Surrogate Markers: Lessons from the Next Gen?
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Abstract

The article by Banerjee and colleagues published in this issue of the journal involving a randomized control prevention trial of ursodeoxycholic acid (UDCA) in Barrett esophagus reported a null outcome despite being well designed and executed. Possible reasons for this null outcome are discussed focusing on use of surrogate endpoints in the trial. The trial is especially topical because it comes at a time when there are calls for a Pre-Cancer Genome Atlas (PCGA) for "understanding the earliest molecular and cellular events associated with cancer initiation..." This commentary discusses current concepts in prevention research including branched evolution that leads to therapeutic resistance. Length bias sampling postulates underdiagnosis is due to rapidly progressing disease that is difficult to detect by screening because it progresses to cancer too rapidly and that overdiagnosis is the result of very slowly or nonprogressing disease that is easy to detect by screening because it persists for a lifetime and the patient dies of unrelated causes. Finally, it also explores study designs, including surrogate endpoints in Barrett esophagus trials, and opportunities and pitfalls for a PCGA in the context of high levels of over and underdiagnosis of Barrett esophagus as well as many other cancers and their precursors. Cancer Prev Res; 9(7) July 2016

The null results of the trial lead to a recurring question in well-designed Barrett esophagus prevention studies: where do we go from here? Animal and human studies have implicated reflux of gastric acid and bile acids in development of Barrett esophagus and esophageal adenocarcinoma (2–4). UDCA has been reported to be associated with a protective effect against oxidative stress and oxidative DNA damage (5) as well as prevention of esophageal adenocarcinoma in a rat model of Barrett esophagus when combined with aspirin (6). In the UDCA trial, 29 patients with Barrett esophagus received daily doses of UDCA for 6 months. This trial was successful in increasing UDCA gastric bile acid concentrations from 18.2% of total gastric bile acids at baseline to 93.39% post intervention. However, there was no effect on the surrogate immunohistochemical markers in the Barrett mucosa: (i) proliferation (Ki67), (ii) oxidative DNA damage (8-hydroxydeoxyguanosine), and (iii) apoptosis (cleaved caspase-3) in formalin-fixed, paraffin-embedded tissue samples. These are all logical surrogates for a cancer prevention trial.

But the question now becomes how do we interpret and move forward from these null results in a well-designed and executed early-phase randomized trial? Is the hypothesis that UDCA is protective against oxidative stress in the esophagus wrong? Was the time span (6 months) of the study too short? Are the surrogate immunohistochemical measures providing misleading information about cancer risk? There is no doubt that apoptosis is an important biologic process that can help protect against cancer, but it also occurs frequently and the affected cells disappear rapidly in the normal body. How sure are we that measuring increased apoptosis in a few biopsies evaluated after a few months reliably reflects a reduced risk of progression from Barrett esophagus to esophageal adenocarcinoma? Similarly, while it is true that cancers may have greater proliferative fractions, Barrett esophagus has a crypt architecture in which most of the proliferating cells detected by Ki67 are sloughed into the lumen and excreted from the body, a process that has long been thought to be tumor-suppressive (7). In the Seattle dietary intervention study, Ki67 staining was also used to assess proliferation as a measure of...
The immunohistochemical results with 8OHdG in the UDCA study raised concern about nonspecific staining so that only moderately or strongly stained nuclei were used in the final analyses. Prevention trials are very challenging, and ideally we would like to learn more from each trial whether or not it is successful so that future trials can be designed to be more successful. As the authors note, few surrogate markers, including those used in this study and our earlier dietary intervention study, have been validated as predictors of progression to esophageal adenocarcinoma in clinical studies.

