



Moving from immune phenotyping of colorectal cancer to mechanistic insights on aspirin use

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Colorectal cancer is the third most common cancer in men and women in the US, with over 95,000 new cases expected in 2016 (1). Many preclinical, epidemiological, and clinical studies have shown that regular aspirin (acetylsalicylic acid) use, even in low-doses, prevents the development of colorectal neoplasia (2-5). In 2015, the US Preventative Services Task Force acknowledged the value of low-dose aspirin in primary prevention of colorectal cancer in adults aged 50-59 years (6). Despite this wealth of evidence, how aspirin reduces colorectal cancer risk is not fully understood. Many mechanisms have been proposed, but the most commonly cited is aspirin's inhibition of PTGS2 (COX-2), a known mediator of inflammation and immunosuppression that is overexpressed in many colorectal neoplasms (7-12).

Another key development in the understanding of colorectal cancer has been evidence supporting the importance of immunological phenotyping in prognosis (13,14). A strong intratumoral lymphocytic reaction has been associated with better clinical outcomes in a wide range of tumors, including colorectal, breast, and ovarian (15). These studies have emphasized tumor progression rather than etiology, but the prognostic value of tumor immune phenotyping (13-17) introduces a potentially useful means of classifying neoplasms. Given the likely immunomodulatory effects of aspirin (7), examination of its effects on colorectal neoplasia with quantification of tumor immune contexture could provide novel mechanistic insights into aspirin's chemoprevention and better inform management of colorectal cancer.

Cao *et al.* explored this relationship in their recent cohort study of aspirin use and its effect on the immunological phenotype of colorectal cancer (18). The authors hypothesized that tumors with low levels of lymphocytic infiltrates would exhibit a stronger inverse relationship with aspirin use when compared to tumors with higher levels of lymphocytic infiltrates. Drawing from two large cohort studies, the Nurses' Health Study and the Health Professionals Follow-up Study, with interval information on aspirin use and confirmed incident cases of colorectal cancer over 30 years of follow-up, the authors evaluated 1,458 tumor specimens for pattern and degree of lymphocytic infiltration. They classified four patterns of lymphocytic reaction with each pattern categorized as low, intermediate, or high. Of the four, tumor-infiltrating lymphocytes (TILs) stood out statistically: regular aspirin use, when compared to nonregular use, was associated with reduced risk of low TIL subgroup tumors (RR 0.72; 95% CI, 0.63-0.81), but not cancers with more extensive TILs. Within the low TIL subgroup, the association was more pronounced with increasing aspirin dose and duration of use. By identifying a subgroup of tumors that may be preferentially targeted by aspirin chemoprevention, these results support the causal relationship between aspirin use and reduced colorectal cancer risk.

This evidence of etiologic heterogeneity by immune phenotype is not an isolated finding, and is paralleled by previous results from this group investigating heterogeneity according to PTGS2 expression. Using the same cohorts, the authors found a stronger inverse association between aspirin use and colorectal cancers overexpressing

PTGS2 when compared to tumors without PTGS2 overexpression (19). Together these two reports suggest distinct subgroups of etiologic significance. Moreover, these results extend the previous findings by showing that stratification on PTGS2 does not alter the inverse relation between aspirin use and low TIL colorectal cancer incidence. This lack of statistical evidence of effect modification could be consistent with a PTGS2 independent mechanism, raising uncertainty about the prevailing mechanistic proposal for aspirin's effect on colorectal cancer development.

These interesting findings underscore the complexity of interpreting tumor immune contexture. Variation in the expression of a tumor biomarker of immune response, such as TILs, may reflect (I) a reaction to a tumor antigen that is present in only a subset of cancers and which may or may not be on the pathway between exposure and disease, (II) a host response that varies in the population with some individuals having a robust response and others showing low infiltration, or (III) a complex interaction of both tumor and host factors. Even during the latent period in which neoplasia is present but not detected, aspirin use may further modulate the tumor-host interaction by acting directly on the innate and adaptive immune responses (20). Disentangling the role of the host and that of the tumor is challenging, particularly when the exposure period overlaps with the latent period, as is the case in this study. Future studies to understand the immune system in cancer etiology may help resolve some of the remaining questions this research has raised. For example, does aspirin target the tumor directly, or does it work synergistically with the host to increase immunosurveillance?

Central to answering such questions is a precise understanding of the timing and duration of aspirin exposure. As it stands, aspirin's point of action on the timeline from polyp (adenoma or serrated polyp) to invasive cancer is unknown. Evidence from multiple randomized trials has suggested an empirical induction period between initiation of aspirin consumption and reduced risk of colorectal cancer: Rothwell *et al.* reported a period of 7–8 years before incidence curves separated in a pooled analysis of five randomized trials of aspirin exposure (4). The Women's Health Study of alternate-day, low-dose aspirin showed no difference in colorectal cancer incidence at the end of the 10-year active intervention period, but observational follow-up at a median of 18 years did indicate reduced risk with aspirin use (5,21). A long empirical induction period, defined as the interval of time from etiological action to disease detection (22), may indicate an

effect of aspirin early in carcinogenesis. Furthermore, since the inverse association of aspirin with colorectal cancer risk may dissipate with cessation of use (23), it is important to consider consistency of use. As Friis *et al.* reported in their nested population-based case-control study of Danish patients with colorectal cancer, discontinuous long-term use was not associated with risk reduction (24).

