatment with 5FU (2 μM), apoptosis was measured by flow cytometry using Annexin-V and propidium iodide double staining. While aspirin-pretreated SW480 cells showed an increase in 5-FU-induced apoptosis, Caco2 cells showed a significant decrease in apoptosis in aspirin-pretreated cells in a pretreatment duration-dependent manner, and DLD1 cells did not show any significant change in the same set of experiments. Graphs represent the combined percentage of early (UR) and late (LR) apoptosis relative to all cells. Data are expressed as means of triplicate determinations (mean ± standard error) and are representative of three independent experiments. $^{*}P<0.05$.

significant (48.56 ± 20.01 months vs. 52.40 ± 17.51 months, *P*=0.052).

In the survival analysis, prediagnostic aspirin use affected the prognosis of stage III colorectal cancer in both the univariate analysis and multivariate analysis (**Figure 1** and **Table 3**). In order to identify the effect of aspirin on DFS and cancer-specific survival, we used Kaplan-Meier curves stratified by aspirin use or the lack of aspirin use. DFS and cancer-specific survival were significantly decreased in prediagnostic aspirin-users than in non-users [*P*=0.002 and *P*=0.003, respectively; **Figure 1A** and **1B**]. In addition, using Cox proportional hazards modeling adjusted for age, sex, comorbidity, medication, chemotherapy, cancer site, initial stage, and pathologic differentiation, DFS was significantly decreased in prediagnostic aspirin users than non-users [HR, 1.525 (1.018-2.286); *P*=0.041]. Also, statin use, initial stage IIIB (vs. IIIA), initial stage IIIC (vs. IIIA), PD pathologic differentiation (vs. well differentiated), primary cancer site, and chemotherapy-affected CRC DFS on the multivariate analysis [HR=0.488 (0.281-0.848), *P*=0.011; HR=3.248 (1.498-7.040), *P*=0.003; HR=6.372 (2.890-14.051), *P*<0.001; HR=3.574 (1.758-7.265), *P*<0.001; HR=1.482 (1.122-1.958), *P*=0.006; and HR=0.462 (0.232-0.921), *P*=0.028, respectively]. However, cancer-specific survival was not significantly different between the prediagnostic aspirin users and the non-users on multivariate analysis (**Table 3**).

*Cell line-specific differences in 5FU-induced apoptosis in long-term aspirin-pretreated CRC cells*

**Figure 2** shows cell line-specific differences in 5FU-induced apoptosis measured with flow cytometry in Caco2, SW480, and DLD-1 cell lines. Three CRC cell lines (Caco2, SW480, and DLD-1) were pretreated with aspirin (1 mM) for 8 or 28 days each, and then were treated with 5-FU (2 μM). After 24 hr of 5FU treatment, apoptosis was measured by flow cytometry using Annexin-V and propidium iodide double staining. While aspirin-pretreated SW480 showed an increase in 5-FU-induced apoptosis, compared with the control without pretreatment of aspirin, Caco2 cells showed a significant decrease in apoptosis in a pretreatment duration-dependent manner, and DLD1 cells did not show any significant change in the same set of experiments (**Figure 2**).

## Discussion

In most studies that have demonstrated the effect of NSAIDs on CRC survival, they presented an overall effect on CRC. However, we focused on the recurrence of CRC, especially to show a possible difference in the behavior of CRC with prediagnostic long-term NSAID exposure, thereby reducing the effect of post-operative adjuvant chemotherapy, and thus finally leading to a difference in tumor recurrence. Therefore, we identified patients with stage III CRC and focused on their recurrence of CRC. As a result, we found that long-term use of aspirin before the diagnosis of stage III colorectal cancer was associated with a poor outcome in terms of recurrence and DFS.

Many reports have shown that NSAIDs, including aspirin, extend the survival of patients with colorectal cancer [7, 9, 10, 14], but these studies showed a benefit mostly in post-diagnostic NSAID users, not in those who were taking NSAIDs regularly prior to diagnosis. Meanwhile, a recent large-scale study recently showed that the regular use of NSAIDs prior to diagnosis is associated with improved colorectal cancer survival, particularly among cases diagnosed with proximal disease [15]. However, recent meta-analysis data showed no prognostic benefit of prediagnostic NSAID use in CRC [16].

The beneficial effects of NSAIDs cannot be generalized in all patients, and there have been some studies of the limited effects of NSAIDs. These studies have suggested that only CRC patients with specific factors such as COX-2 overexpression or specific mutations in genes like *SMAD7* and *PIK3CA* would benefit from NSAID use [7, 17, 18]. Chan, et al. reported that, among patients with colorectal cancer participating in a large cohort study, aspirin users had a 29% lower cancer-specific mortality and a 21% lower overall mortality than non-users. The reduction in mortality was even greater among patients who initiated aspirin use after cancer diagnosis than among those who used it before, and the benefit was limited to those with tumors that overexpressed COX-2 [7]. Another study showed that regular use of aspirin after diagnosis was associated with superior CRC-specific survival in patients with mutated *PIK3CA* CRC, but not in patients with wild-type *PIK3CA*, proposing it as a predictive molecular biomarker for adjuvant aspirin therapy [17].

Moreover, a large case-control study undertaken to extend the Genome-wide Association Study (GWAS) findings noted a statistically significant interaction between *SMAD7* variants and use of NSAIDs two years prior to the diagnosis of colorectal cancer. This study also showed that CRC-specific survival differed according to the genotype of *SMAD7* variants. Specifically, each minor allele of rs4939827 was associated with worse survival, and each minor allele of rs4464148 was associated with better survival [19].