The question of the validity of surrogate measures is becoming fundamental to the design of early-stage prevention trials. Rigorous standards for using surrogate biomarker endpoints have been well-established for more than 2 decades (6, 9). Valid surrogate markers should be in key causal pathways to esophageal adenocarcinoma, have substantial ability to distinguish patients who progress to cancer from those who do not, and be objectively measured (7). Multiple studies in Barrett esophagus meet all or most of these requirements, including a cancer incidence end-point although measures of dysplasia as predictors continue to not be “objectively measured” (10–18). Although it has become increasingly difficult to meet all of these standards, alternative study designs have emerged that preserve much of the original intent of the guidelines and are consistent with the results of other rigorous studies (19). Study designs that “map” the spatial distribution of genomic abnormalities in Barrett esophagus surrounding esophageal adenocarcinomas can provide highly useful information on the process by which Barrett esophagus evolves to esophageal adenocarcinoma (20–22). However, these studies must be interpreted cautiously because they lack nonprogressing control populations and can easily lead to overdiagnosis and overtreatment. A further limitation of spatial study designs is that none of them address the critical element of time: how fast do the changes occur that lead to esophageal adenocarcinoma in general and underdiagnosis of esophageal adenocarcinoma specifically?

Ki67, which detects actively proliferating cells in G1, S, and G2–M, is one of the oldest measures of proliferation in Barrett esophagus and many other conditions, yet it has rarely been tested in prospective studies to determine the extent to which it actually discriminates between individuals who will and will not progress to cancer. One study used DNA content/Ki67 multiparameter flow cytometry in 853 diploid biopsies from 362 patients with Barrett esophagus (23). Of these patients, 276 were followed prospectively for an average of 6.3 years with 29 developing esophageal adenocarcinoma. Neither total Ki67 positivity nor diploid Ki67 G1 fractions, which account for the bulk of Ki67-positive cells, were predictive of progression to esophageal adenocarcinoma (P = 0.13 and 0.15, respectively). Ki67-positive cells in S-phase were somewhat predictive (P < 0.03). However, increased 4N fractions, which at the time were termed “G2/tetraploid” were much more strongly associated with progression to esophageal adenocarcinoma (P < 0.0001). These increased 4N fractions are now recognized to be early manifestations of whole-genome doublings that are strongly predictive of progression from Barrett esophagus to esophageal adenocarcinoma (10, 11). Whole genome doublings in Barrett esophagus have been associated with biallelic inactivation of TP53 by mutation and LOH and are highly predictive of progression to aneuploidy as well as esophageal adenocarcinoma (10–12). Interestingly, a screen to detect selective killing of tetraploid cells led to identification of resveratrol and salicylate as potential anticancer chemoprevention by eliminating tetraploid cells (24). This concept of chemoprevention acting against cells with chromosome instability and genome doublings is further supported by a study that reported aspirin and other nonsteroidal anti-inflammatory drugs (NSAID) are associated with a decrease in esophageal adenocarcinoma and DNA content aneuploidy and tetraploidy in patients with Barrett esophagus (25). Similarly, aspirin and other NSAIDs have also been reported to decrease progression to esophageal adenocarcinoma in a prospective cohort study of patients with multiple high-risk somatic genome abnormalities including 17p LOH, DNA content aneuploidy, and tetraploidy (16).

Whole genome doublings, which have been termed “increased 4N fractions” and “increased G2/tetraploid” in the DNA content literature, and DNA content aneuploidy have consistently been reported in esophageal adenocarcinoma and in Barrett esophagus that progresses to esophageal adenocarcinoma using a number of different platforms, including DNA content flow cytometry (10, 11, 26, 27). The historical conflict between flow and image cytometry seems to have abated with recent results showing that both methodologies provide comparable information when the proper prep is used and the analyses are adjusted for clumping of nuclei that could lead to false-positive 4N cells (28). At the present time, DNA content cytometry is the only method that has been shown to reliably detect genome doublings at early stages when they constitute only a small proportion of the cells in the biopsy (~6–15%) especially in the presence of one or more aneuploid cell populations in the same biopsy (11, 11). Advances in bioinformatics employing collaborations between scientists with genome and cytometric expertise are likely to improve detection of whole-genome doublings as important predictors of progression in Barrett esophagus using DNA sequencing and SNP arrays just as advances in DNA content cytometric sample preparation were made by collaborations between investigators using image and flow cytometric methods (28).

