Zinc deficiency activates S100A8 inflammation in the absence of COX-2 and promotes murine oral-esophageal tumor progression

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Zinc (Zn)-deficiency (ZD) is implicated in the pathogenesis of human oral-esophageal cancers. Previously, we showed that in ZD mice genetic deletion of cyclooxygenase-2 (Cox-2) enhances N-nitrosomethylbenzylamine-induced forestomach carcinogenesis. By contrast, Cox-2 deletion offers protection in Zn-sufficient (ZS) mice. We hypothesize that ZD activates pathways insensitive to COX-2 inhibition, thereby promoting carcinogenesis. This hypothesis is tested in a Cox-2-/- mouse tongue cancer model that mimics pharmacologic blockade of COX-2 by firstly examining transcriptome profiles of forestomach mucosa from Cox-2-/- and wild-type mice on a ZD vs. ZS diet, and secondly investigating the roles of identified markers in mouse forestomach/tongue preneoplasia and carcinomas. In Cox-2-/- mice exposed to the tongue carcinogen 4-nitroquinoline 1-oxide, dietary ZD elicited tongue/esophagus/forestomach carcinomas that were prevented by ZS. The precancerous ZD:Cox-2-/- vs. ZS:Cox-2-/- forestomach had an inflammatory signature with upregulation of the proinflammation genes S100a8 and S100a9. Bioinformatics analysis revealed overrepresentation of inflammation processes comprising S100a8/a9 and an nuclear factor (NF)-κB network with connectivity to S100A8. Immunohistochemistry revealed co-overexpression of S100A8, its heterodimeric partner S100A9, the receptor for advanced glycation end-products (RAGE), NF-κB p65, and cyclin D1, in ZD:Cox-2-/- forestomach/tongue preneoplasia and carcinomas, evidence for the activation of a RAGE-S100A8/A9 inflammatory pathway. Accumulation of p53 in these carcinomas indicated activation of additional inflammatory pathways. Zn-replenishment in ZD:Cox-2-/- mice reversed the inflammation and inhibited carcinogenesis. Thus, ZD activates alternative inflammation-associated cancer pathways that fuel tumor progression and bypass the antitumor effect of Cox-2 ablation. These findings have important clinical implications, as combination cancer therapy that includes Zn may improve efficacy.

Oral-esophageal squamous cell carcinomas (SCCs) are a major cause of cancer deaths worldwide. Oral cancer, the major site being the tongue, causes a high mortality rate because of frequent development of a second primary esophageal cancer because of field carcinization effects. Risk factors include alcohol consumption, tobacco and human papillomavirus (HPV). The incidence of oral cancer is increasing, particularly in young adults without documented risk.

Key words: zinc deficiency, transcriptome profiling, Cox-2 null mice, S100A8 inflammation, tongue cancer prevention

Abbreviations: COX-2, cyclooxygenase-2; DAVID, database for annotation, visualization and integrated discovery; GO, gene ontology; HNSCC, head and neck squamous cell cancer; IHC, immunohistochemistry; IPA, ingenuity pathway analysis; NMBA, N-nitrosomethylbenzylamine; NQO, 4-nitroquinoline 1-oxide; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; RAGE, receptor for advanced glycation end-products; S100a8, S100 calcium binding protein A8; S100a9, S100 calcium binding protein A9; SCC, squamous cell carcinoma; NF-κB, nuclear factor-κB; PCNA, proliferating cell nuclear antigen; Zn, zinc; ZD, Zn-deficiency; ZR, Zn-replenishment; ZS, Zn-sufficiency.

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Grant sponsors: NIH Grants R01CA118560 (LYBF) and R01 CA115965 (CMC)
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DOI: 10.1002/ijc.25688

History: Received 6 Aug 2010; Accepted 10 Sep 2010; Online 20 Sep 2010

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20 The report that deletion of the and is induced by factors implicated in carcinogenesis, 

Consequently, ZD predisposes to disease by adversely affecting immune system, by increasing oxidative stress, and by increasing the generation of inflammatory cytokines. 

In the rat, a ZD diet creates a precancerous condition in the upper digestive tract, including tongue, esophagus and stomach (an expanded lower esophagus), by inducing proliferation and gene expression changes, including overexpression of cyclooxygenase-2 (Cox-2) and the proinflammatory genes S100 calcium binding protein a8 (S100a8) and a9 (S100a9). ZD rats rapidly develop esophageal tumors after a single exposure to the environmental carcinogen N-nitrosomethylbenzylamine (NMBA) and concurrent tongue, esophageal and stomach tumors with exposure to the tongue carcinogen 4-nitroquinoline 1-oxide (NQO). Zn-replenishment (ZR) reverses cell proliferation, corrects gene expression and inhibits carcinogenesis.

Targeted therapies that block molecules crucial to tumor growth are being explored in attempts to prevent or cure cancer. The rationale for targeting the COX-2 pathway is supported by numerous studies. COX-2 is overexpressed in many human cancers, including esophageal and tongue SCC. COX-2 catalyzes the formation of prostaglandins and is induced by factors implicated in carcinogenesis, including growth factors, inflammatory stimuli, oncogenes and tumor promoters. The report that deletion of the Cox-2 gene in Apc knockout mice greatly reduces intestinal polyp formation provides genetic evidence that COX-2 plays a key role in tumorigenesis. COX-2 selective inhibitors, celecoxib in particular, are being tested in clinical trials for the prevention of several cancers, including esophageal cancer. Although such targeted therapies have shown promising results in several cancers, their efficacy in oral-esophageal cancer has been limited.

Our previous work showed that in ZD rats pharmacologic COX-2 inhibition by the drug celecoxib did not prevent tongue carcinogenesis, and in ZD mice genetic Cox-2 deletion actually enhanced NMBA-induced forestomach tumorigenesis. Aside from the result that ZD:Cox-2-/- mice forestomach overexpressed leukotriene A4 hydrolase protein, indicating a shift of arachidonic acid to the 5-lipoxygenase pathway, mechanisms underlying this effect of ZD were not elucidated. We hypothesized that ZD adversely affects treatment outcome by stimulating pathways not inhibited by the pharmacologic blockade of COX-2. We tested this hypothesis in a ZD:Cox-2-/- mouse oral-esophageal cancer model that mimics pharmacologic COX-2 blockade, using techniques that included transcriptome profiling, bioinformatics analyses, and investigation of the pathobiological roles of identified markers in murine tongue/forestromach preneoplasia and neoplasia.