Given the challenges of temporality in this type of investigation, it is difficult to draw etiologic inferences based on the exposure reported by Cao *et al.* The authors defined regular aspirin users as participants who reported consumption of 2 or more standard-dose (325 mg) aspirin tablets per week, but it is unclear whether these were “ever regular” users—participants who took aspirin regularly at any point in time—or regular users at the time of colorectal cancer diagnosis. Without this distinction it is impossible to determine a lag time in aspirin's protective effect or potential effect modification by discontinuity of use. Future work comparing these findings to those of clinical trials, where an early etiologic endpoint, such as adenoma, is measured and where exposure temporality is more firmly controlled could help resolve these uncertainties.

Another aspect of data collection worthy of critical examination is how the authors quantified and stratified TIL subgroups. Tumor immune contexture is well recognized as a prognostic indicator in colorectal cancer, as well as a variety of other neoplasms (15). One challenge in implementing this assessment tool is establishing a standardized method for histopathological analysis, a process that, as it stands, is somewhat subjective. Cao *et al.* report only moderate concordance in TIL assessment between independent review of more than 400 cases by two separate pathologists (18), so some degree of misclassification is inevitable. This variability may mask differences across TIL strata, as well as obscure trends in other lymphocyte reaction groups. Moving forward, a reproducible, systematic approach to histopathological analysis will be valuable in elucidating the aspirin-tumor relation by tumor subgroup. Some challenges to this will be establishing tumor region selection criteria and achieving cross-laboratory validation of protocols that avoid overburdening pathologists (13). One potential solution could be implementing image analysis software to eliminate subjectivity and streamline analysis.

In addition to assessing TILs, Cao *et al.* measured specific types of lymphocytes and found an inverse relation between aspirin use and colorectal cancer risk for tumors with low densities of CD3+, CD8+, and CD45RO+ cells,

but not for tumors with high densities. The associations are similar to those for TILs and suggest that the more specific lymphocyte markers add little to the TIL associations. Other studies show that such granularity in lymphocyte quantification is not necessary to achieve prognostic utility. A meta-analysis of 30 studies examining associations between colorectal cancer mortality and tumor inflammatory infiltrates indicated that generalized high inflammatory infiltrate density correlated with better disease outcomes while subsets of inflammatory cells stratified by tumor location exhibited significant heterogeneity (14). Rozek *et al.* examined lymphocyte infiltrates in their 2016 analysis of 2,369 colorectal tumors and also found generalized subtyping of TILs by quantity to be prognostic (16). These findings are heartening for the future of immune phenotyping in colorectal cancer prognosis, as they suggest that nonspecific measurements may be as or more useful than fine-toothed subtyping, thereby lowering the barrier to incorporation of immune grading in clinical colorectal cancer staging. However, continued work to characterize and validate immune markers of tumor heterogeneity is needed to conclusively determine best practices.

Finally, it is important to consider the broader clinical implications of TIL subtyping with regard to aspirin chemoprevention. Because aspirin use carries a risk for gastrointestinal and intracerebral bleeding, any benefits must be weighed against harms, particularly in the setting of long-term use. Cao *et al.* found a stronger inverse relation between risk of low TIL tumors and increasing aspirin dose, but multiple clinical studies suggest that low dose aspirin consumption is effective in preventing colorectal cancer, which may minimize the risk of bleeding when compared to higher dosing regimens (4,5). With a better understanding of the mechanism by which aspirin prevents neoplasia, it may be possible to further optimize the timing and dosing of chemoprevention. From a prognostic standpoint, the observation of worse outcomes from tumors with low levels of immune infiltrates warrants a closer look at aspirin's role in preventing this tumor subtype that carries a relatively poor prognosis (13-17). Studies of overall colorectal cancer incidence may underestimate the influence of aspirin on these subtypes. By stratifying tumor immune phenotype, Cao *et al.* have opened the door to a greater understanding of how aspirin chemoprevention affects colorectal cancer morbidity and mortality.

Looking forward, several ongoing randomized controlled trials of aspirin use will provide more data to further elucidate the complexities of aspirin chemoprevention. The Aspirin for Dukes C and High Risk Dukes B Colorectal

Cancers (ASCOLT) trial will investigate the role of aspirin as adjuvant therapy following colorectal cancer resection and the Aspirin Intervention for the Reduction of Colorectal Cancer Risk (ASPIRED) trial will seek to identify the effect of aspirin on cancer-related biomarkers in participants with recent adenoma removal (25). Trials such as these, paired with the continued secondary analyses and biomarkers studies similar to those of Cao *et al.*, will help address the uncertainties that remain with regards to host versus tumor interactions with aspirin.

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Footnote

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Ethical Statement: The authors are accountable for all

aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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