Recently, there has been a call for a “Pre-Cancer Genome Atlas” (PCGA; ref. 29). We have learned much from The Cancer Genome Atlas (TCGA), but it was a case-only study design. This case-only design was appropriate to determine the atlas of genomic alterations in advanced cancers to guide targeted therapy. However, such a case-only study design will not be appropriate for precursors of cancer. Any PCGA will need well-designed studies that include nonprogressing controls to avoid exacerbating overdiagnosis and overtreatment (30, 31). For example, a recent study of healthy, sun-exposed skin reported an unanticipated abundance of somatic mutations, including purported drivers of skin carcinogenesis such as NOTCH1 (32).

Properly designed intervention studies will have to include many of the elements of the Banerjee UDCA study including unbiased protocol biopsy sampling in space and time to determine the evolutionary trajectory of different genomic alterations and robust, reproducible measures of genomic alterations that determine whether the patient will progress to cancer or not. The authors of this UDCA study have wisely avoided dysplasia grade, which meets none of the standards for surrogate markers (7).

A recent meta-analysis reported that 25% of all esophageal adenocarcinomas are missed after initial screen detection of Barrett esophagus, including esophageal adenocarcinomas detected within a year (33). Thus, both clinical and prevention research communities share a need for more robust measures of efficacy for the dietary intervention, and the results were also null (1).
risk for progression to esophageal adenocarcinoma than are currently being used. A combination of recent research advances and rediscovery of an ancient pathology literature is shedding light on why most Barrett esophagus remain stable for a lifetime ("overdiagnosis") while a minority of Barrett esophagus rapidly progress to esophageal adenocarcinoma ("underdiagnosis").

First, several lines of evidence indicate that Barrett esophagus has a large number of physiologic, expression, and proteomic functions that provide epithelial cell protection in the harsh environment of gastroesophageal reflux disease (7, 34–36). Second, recent studies in model organisms and human autopsy studies from the 1950s and earlier indicate that both the embryonic human and mouse esophagus have a columnar lining that is replaced by a squamous lining during fetal development and that these cells can be identified in the adult (37–39). Third, the expression profile of the columnar lining in the embryonic mouse is very similar to that of adult human Barrett esophagus (39). Reappearance of a latent embryonic program that arises as a protective adaptation in the presence of severe reflux disease may help explain why so few cases of Barrett esophagus progress to esophageal adenocarcinoma (7).

In contrast, esophageal adenocarcinoma have evidence of massive genomic alterations. For example, the transition from Barrett esophagus to esophageal adenocarcinoma in many patients occurs rapidly through chromosome instability (gain or loss of whole chromosomes or large regions of chromosomes) and whole-genome doubling with a 2- to 4-year window of opportunity for early detection of esophageal adenocarcinoma using a single endoscopic biopsy every 2 cm in the Barrett esophagus segment (17). Whole-genome sequencing studies have reported a median of 26,161 mutations (range, 18,881–66,225) per esophageal adenocarcinoma (40). It may be that the light microscope is not the optimal method for detecting the complex processes that lead to evolution of Barrett esophagus to esophageal adenocarcinoma.

Reliance on selective sampling based on nonreproducible, nonrobust clinical assessments such as histopathologic interpretation of dysplasia in Barrett esophagus will likely undermine the goals of any PCGA. Temporal assessment of the trajectory will be essential because length bias sampling is believed to underlie over- and underdiagnosis in screening so that nonprogressing conditions that remain stable are more easily detected while rapidly progressing neoplasms are selectively missed by screening [see Figs. 1 and 3 in Reid and colleagues 2015 (41) for more detailed discussion of this concept].

One example of such an approach is a recent case–cohort study with 248 patients with Barrett esophagus, including 79 who progressed to esophageal adenocarcinoma and 169 who did not progress to esophageal adenocarcinoma using 1M SNP arrays (17). The study reported that there was a 2- to 4-year window of opportunity for early detection and prevention of esophageal adenocarcinoma in progressors. Progression was characterized by gain or loss of whole chromosomes or large regions of chromosomes beginning about 48 months before the diagnosis of esophageal adenocarcinoma followed by whole-genome doublings in the 24 months before diagnosis of esophageal adenocarcinoma. In contrast, nonprogressors were typically characterized by evolutionary "stasis" with few manifestations of chromosome instability over prolonged times up to or more than 20 years.