Material and Methods

Mice, diets and carcinogens

We bred heterozygous B6;129S7-Ptg2tm1Jed males to generate Cox-2-/-, Cox-2+/+ and Cox-2+/+ (WT) littermates. Custom-formulated ZD and Zn-sufficient (ZS) diets (Harlan Teklad, Madison, WI) were identical except for the Zn content. NQO was from Wako Chemicals (Richmond, VA) and NMBA from Midwest Research Institute (Kansas City, MI).

NQO-induced tongue carcinogenesis

The mouse studies were approved by The Ohio State University Animal Use Committee. Four-week old littermates were fed ZD or ZS diets to form six groups, namely, ZD:Cox-2-/- (n = 14), ZD:Cox-2-/- (n = 46), ZD:WT (n = 19), ZS:Cox-2-/- (n = 16), ZS:Cox-2-/- (n = 37) and ZS:WT (n = 25). After 4 weeks the mice were administered NQO in deionized water for tongue tumor induction (20 ppm for 19 weeks followed by 30 ppm for 7 weeks). At 26 weeks, the animals were sacrificed for tumor incidence analysis.

Expression profiling and related studies

Weaning Cox-2-/- and Cox-2+/+ mice were fed ZD or ZS diets to form four groups, namely, ZD:Cox-2-/- (n = 20), ZS:Cox-2-/- (n = 12), ZD:WT (n = 12) and ZS:WT (n = 12). After 9 weeks, 8 ZD:Cox-2-/- mice were switched to a ZS diet to form the ZR:Cox-2-/- group. After a week, all mice were sacrificed. This experimental regimen produced unbrided cell proliferation in ZD:Cox-2-/- forestomach. Tongue and forestomach were isolated and cut into two portions. One portion was formalin-fixed and paraffin-embedded for immunohistochemical (IHC) studies. Forestomach epithelia for expression profiling studies were prepared from the remaining portion by using a blade to strip off the submucosal layers and snap-frozen in liquid nitrogen.

We performed expression profiling of forestomach mucosa from ZD:Cox-2-/-, ZS:Cox-2-/-, ZD:WT mice and ZS:WT mice after 10 weeks of ZD or ZS diets (n = 4 mice/group), using GeneChip® Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA). Total RNA was extracted from forestomach mucosa using TRIZOL reagent (Invitrogen, Carlsbad, CA). Five micrograms of total RNA was reverse transcribed into cDNA followed by in vitro transcription and labeling to
produce biotin-labeled cRNA. The cRNA was hybridized to the arrays as described.14

Expression data analysis
We used the Class Comparison analysis of BRB-Array Tools software version 3.7.0 (Biometric Research Branch, NCI) to identify differentially expressed mRNAs. The Robust Multichip Average method was performed. The array data were submitted to ArrayExpress (Accession number: E-TABM-778).

Gene ontology and pathway analyses
We used DAVID (Database for Annotation, Visualization and Integrated Discovery)25 bioinformatics to identify relevant biological processes/functions from expression data captured by transcriptome analysis. Based on gene ontology, differentially expressed genes were grouped by scoring the statistical significance of predefined functional gene groups according to their functional similarity.

We used Ingenuity Pathway Analysis software (IPA, http://www.ingenuity.com) to analyze probable network/pathway and functional group enrichment. For each data set, the selected genes were uploaded into the IPA application. Networks were then algorithmically generated based on gene-gene connectivity.

ZR and forestomach carcinogenesis in ZD:Cox-2−/− mice
This mouse study was approved by the Thomas Jefferson University Animal Use Committee. Thirty-nine 4-week old Cox-2−/− mice were fed a ZD diet to form the ZD:Cox-2−/− group. After 4 weeks, the mice received three intragastric doses of NMBA (2 mg/kg body weight, twice weekly), a regimen that produced a high tumor outcome in ZD:Cox-2−/− mice.16 A day after the 3rd dose, 18 mice were switched to a ZS diet to form the ZR:Cox-2−/− group, which were given an intragastric dose of Zn gluconate weekly for 14 weeks (0.04 mg Zn). The remaining ZD:Cox-2−/− mice continued on ZD diet. All mice were sacrificed for tumor outcome analysis at 14 weeks of Zn intervention.

Tumor analysis
At autopsy, tongue, esophagus and forestomach were excised. Tumors greater than 0.5 mm were mapped. Tissues were formalin-fixed and paraffin-embedded for histopathologic/IHC studies.

Quantitative reverse transcriptase-polymerase chain reaction
Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed using the comparative Ct method and predesigned probes on 7300 Real-time PCR System (Applied Biosystems, Foster City, CA). GAPDH was used to normalize RNA samples.14

Immunoblotting
Proteins were separated by 10–14% Tris-HCl gel (Bio-Rad, Hercules, CA) as described.13 GAPDH (Calbiochem, San Diego, CA) was used as a loading control.

IHC
IHC was performed as described.13–16 The following antisera were used: mouse anti-proliferating cell nuclear antigen (PCNA) monoclonal (Thermo Scientific); rat anti-S100A8 monoclonal, goat anti-S100A9 monoclonal, and rat anti-receptor for advanced glycation end-products (RAGE) monoclonal (R&D Systems, Minneapolis, MN); rabbit anti-nuclear factor (NF)-κB p65 polyclonal (Abcam, Cambridge, MA), rabbit anti-NF-κB phospho-p65 (serine 276) polyclonal and rabbit anti-cyclin D1 monoclonal antisera (Cell Signaling, Danvers, MA) or rabbit anti-p53 polyclonal antisera (detects both mutated and wild-type proteins) (Leica Microsystems, Bannockburn, IL). Protein was localized by incubation with 3-amino-9-ethylcarbazole substrate-chromogen (AEC) (Dako, Carpinteria, CA) or 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, St. Louis, MO).

Immunoreactive scores were calculated by multiplying the percentage of positive cells by the grade of staining intensity.15 The percentage of positive cells was evaluated as follows: 0 = 0–5%, 1 = 6–25%, 2 = 26–50%, 3 = 51–75% and 4 = 76–100%. The intensity of immunostaining was graded as follows: 0 = none, 1 = weak, 2 = moderate and 3 = intense. The PCNA labeling index (%) was calculated by dividing the number of PCNA-labeled nuclei by the total number of cells counted.