In addition to these molecular or genetic factors of tumors, the different characteristics of included patients and tumors could be contributing factors to the heterogeneity observed in the prognostic effect of NSAID use in CRC. For example, Coghill, et al. reported a significant beneficial effect of prediagnostic NSAIDs only in right colon cancer patients [15]. Compared to this study, our study included fewer right colon cancer patients (38.7 vs. 20.9%), suggesting an explanation for the lower beneficial effect of NSAID use in our study.

The different stages of CRC in previous studies also could be an important factor related to the heterogeneity observed in the prognostic role of prediagnostic NSAID use. Most previous studies included all stages of CRC or the non-advanced stages of CRC, and showed earlier stages at diagnosis in NSAID users than in non-users [15], suggesting the possibility of reduced tumor progression and better survival due to NSAIDs. However, our study included only stage III patients to focus on the recurrence of CRC after adjuvant chemotherapy, and excluded the influence of different stages at diagnosis on prognosis. Even in the study that showed a beneficial survival effect of prediagnostic aspirin use, the survival benefit was attenuated and disappeared after stage adjustment [15]. Our other data, including stage IV CRC, showed that long-term aspirin use before the diagnosis of stage IV CRC was associated with a lower CRCspecific survival, compared to aspirin non-users (64.2 ± 2.5 months vs. 72.6 ± 1.0 months; $P$=0.002, unpublished).

The duration of NSAID exposure also might lead to different results in terms of CRC prognosis. Although a recent large study showed that the beneficial prognostic role of prediagnostic NSAID use is dependent on the duration of NSAID use, a very long-term user (>7 years) did not follow this duration-dependent trend [15]. The patients in our study had a relatively longer period of prediagnostic aspirin-use (average, 61.7 months), compared with the previous study (average, 36 months) [19], and the proportion of patients with long duration (≥2 years) of aspirin use was higher in our study than in another study (82.6% vs. 50%) [15]. The early exposure to NSAIDs during an average CRC carcinogenesis period of about 10 years and subsequently escaping this continuous tumor suppressive pressure for the whole tumor progression period might induce the development of different carcinogenic pathways, leading to different CRC characteristics such as resistance or poor response to adjuvant chemotherapy.

In this aspect, our experiments simulating the conditions of post-operative adjuvant chemotherapy in prediagnostic aspirin-exposed CRC (through the use of aspirin pretreatment and then treatment with chemotherapeutic agents in CRC cell lines) demonstrated that in the aspirin-exposed condition for a short or long duration, the three CRC cell lines showed different apoptotic responses to chemotherapeutic agents, especially aspirin exposure duration-dependent resistance of apoptotic response to a chemotherapeutic agent in Caco2 cells. There have been many reports about enhanced tumor regression with the combination of NSAIDs and chemotherapeutic agents [20-22]. However, as prolonged exposure to specific chemotherapy induces resistance to that agent or select chemoresistant clones in tumor cells [23, 24], prolonged exposure to NSAIDs can results in the development of NSAID-resistant tumors in CRC carcinogenesis. In this aspect, for the first time, we showed that in some cell lines the long-term pretreatment of aspirin could induce the poor response to a chemotherapeutic agent. This suggests that NSAID-resistant cells could be an important cause of recurrence in some subtypes of stage III CRC with long-term prediagnostic NSAID use. Therefore, identification of molecular mechanisms underlying this cell-line specific differential response could provide surrogate markers that might indicate a favorable NSAID response.

Although some studies showing the beneficial survival effect of prediagnostic NSAID use explain that this benefit may be based on the

development of less aggressive tumors expressing lower level of COX-2 by NSAIDs, our experiments showed the possibility of the development of differential characteristics of CRCs showing poor response to a chemotherapeutic drug through long-term exposure of aspirin prior to CRC diagnosis. In the future, we need to elucidate the detailed molecular characteristics of CRC with long-term prediagnostic exposure to NSAIDs.

The limitation of our study is its retrospective nature, including its insufficiency in record-keeping. Therefore, a prospective cohort study of a large number of patients is needed to identify the effect of aspirin on the prognosis of stage III colorectal cancer when used over a certain period of time before cancer diagnosis.

Nevertheless, this is the first study to investigate a relationship between prediagnostic aspirin-use and prognosis of stage III colorectal cancer. Its results suggest that if a particular tumor-suppressing agent, such as NSAIDs, is used for a significant duration from the initial tumor development, resistance might occur and the tumor could evade the anti-tumor effects of that agent, affecting tumor biology, the response to other anti-tumor agents, and the prognosis of the tumor.

However, other factors related to NSAID response including the previously mentioned characteristics of patients, such as tumor location, tumor genetic variants, duration of NSAID use, and stage, etc., should be also considered to interpret the effect of prediagnostic NSAID use on CRC survival in future studies.

In conclusion, our results show that long-term aspirin use before diagnosis could be a negative prognostic factor in stage III colorectal cancer. In order to confirm and further elucidate the mechanism of this, a prospective cohort study with a large number of patients is needed. Furthermore, to identify a molecular mechanism and biomarker related to heterogeneity of the long-term prediagnostic NSAID effect on the tumor, molecular epidemiologic and experimental studies are needed in the future.

## Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Tae Il Kim, Department of Internal Medicine, Division of Gastroenterology, Yonsei University College of Medicine, 50 Yonseiro, Seodaemun-gu, Seoul 120-752, Korea. Tel: (82-2) 2228-1965; Fax: (82-2) 393-6884; E-mail: taeilkim@yuhs.ac

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