It has been known since the 1990s that somatic genome evolution in Barrett esophagus is branched, not linear (42), and that there is no linear order of the events that lead to inactivation of CDKN2A and TP53 (43). Although our understanding of the evolution of esophageal adenocarcinoma has advanced rapidly over the last few years as a result of genomic advances including SNP arrays, exome, and whole-genome sequencing (17, 19, 21, 22, 40, 44), the fundamental concepts of branched evolution and nonlinearity have not changed. However, many investigators and clinicians still conceive of neoplastic progression as a linear pathway, and a massive continuing medical education process will be necessary to make the transition to the recognition that cancer develops through branched evolution pathways that are being elucidated during the genomic era (ref. 41; Fig. 1).

TP53 appears to be the only gene mutated at sufficiently high frequency (~70%) in esophageal adenocarcinoma and Barrett esophagus that progresses to esophageal adenocarcinoma to be highly informative for targeted early detection and prevention, although assessment of chromosome instability and genome doublings are likely to be useful in early detection and prevention as well (17–19, 40). These results raise the question of whether we should consider genomic alterations such as TP53 mutations, chromosome instability, and whole-genome doublings and aneuploidy as markers of risk in prevention and early detection studies. A risk prediction model based on SNP array results in a single biopsy every 2 cm in the Barrett esophagus segment was highly predictive of progression to esophageal adenocarcinoma (area under the curve or AUC = 0.94) and outperformed histopathology on the basis of four biopsies every 2 cm (AUC = 0.81 for high-grade dysplasia and 0.78 for high- and low-grade dysplasia combined; ref. 18). Interestingly, the AUC for DNA content flow cytometry (0.79) was essentially equivalent to dysplasia classification even though only one biopsy was evaluated at each 2 cm interval compared to 4 for histopathology. The window of opportunity afforded by measuring these genomic alterations may allow more effective design of prevention studies in Barrett esophagus, potentially using interventions such as resveratrol and aspirin or other NSAIDs that have already shown promise in controlling some of these processes including whole-genome doublings and aneuploidy as endpoints (16, 24, 25).

Biopsies should not be selected for analyses in genomic studies on the basis of dysplasia classification, which is subject to observer variation (45–47). Lack of reproducibility of dysplasia interpretations can undermine progress to developing more robust indices of risk. This can become especially problematic if the nonreproducibility of dysplasia interpretation leads to the perception that the genomic/genetic results are not reproducible because two or more pathologists interpret the dysplasia status of biopsies differently.

These comments should not be considered a criticism of the pathology community that has worked very hard to improve the current dysplasia classification system. In many centers, Barrett esophagus cases may receive input from two or more specialty pathologists and difficult cases are frequently referred to centers with special expertise in Barrett esophagus, where multiple gastrointestinal pathologists participate in the final review. Yet, the problems persist despite the best efforts of both gastrointestinal and pathology communities, who in general establish close working relationships to try to provide the best possible care. This seems to be the best we can do with the current tools, and these tools and the second opinion options are far better than they were 30 years ago. However, there is still room for improvement as witnessed by the recent report that 25% of esophageal...
pathways. This model predicts that treatments that affect one branch may initially appear successful (e.g., lower progression pathway leading to EA1), but continued populations. This branched evolution model, originally proposed by Nowell in 1976, makes the prediction that neoplastic evolution can follow multiple strategies (17, 22).