Zn measurement
Hair Zn content was determined by atomic absorption spectrometry.16 Invariably, Zn levels were significantly lower in ZD than ZS samples. As examples, hair Zn levels were significantly lower in ZD:Cox-2−/− than ZS:Cox-2−/− mice at 10 weeks (array study) [130 μg/g (95% confidence interval [CI] = 124–135) vs. 172 μg/g (95% CI = 165–179), p = 0.002, n = 10/group] and at 26 weeks (NQO study); 111 μg/g (95% CI = 99–122) vs. 157 μg/g (95% CI = 146–168), n = 14 mice/group, p < 0.001].

Statistical analysis
Tumor multiplicity was analyzed by two-way analysis of variance (ANOVA). Differences among the groups were assessed using the Tukey-HSD post hoc t-tests for multiple comparisons. Tumor and carcinoma incidence rates were assessed by Fisher’s exact test. CIs for the differences in incidence rates were calculated using the Wilson Score Method.26 Statistical tests were two-sided and considered significant at p < 0.05.

Results
ZD enhances tongue carcinogenesis in Cox-2 deficient mice
NQO is a DNA adduct-forming agent that serves as a surrogate of tobacco exposure.27 Nutritional complete WT mice exposed to 10 ppm of NQO for 50 weeks did not develop tongue lesions.28 At a high concentration of 100 ppm,
however, WT mice developed malignant tongue and esophageal tumors.29

To investigate whether a Zn-deficient condition eliminates the antitumor effect of genetic Cox-2 disruption in NQO-induced tongue carcinogenesis as it does in NMBA-induced forestomach carcinogenesis,16 Cox-2−/−, Cox-2+/−, and WT mice on ZD vs. ZS diets were exposed to drinking water containing 20 ppm of NQO for 19 weeks followed by 30 ppm for another 7 weeks. At week 26, ZS:Cox-2−/− and ZS:Cox-2+/− mice had significantly lower tongue/forestomach tumor incidence than ZS:WT littermates (Fig. 1a, statistical data in Supporting Information Table 1). This result is consistent with those reported in nutritionally complete mice showing that Cox-2 absence protects against carcinogenesis.16,21,30 Conversely, in ZD mice, genetic Cox-2 did not protect against carcinogenesis. ZD:Cox-2−/− mice had significantly higher tongue/esophageal tumor incidence than ZD:WT littermates, and ZD:Cox-2−/− and ZD:Cox-2+/− mice showed significantly higher tumor multiplicity in all three sites (tongue, esophagus and forestomach) than ZD:WT controls (incidence, Fig. 1c) and malignant progression of tongue/esophageal/forestomach tumors in ZD:Cox-2−/− and ZD:Cox-2+/− mice compared with ZD:WT mice (Fig. 1d), with statistical significance achieved for tongue SCC (ZD:Cox-2−/− vs. ZD:WT, 35.7% [5 of 14] vs. 0% [0 of 19], p < 0.01; ZD:Cox-2+/− vs. ZD:WT, 32.6% [15 of 46] vs. 0% [0 of 19], p < 0.01) (Fig. 1a, Supporting Information Table 1). These data demonstrated that prolonged ZD abolished the antitumor effect of COX-2 blockade in tongue tumor prevention and elicited tumors in multiple sites with progression to malignancy.

Among mice of the same genetic background, tongue tumor incidence/multiplicity and carcinoma incidence were significantly higher in homozygous ZD:Cox-2−/− vs. ZS:Cox-2−/− or heterozygous ZD:Cox-2−/− vs. ZS:Cox-2+/− mice, but not in ZD:WT vs. ZS:WT mice (Fig. 1a and b, Supporting Information Table 1), demonstrating that combined ZD and Cox-2 ablation led to a worse tumor outcome. These results are consistent with and extend our previous study in NMBA-induced forestomach carcinogenesis.16

**ZD per se induces an inflammatory gene signature in ZD:Cox-2−/− forestomach**

To test the hypothesis that ZD promotes carcinogenesis by activating cancer pathways not inhibited by genetic Cox-2 ablation, we performed transcriptome profiling of forestomach mucosa from ZD:Cox-2−/−, ZS:Cox-2−/−, ZD:WT and ZS:WT mice (n = 4/group). We used forestomach rather than tongue because its epithelia can be readily separated from the muscularis layers without enzymatic digestion.

First, we examined the effect of ZD on gene expression changes in Cox-2−/− forestomach and WT forestomach. By using a cutoff of p ≤ 0.05 and 2-fold difference in expression levels, we found 314 dysregulated probe sets in ZD:Cox-2−/− vs. ZS:Cox-2−/− forestomach (Supporting Information Table 2) but only 67 in ZD:WT vs. ZS:WT forestomach (Supporting Information Table 3). Thus, dietary ZD causes more extensive changes in gene expression in Cox-2−/− than WT forestomach. A cohort of 36 genes, including the proinflammation mediators S100a8/a9, small proline-rich protein 2 Spr2lf2h, and keratins Krt6a8/19, was common to both class comparisons, indicating that these genes were induced by ZD regardless of genotype.

Next, we compared the effect of Cox-2 deletion on gene expression changes in ZD forestomach and in ZS forestomach. With a cutoff of 2-fold difference, we found 90 dysregulated genes in ZD:Cox-2−/− vs. ZD:WT forestomach (Supporting Information Table 4) but only 17 in ZS:Cox-2−/− vs. ZS:WT forestomach (Supporting Information Table 5). There are no common changes in gene expression between these two class comparisons, and Cox-2 deletion causes fewer changes in ZS than ZD forestomach.

Our qRT-PCR data validated a total 12 selected genes for ZD:Cox-2−/− vs. ZS:Cox-2−/− forestomach; six genes for ZD:WT vs. ZS:WT, 7 genes for ZD:Cox-2−/− vs. ZD:WT, and three genes for ZS:Cox-2−/− vs. ZS:WT forestomach (Supporting Information Table 6).