Barrett esophagus can acquire genomic alterations that promote properties essential for progression to a lethal cancer [autonomous cell proliferation, invasion of surrounding tissues and metastasis to distant sites (ref. 51), http://www.cancer.gov/publications/dictionaries/cancer-terms] or increase genomic instability to facilitate the acquisition of these properties (circles filled red). Circles with red Xs represent clonal populations that either die or are outcompeted by surrounding populations. This branched evolution model, originally proposed by Nowell in 1976, makes the prediction that neoplastic evolution can follow multiple pathways. This model predicts that treatments that affect one branch may initially appear successful (e.g., lower progression pathway leading to EA1), but continued evolution with selection of resistant clones can lead to relapse and therapeutic failure (e.g., in the upper progression pathway leading to EA2).

Adenocarcinomas are missed by current screening and surveillance practices (33). Two recent genomic studies have provided data indicating that whole-genome doublings may evolve so rapidly that they are missed by current screening and surveillance strategies (17, 22).

To some extent, the challenges the field faces with overdiagnosis of benign Barrett esophagus and underdiagnosis of early, curable esophageal adenocarcinoma is shared by many other cancers including those of the breast, prostate, lung, and many other organs (30). It is of interest that the sequence of chromosome instability followed by whole-genome doubling observed in Barrett esophagus has been observed in many cancer types in addition to esophageal adenocarcinoma, including breast, lung, colon, and ovarian (26). Extension of this type of research will be best accomplished using biorepositories that have established standardized biopsy protocols that obtain biospecimens at defined points in space and time such as described in Banerjee and colleagues Thus, the advances of TCGA have provided potential clues for early diagnosis and prevention of multiple different cancer types in different organ systems that might be pursued in well-designed PCGA studies.

Recent DNA sequencing studies of esophageal adenocarcinoma have also reported breakage-fusion bridge cycles in advanced esophageal adenocarcinoma that result from telomere attrition (44). Short telomeres have been reported in the blood of patients who progress from Barrett esophagus to esophageal adenocarcinoma (48), in Barrett esophagus tissue with evidence of chromosome instability (49) and in esophageal adenocarcinoma (50). What would be the implications of developing sequence-based biomarkers for telomere length for novel surrogates in cancer prevention and early detection research?

Other recent studies have reported results that may open new avenues for future studies, or potentially even for studies already completed provided sufficient biospecimens have been preserved for statistical power. For example, TCGA and other sequencing studies have reported a novel mutation signature that is found in gastric and esophageal adenocarcinoma (40, 44). The mutagen that causes this mutation signature is currently unknown, but its identification could lead to prevention strategies targeting the mutagen or reanalysis of biospecimens from previous trials. Recent studies, including esophageal adenocarcinoma sequencing studies, have also opened the door to reconsidering the etiology of Barrett esophagus and esophageal adenocarcinoma. Genome sequencing studies of esophageal adenocarcinoma are notable for lack of the tobacco signature, which has not been reported in esophageal adenocarcinoma even though tobacco smoking has been reported to be a risk factor in esophageal adenocarcinoma in association studies (40, 44). This finding in combination with population trends showing that lung cancer mortality declined following the decrease in tobacco smoking in the United States, whereas the rates and mortality of esophageal adenocarcinoma increased rapidly suggest that our understanding of the environmental insults that are etiologic for esophageal adenocarcinoma are incomplete. What role does tobacco play in the increased incidence of esophageal adenocarcinoma? Have
other as yet unknown mutagens slipped into the environment? Can we use DNA mutation signatures to track these carcinogens? Once they are identified, can we target the carcinogens for prevention of esophageal adenocarcinoma?

The vast majority of the above genomic results were reported after the Banerjee study was designed, and these possibilities were largely unknown four or five years ago. The Banerjee trial was well-designed and conducted yet the difficulty in interpreting the surrogate immunohistochemical biomarkers limits our understanding of the null results. Are we using measures that make biological sense (increased proliferation, decreased apoptosis, DNA damage) but haven’t yet been validated as surrogate markers? Are current assays not sufficiently sensitive and specific to detect differences associated with progression? This raises questions for the field to answer, not a single individual. However, these and other questions should be discussed before undertaking a massive PCGA study. Now may be the time to take stock of surrogate markers being used in early-phase cancer prevention studies and to address the implications of such surrogate markers on the interpretation of the results, possibly through a conference or multidisciplinary think tank.

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