Among the four class comparisons (Supporting Information Tables 2–5), ZD:Cox-2−/− vs. ZS:Cox-2−/− forestomach showed the most extensive changes in gene expression, a result consistent with their divergent tumorigenic potential16 (Fig. 1). Hierarchical clustering analysis of 45,000 transcripts revealed distinct expression patterns (Fig. 2a) between hyperplastic ZD:Cox-2−/− and nonproliferative ZS:Cox-2−/− forestomach (Fig. 2b). By further filtering the data using a cutoff of 4-fold difference, we identified a group of 63 genes (62 up- and 1 downregulated; Table 1). The most upregulated genes are implicated in the following processes: Spr2lf2h/2f and Krt6a8/16 in cytoskeleton metabolism and S100a8 and S100a9 (upregulated 24- and 2.2-fold) in inflammatory/defense/immune responses. Interestingly, S100a8/a9 were also upregulated 4.2- and 2.4-fold in ZD:WT vs. ZS:WT forestomach (Supporting Information Table 3). Because S100a8/a9 overexpression is associated with ZD-induced rat esophageal preneoplasia,14 the data that these same genes were upregulated by ZD in hyperplastic ZD:Cox-2−/− forestomach indicate that they are relevant ZD-induced markers in early forestomach carcinogenesis.

**DAVID bioinformatics reveals overrepresentation of inflammatory processes**

To define the biological significance of the large lists of differentially expressed genes captured by our transcriptome profiling (Supporting Information Tables 2–5), we performed gene ontology functional group analyses for the four class comparisons using DAVID resources.25 In preneoplastic ZD:Cox-2−/− vs. ZS:Cox-2−/− forestomach (Supporting Information Table 7a), we found significantly overrepresented biological processes only among the upregulated genes; including
Figure 1. Zn-deficiency abolishes the antitumor effects of genetic Cox-2 blockade in NQO-induced tongue carcinogenesis. a. Tumor incidence (%). ZS:Cox-2⁻/⁻ and ZS:Cox-2⁺/⁻ mice had significantly lower tongue/forestomach tumor incidence than ZS:WT mice (*p < 0.05, **p < 0.001). By contrast, ZD:Cox-2⁻/⁻ had greater tongue/esophagus tumor incidence than ZD:WT mice (*p < 0.05). ZD:Cox-2⁻/⁻ and ZD:Cox-2⁺/⁻ mice had greater tongue/esophagus/forestomach tumor incidence than ZS:Cox-2⁻/⁻ and ZS:Cox-2⁺/⁻ mice (††p < 0.01, †††p < 0.001). Carcinoma incidence (%): ZD:Cox-2⁻/⁻ and ZD:Cox-2⁺/⁻ mice had higher tongue carcinoma incidence than ZD:WT mice (**p < 0.01). Also, ZD:Cox-2⁻/⁻ and ZD:Cox-2⁺/⁻ mice had greater tongue carcinoma incidence than respective ZS:Cox-2⁻/⁻ and ZS:Cox-2⁺/⁻ (††p < 0.01, †††p < 0.001). b. Tumor multiplicity (error bars = SEM). ZD:Cox-2⁻/⁻ and ZD:Cox-2⁺/⁻ mice had significantly more tumors/site (tongue, esophagus, forestomach) than ZD:WT counterpart (*p < 0.05, **p < 0.01, and ***p < 0.001). In addition, ZD:Cox-2⁻/⁻ and ZD:Cox-2⁺/⁻ mice had significantly more tumors/site than ZS:Cox-2⁻/⁻ and ZS:Cox-2⁺/⁻ counterpart (††p < 0.01, †††p < 0.001). c. Gross anatomy of tongue/esophagus/forestomach. Scale bars, 5 mm. d. H&E-stained sections showing tongue/esophagael/forestomach carcinomas from ZD:Cox-2⁻/⁻ and ZD:Cox-2⁺/⁻ and thickened mucosa from ZS:Cox-2⁻/⁻ mice. Scale bar = 100 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
in particular, response to external stimulus comprising S100a8/a9 and 14 genes \((p = 3.98 \times 10^{-4})\) and response to stimulus comprising S100a8/a9 and 34 genes \((p = 5.04 \times 10^{-4})\). Thus, DAVID supports the premise that S100a8/a9 are relevant markers associated with ZD-induced hyperplasia in ZD: Cox-2−/− forestomach. Similarly, in ZD:WT vs. ZS:WT (Supporting Information Table 7b) and ZD:Cox-2−/− vs. ZD:WT forestomach (Supporting Information Table 7c), significantly overrepresented processes were found only among the upregulated genes, including cytoskeleton and chemotaxis processes. By contrast, in ZS:Cox-2−/− vs. ZS:WT forestomach (Supporting Information Table 7d), significantly overrepresented processes were found only among the downregulated genes that negatively modulated cell cycle and cytoskeleton processes. Together, the data revealed that dietary ZD and sufficiency led to distinct regulated processes in proliferation in Cox-2−/− forestomach, a finding consistent with the divergent tumorigenic potential of ZD:Cox-2−/− vs. ZS:Cox-2−/− forestomach.

IPA reveals a NF-κB—centric network
To understand gene expression interactions in ZD:Cox-2−/− vs. ZS:Cox-2−/− forestomach (Table 1) in the context of signaling pathways, we performed pathway analysis using IPA. We identified a nuclear factor (NF)-κB centric network of 35 genes, with 60% of the genes (21 of 35) from the upregulated genes that included S100a8 (Fig. 2c). Because NF-κB is a transcription factor that regulates immune responses/cell proliferation and it is a link between inflammation and cancer development/progression,31 our result that NF-κB showed connectivity to S100a8 predicted activation of a S100A8-NF-κB inflammatory pathway in ZD:Cox-2−/− forestomach.

ZD activates S100A8 inflammatory signaling in preneoplastic ZD:Cox-2−/− forestomach
We focused our study on S100A8 and its heterodimeric partner S100A9 because of their role in inflammation and cancer,14,32 and their prominence among ZD-induced
Table 1. Gene expression signature in hyperplastic ZD:Cox-2\(^{-/-}\) vs non-proliferative ZS:Cox-2\(^{-/-}\) mouse forestomach

| Gene symbol | Affymetrix id   | p       | FDR   | Fold-change, ZD:Cox-2\(^{-/-}\) vs ZS:Cox-2\(^{-/-}\) | Gene title                          | Function                              |
|-------------|----------------|---------|-------|--------------------------------------------------|--------------------------------------|---------------------------------------|
| Sprr2h      | 1422240_s_at   | 0.00005 | 0.17  | 64                                               | Small proline-rich protein 2H       | Keratinocyte differentiation           |
| Sprr2f      | 1449833_at     | 0.00009 | 0.21  | 49                                               | Small proline-rich protein 2F       | Epithelial to mesenchymal transition  |
| Krt16       | 1448932_at     | 0.00006 | 0.17  | 47                                               | Keratin 16                         | Intermediate filament cytoskeleton     |
| Krt6a       | 1422784_at     | 0.00056 | 0.26  | 45                                               | Keratin 6A                         | Intermediate filament organization     |
| Chi3l4      | 1425450_at     | 0.00431 | 0.28  | 39                                               | Chitinase 3-like 4                  | Inflammatory response                 |
| Defb3       | 1421806_at     | 0.00000 | 0.02  | 29                                               | Defensin beta 3                    | Defense response                      |
| Dmbt1       | 1418287_a_at   | 0.00015 | 0.23  | 27                                               | Deleted in malignant brain tumors 1| Positive regulation of epithelial cell|
| *S100a8     | 1419394_s_at   | 0.00000 | 0.06  | 24                                               | S100 calcium binding protein A8 (calgranulin A)| Chemotaxis                           |
| Mcpt2       | 1449989_at     | 0.00071 | 0.26  | 23                                               | Mast cell protease 2               | Proteolysis                           |
| Pigr        | 1450060_at     | 0.00259 | 0.27  | 17                                               | Polymeric immunoglobulin receptor  | Receptor activity                     |
| U46068      | 1423719_at     | 0.00309 | 0.28  | 17                                               | cDNA sequence U46068               | Lipid binding                         |
| Chi3l3      | 1419764_at     | 0.00096 | 0.26  | 17                                               | Chitinase 3-like 3                 | Inflammatory response                 |
| Ltf         | 1450009_at     | 0.00096 | 0.26  | 15                                               | Lactotransferrin                   | Cellular iron ion homeostasis         |
| LOC67527    | 1431213_a_at   | 0.00028 | 0.26  | 14                                               | Murine leukemia retrovirus         | NA                                    |
| Mcpt1       | 1422352_at     | 0.00043 | 0.26  | 14                                               | Mast cell protease 1               | Proteolysis                           |
| A130040M12Rik| 1428909_at   | 0.00042 | 0.26  | 13                                               | RIKEN cDNA A130040M12 gene         | NA                                    |
| 1190003M12Rik| 1429286_at  | 0.00310 | 0.28  | 12                                               | RIKEN cDNA 1190003M12 gene         | NA                                    |
| Tff2        | 1422448_at     | 0.00984 | 0.30  | 12                                               | Trefoil factor 2 (spasmolytic protein 1)| Mucosal defense                      |
| Krt17       | 1423227_at     | 0.00100 | 0.26  | 11                                               | Keratin 17                         | Intermediate filament organization     |
| Agr2        | 1419268_at     | 0.00242 | 0.26  | 10                                               | Anterior gradient 2 (Xenopus laevis)| Mucosal defense                      |
| 2310046K23Rik| 1454264_at   | 0.00317 | 0.28  | 9.3                                              | RIKEN cDNA 2310046K23 gene         | NA                                    |
| Krt19       | 1417156_at     | 0.00878 | 0.30  | 8.9                                              | Keratin 19                         | Intermediate filament                 |
| C10a3       | 1416306_at     | 0.01335 | 0.32  | 8.3                                              | Chloride channel calcium activated 3| Calcium ion transport                 |
| Anxa10      | 1449426_a_at   | 0.03486 | 0.35  | 8.2                                              | Annexin A10                        | Calcium-dependent phospholipid binding|
| Defb4       | 1419600_at     | 0.00152 | 0.26  | 8.1                                              | Defensin beta 4                    | Defense response                      |
| Stfa1       | 1435761_at     | 0.00228 | 0.26  | 8.0                                              | Stefin A1                          | Cysteine protease inhibitor activity  |
| Cfi         | 1418724_at     | 0.00192 | 0.26  | 7.9                                              | Complement component factor i      | Innate immune response                |
| Spp1        | 1449254_at     | 0.00022 | 0.23  | 7.9                                              | Secreted phosphoprotein 1          | Cell adhesion                         |
| Krt18       | 1448169_at     | 0.00374 | 0.28  | 7.9                                              | Keratin 18                         | Intermediate filament                 |
Table 1. Gene expression signature in hyperplastic ZD:Cox-2-/- vs non-proliferative ZS:Cox-2-/- mouse forestomach (Continued)

| Gene symbol | Affymetrix id | p  | FDR  | Fold-change, ZD:Cox-2-/- vs ZS:Cox-2-/- | Gene title | Function |
|-------------|---------------|----|------|----------------------------------------|------------|----------|
| Krt8        | 1420647_a_at  | 0.01074 | 0.31 | 7.1                                    | Keratin 8  | Intermediate filament |
| Idi2        | 1440852_at    | 0.02160 | 0.33 | 6.8                                    | Isopentenyl-diphosphate delta isomerase 2 | Isoprenoid biosynthetic process |
| Crisp1      | 1416325_at    | 0.00134 | 0.26 | 6.7                                    | Cysteine-rich secretory protein 1 | NA |
| Cfr         | 1420579_s_at  | 0.00236 | 0.26 | 6.6                                    | Cystic fibrosis transmembrane conductance regulator homolog | Ion transport |
| Igh         | 1421653_a_at  | 0.00387 | 0.28 | 6.0                                    | Immunoglobulin heavy chain complex | Immune response |
| Tspan8      | 1424649_a_at  | 0.02129 | 0.33 | 5.9                                    | Tetranspanin 8 | Signal transduction |
| Csect        | 1418989_at    | 0.03346 | 0.35 | 5.9                                    | Cathepsin E | Aspartyl protease activity |
| Dsc2        | 1421156_a_at  | 0.00036 | 0.26 | 5.9                                    | Desmocollin 2 | Cell adhesion |
| 2610528A11Rik | 1435639_at   | 0.00555 | 0.28 | 5.8                                    | RIKEN cDNA 2610528A11 gene | NA |
| Ifi202b     | 1421551_s_at  | 0.00020 | 0.23 | 5.6                                    | Interferon activated gene 202B | Proteolysis |
| Kik1        | 1415837_at    | 0.00503 | 0.28 | 5.5                                    | Kallikrein 1 | Proteolysis |
| Ghrl        | 1448980_at    | 0.02208 | 0.33 | 5.5                                    | Ghrelin | Dendrite development |
| Krt20       | 1426284_at    | 0.00750 | 0.29 | 5.4                                    | Keratin 20 | Cellular response to stress |
| Gcnt3       | 1440409_at    | 0.01574 | 0.32 | 5.4                                    | Glucosaminyl (N-acetyl) transferase 3, mucin type | Transferase activity |
| Clcn2       | 1417231_at    | 0.00229 | 0.26 | 5.3                                    | Claudin 2 | Calcium-independent cell-cell adhesion |
| Clcn7       | 1448393_at    | 0.00018 | 0.23 | 4.8                                    | Claudin 7 | Structural molecule activity |
| Clcn18      | 1449428_at    | 0.04404 | 0.36 | 4.7                                    | Claudin 18 | Tight junction |
| 1200016E24Rik | 1435137_s_at  | 0.00003 | 0.16 | 4.7                                    | RIKEN cDNA 1200016E24 gene | NA |
| Krt6b       | 1422588_at    | 0.00143 | 0.26 | 4.6                                    | Keratin 6B | Intermediate filament |
| Ceacam1     | 1425538_x_at  | 0.00140 | 0.26 | 4.6                                    | Carcinoembryonic antigen-related cell adhesion molecule 1 | Positive regulation of MAP kinase activity |
| Scd2        | 1415824_at    | 0.00166 | 0.26 | 4.5                                    | Stearoyl-Coenzyme A desaturase 2 | Lipid metabolic process |
| Muc1        | 1449199_at    | 0.01496 | 0.32 | 4.4                                    | Mucin 1, transmembrane | Cytoskeleton |
| Lcn2        | 1427747_at    | 0.02630 | 0.34 | 4.4                                    | Lipocalin 2 | Transporter activity |
| Prom1       | 1419700_a_at  | 0.01526 | 0.32 | 4.3                                    | Prominin 1 | maintaining stem cell properties |
| Anxa8       | 1417732_at    | 0.02923 | 0.34 | 4.2                                    | Annexin A8 | Calcium-dependent phospholipid binding |
| Aqp5        | 1418818_at    | 0.00460 | 0.28 | 4.2                                    | Aquaporin 5 | Transporter activity |
| Muc4        | 1438555_x_at  | 0.01402 | 0.32 | 4.1                                    | Mucin 4 | Cell adhesion |
| Tcrb-J      | 1452205_x_at  | 0.00043 | 0.26 | 4.1                                    | T-cell receptor beta, joining region | NA |
| C130090K23Rik | 1426268_at  | 0.00725 | 0.29 | 4.1                                    | RIKEN cDNA C130090K23 gene | NA |
| Ppbp        | 1418480_at    | 0.00167 | 0.26 | 4.0                                    | Pro-platelet basic protein | Chemotaxis |
proinflammation markers in ZD:Cox-2/−/− forestomach (Supporting Information Table 7a). S100a8/a9 genes encode the S100 family member calcium binding proteins. Interaction of S100A8/A9 ligands with their receptor RAGE triggers an intracellular NF-κB signaling cascade.\(^1\),\(^3\),\(^2\) To determine if there is a link between S100A8 overexpression and downstream NF-κB signaling in preneoplastic ZD:Cox-2/−/− vs. ZS:Cox-2/−/− forestomach as predicted by IPA (Fig. 2c), we analyzed expression of five signaling markers, namely, S100A8, S100A9, the RAGE receptor, NF-κB p65, and cyclin D1, by IHC. The cyclin D1 gene is a target of NF-κB activation.\(^3\)\(^3\) IHC showed that all five markers were more strongly expressed in hyperplastic ZD:Cox-2/−/− forestomach than wildtype ZS:Cox-2/−/− forestomach (Fig. 3a). The semi-quantitative mean immunoreactive scores of S100A8 and S100A9 protein were significantly higher in ZD:Cox-2/−/− than ZS:Cox-2/−/− forestomach (S100A8, 7.3 (95% CI = 5.5–9.1) vs. 1.6 (95% CI = 0.9–2.3), \(p < 0.001\); S100A9, 6.6 (95% CI = 3.7–9.5) vs. 1.5 (95% CI = 0.9–2.1), \(p < 0.001\), \(n = 8\) mice/group). In addition, ZD:Cox-2/−/− forestomach overexpressed phospho-NF-κB p65, indicating activation and nuclear translocation of NF-κB p65 (Supporting Information Fig. 1). These data show that at the earliest stages of forestomach carcinogenesis ZD activates an alternative S100A8 inflammatory pathway not affected by genetic Cox-2 inhibition.

**ZR reverses S100A8 inflammatory signaling and hyperplasia in ZD:Cox-2/−/− forestomach**

Because ZR attenuates inflammation and reverses hyperplasia in early rat esophageal carcinogenesis,\(^14\) we investigated this effect in ZD:Cox-2/−/− mice one week after switching to a ZS diet. In contrast to ZD:Cox-2/−/− forestomach that showed strong expression of all five S100A8 inflammatory signaling markers (Fig. 3a [left column]), ZR:Cox-2/−/− mouse forestomach had reduced or absent immunostaining of the same five markers (Fig. 3b). Additionally, qRT-PCR analysis shows that S100a8 and S100a9 mRNA expression was significantly reduced in ZR:Cox-2/−/− vs. ZD:Cox-2/−/− forestomach (Fig. 3c).

In addition, we determined the rate of cell proliferation in ZR:Cox-2/−/− vs. ZD:Cox-2/−/− forestomach by quantitative PCNA-IHC (PCNA: Fig. 3b vs. 2b). PCNA is an endogenous cell proliferation marker. The PCNA-labeling index (%) was significantly lower in ZR:Cox-2/−/− than ZD:Cox-2/−/− forestomach (Fig. 3d). Together, these data (Fig. 3b–d) document that ZR effectively attenuated S100A8 inflammation and reversed the hyperplastic ZD:Cox-2/−/− phenotype.

**ZR upregulates S100a8/a9 expression in preneoplastic ZD:Cox-2/−/− tongue**

To determine whether ZD:Cox-2/−/− tongue, which shows high tumorigenic potential as does ZD:Cox-2/−/− forestomach\(^16\) (Fig. 1), also overexpresses the proinflammation genes S100a8/a9 discovered in forestomach, we determined S100a8/a9 mRNA and protein expression levels in tongue and forestomach from the four mouse groups (profiling studies) by qRT-PCR and immunoblotting. As in forestomach, S100a8/a9 mRNA expression was strongest in ZD:Cox-2/−/− tongue, followed by ZD:WT tongue, and negligible in ZS:Cox-2/−/− and ZS:WT tongue (Fig. 3e, top). In parallel, S100A8/A9

| Gene symbol | Affymetrix id | \(p\)  | FDR | Fold-change, ZD:Cox-2/−/− vs ZS:Cox-2/−/− | Gene title | Function |
|-------------|---------------|-------|-----|------------------------------------------|------------|----------|
| Igk-V1      | 1427660_x_at  | 0.00994 | 0.30 | 4.0                                      | Immunoglobulin kappa chain variable 1 (V1) | NA       |
| AA667197    | 1434046_at    | 0.01196 | 0.31 | 4.0                                      | Expressed sequence AA667197               | NA       |
| F5          | 1418907_at    | 0.00510 | 0.28 | 4.0                                      | Coagulation factor V                      | Blood circulation |
| EG668468    | 1451699_at    | 0.00347 | 0.28 | 3.7                                      | predicted gene, EG668468                | NA       |
| 100042999   | 1437258_at    | 0.00395 | 0.28 | 3.7                                      | Predicted gene, 100042999                 | NA       |
| Chad        | 1420569_at    | 0.01608 | 0.32 | 3.7                                      | Chondroadherin                           | Protein binding |
| S100a9      | 1448756_at    | 0.00329 | 0.28 | 2.2                                      | S100 calcium binding protein A9 (calgranulin B) | Chemotaxis |

The list includes genes that are significantly upregulated or downregulated \(\geq 4\)-fold in ZD:Cox-2/−/− vs ZS:Cox-2/−/− mouse forestomach. Multiple sets for the same gene are excluded.

\(*S100a9\) (up 2.2-fold), a heterodimer of \(*S100a8\), is included for reference.

\[8521416612\_at 0.00285 0.28 0.16 Cytochrome P450, family 1, subfamily a, polypeptide 1 | Toxin metabolic process]
protein expression was strong in both ZD:Cox-2⁻/⁻ tongue and forestomach but weak or absent in similar tissues of other mouse groups (Fig. 3e, bottom). These data suggests that in tongue and forestomach ZD activates similar inflammatory pathways that are not affected by COX-2 inhibition.

Activation of S100A8 and p53 inflammatory pathways accompanies malignant tumor progression in ZD:Cox-2⁻/⁻ and ZD:Cox-2⁺/⁺ mice

We then went on to investigate whether during malignant tongue/forestomach tumor progression (Fig. 1) S100A8 inflammatory signaling is in fact activated. In addition, we determined whether these carcinomas overexpress PCNA and p53 protein, two prognostic factors in human oral cancers.34,35 The p53 tumor suppressor gene is mutated in approximately 50% of all human cancers, including oral-esophageal cancers36 and divergent carcinogenic pathways mediated separately by NF-kB and p53 were reported in oral cancer.57 Using IHC we examined expression of seven markers: PCNA, p53, and five S100A8—NF-kB signaling markers (S100A8, S100A9, RAGE, NF-kB p65, and cyclin D1). We analyzed a total of 15 ZD:Cox-2⁻/⁻ tongue SCC and 6 ZD:Cox-2⁺/⁺ forestomach SCC, as well as non-neoplastic ZS:Cox-2⁻/⁻ tongue SCC and ZS:Cox-2⁻/⁻ forestomach (n = 10/group).

ZD:Cox-2⁻/⁻ tongue SCC and ZD:Cox-2⁺/⁺ forestomach SCC showed high proliferative activity with abundant PCNA-positive nuclei in tumor areas and prominent accumulation of intensely stained p53-positive nuclei. Concurrently, these carcinomas displayed strong co-overexpression of all five S100A8—NF-kB signaling markers (Fig. 4). In addition, these carcinomas overexpressed phospho-NF-kB p65 (Supporting Information Fig. 1), indicating activation and nuclear translocation of NF-kB p65. Collectively, these data demonstrate that under complete or partial genetic Cox-2 ablation, ZD stimulated RAGE-S100A8 inflammatory signaling cancer-and p53-associated response pathways, thereby driving malignant growth.
Figure 4. Zn-deficiency activates RAGE-S100A8/9 signaling and p53 accumulation in ZD:Cox-2<sup>+/−</sup> and ZD:Cox-2<sup>−/−</sup> tongue/forestomach carcinomas. IHC of 7 markers: PCNA, p53, S100A8, S100A9, RAGE, NF-κB p65 and cyclin D1 (red, AEC; brown, DAB; black, DAB with cobalt chloride). Scale bars, 50 μm (PCNA); 25 μm (all other markers). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
tumor progression and bypassing the antitumor effect of COX-2 blockade.

In sharp contrast, non-neoplastic ZS: Cox-2−/− tongue and ZS: Cox-2−/− forestomach showed basal cell proliferation with isolated occurrence of p53 protein, as well as low levels of expression of the same five S100A8 signaling markers (Fig. 4), providing evidence that inflammatory pathways were not activated under conditions of COX-2 pathway blockade and ZS that protected against carcinogenesis.

**ZR attenuates the inflammation and restores the antitumor effect of COX-2 blockade in cancer prevention**

Finally, we investigated whether replenishing Zn can restore the antitumor effect of COX-2 blockade in tumor prevention. In a NMBA-induced forestomach carcinogenesis study, we showed that 14 weeks after ZR, ZR: Cox-2−/− mice had significantly lower forestomach tumor incidence and multiplicity than ZD: Cox-2−/− mice (Fig. 5a). In addition, S100a8/a9 mRNA expression was significantly lower in ZR:Cox-2−/− vs. ZD:Cox-2−/− forestomach (15 mice/group were analyzed). Scale bars, 100 μm (H&E); 50 μm (PCNA; ZD:Cox-2−/−: p53, S100A8, S100A9, cyclin D1); 25 μm (ZR:Cox-2−/−: p53, S100A8, S100A9, cyclin D1). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

In a NMBA-induced forestomach carcinogenesis study, we showed that 14 weeks after ZR, ZR:Cox-2−/− mice had significantly lower forestomach tumor incidence and multiplicity than ZD:Cox-2−/− mice (Fig. 5a). In addition, S100a8/a9 mRNA expression was significantly lower in ZR:Cox-2−/− vs. ZD:Cox-2−/− forestomach (Fig. 5b); S100A8/A9 protein expression was absent in ZR:Cox-2−/− but strongly expressed in ZD:Cox-2−/− forestomach (Fig. 5c). Histopathologic and IHC studies show that ZR:Cox-2−/− forestomach mucosa was typically thin, with PCNA-positive nuclei mainly in basal cells and weak to negligible immunostaining of the 4 inflammation-associated markers S100A8, S100A9, p53, and cyclin D1 (Fig. 5d). In contrast, neoplastic ZD:Cox-2−/− forestomach was highly proliferative, with PCNA-positive nuclei in

| Mouse group (n) | Hair Zn level ppm (95% CI) | Tumor incidence (%) | Number of tumors per forestomach (95% CI) |
|----------------|---------------------------|---------------------|----------------------------------------|
| ZR: Cox-2−/− (18) | 165 (159 to 172) | 6/18 (33) | 0.83 (0.21 to 1.45) |
| ZD: Cox-2−/− (21) | 140 (134 to 146) | 17/21 (81) | 3.1 (2.0 to 4.2) |
| P-value | 0.001 | 0.004 | 0.0008 |

**Figure 5.** Zn-replenishment reverses inflammatory responses and inhibits NMBA-induced forestomach carcinogenesis. a. Tumor data. b. qRT-PCR analysis of S100a8/a9 mRNA expression in ZR:Cox-2−/− and ZD:Cox-2−/− forestomach (error bars = SEM; n = 6 mice/group). c. Immunoblotting of S100A8/A9 protein expression in ZR:Cox-2−/− and ZD:Cox-2−/− forestomach. d. H&E and IHC of PCNA, p53, S100A8, S100A9 and cyclin D1, in ZR:Cox-2−/− vs. ZD:Cox-2−/− forestomach (15 mice/group were analyzed). Scale bars, 100 μm (H&E); 50 μm (PCNA; ZD:Cox-2−/−: p53, S100A8, S100A9, cyclin D1); 25 μm (ZR:Cox-2−/−: p53, S100A8, S100A9, cyclin D1). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
many cell layers and strong overexpression of the same inflammation-associated markers (Fig. 5d). Thus, ZR reverses preneoplasia (Figs. 3b–3d), and effectively restores the antitumor effect of Cox-2 ablation (Fig. 5) by attenuating the inflammation.

Discussion

Increasingly cancers are treated with drugs that target specific pathways shown to be of pathogenetic significance. Our study shows that the antitumor effect of genetic disruption of Cox-2 in tongue cancer prevention is bypassed by Zn depletion (Fig. 1), owing to activation of an alternative preneoplastic pathway that is not affected by COX-2 inhibition. Using a combination of techniques that included expression profiling, bioinformatics and investigation of identified markers in ZD:Cox-2+/− mouse models of oral-esophageal cancers, our data document a mechanism for the inability of COX-2 blockade to prevent tumor growth under ZD conditions.

The hyperplastic ZD:Cox-2+/− vs. ZS:Cox-2+/− forestomach has a distinct signature (Table 1). The pro-inflammation mediators S100 a8 and S100 a9 are upregulated 24-fold and 2.2-fold. In addition, the typical genes of the cornified envelope Sprz2h/2f and Krt6A/16/17/8/20 are upregulated 64- to 5.5-fold. Because simultaneous upregulation of S100A8/A9, SPRR2 and KRT6A/16/17 is a common feature of human inflammatory skin diseases such as psoriasis, and atopic dermatitis, this same signature in ZD:Cox-2+/− forestomach indicates an association between inflammation and its highly hyperplastic phenotype. Our conclusion that S100a8/a9 are relevant ZD-induced markers belonging to an inflammatory pathway that drives forestomach cell proliferation rather than an epiphenomenon of this process or of dietary Zn-deficit is supported by DAVID bioinformatics (Table 7a).

S100A8/A9 have emerged as important markers for inflammation-associated cancers. They are overexpressed in many human cancers, including lung, colorectal, prostate, skin cancer, as well as HPV18-infected oral SCC. The mechanistic role of S100A8/A9 in tumor biology is emerging. In a mouse skin cancer model, Gebhardt et al. provided genetic evidence that S100A8/A9 binds to RAGE, and RAGE signaling sustains skin inflammation and promotes tumorigenesis. In the lung, S100A8/A9 induces the activation of serotonin amyloid A that activates NF-kB, and facilitates metastasis. In a colitis-induced mouse cancer model, S100A8/A9 and RAGE augment carcinogenesis and in an inflammation-associated liver cancer model, S100A8/A9 are identified as NF-kB target genes and their overexpression promotes malignant progression. Conversely, blockade of RAGE suppresses tumor growth and metastasis.

Our IHC data in ZD:Cox-2+/− tongue and ZD:Cox-2+/− forestomach carcinomas (Fig. 4) demonstrate that under complete or partial blockade of COX-2 pathway dietary ZD activates an alternative cancer-associated RAGE-S100A8 inflammatory pathway. The finding that these same carcinomas showed high PCNA proliferative activity and prominent accumulation of p53 protein indicates that additional inflammation-associated cancer pathways are activated. The p53 tumor suppressor gene is mutated in human oral and esophageal cancer. Mutated p53 protein has a prolonged half-life that leads to its accumulation in the nucleus. In this regard, human head and neck squamous cell cancer (HNSCC), which is a highly inflammatory, proliferative and aggressive cancer, exhibits high levels of p53 expression, abundant cell proliferative activity, as well as divergent carcinogenic pathways mediated separately by NF-kB and p53.

Chronic inflammation contributes to the development of ~20% of all human cancers. The causes of inflammation are often unknown. Our recent report in rat esophagus that dietary Zn regulates S100A8 expression and modulates the link between S100A8-RAGE and downstream NF-kB/COX-2 provides the first evidence that Zn has an inflammation-modulating role in esophageal cancer initiation/reversal. Here we demonstrate that with COX-2 pathway blockade prolonged dietary ZD causes chronic inflammation in the tongue/forestomach by activating alternative inflammatory RAGE-S100A8/A9 and p53 response pathways, thereby fueling tumor progression and bypassing the antitumor effect of Cox-2 deletion. These new data provide a likely mechanism to explain the inefficacy of such targeted cancer therapy in oral-cancer patients, since many of these patients are frequently Zn-deficient.

Recent studies reported that Zn supplementation improves clinical outcomes in patients receiving radiotherapy for HNSCC, as well as concomitant chemotherapy and radiotherapy for advanced nasopharyngeal carcinoma. The present finding that ZR attenuates the inflammatory response and restores the antitumor effect of COX-2 blockade has important clinical implications. Thus, stratification of patients by Zn status would be useful, and a personalized cancer therapeutic paradigm that includes Zn may improve efficacy.

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