Research Article

Cancer Prevention Research

Chemopreventive and Antitumor Efficacy of Curcumin in a Spontaneously Developing Hen Ovarian Cancer Model

Kazim Sahin[1], Cemal Orhan[1], Mehmet Tuzcu[2], Nurhan Sahin[1], Hakkı Tastan[3], İbrahim Hanifi Özercan[4], Osman Güler[5], Nermin Kahraman[6], Omer Kucuk[7], and Bulent Ozpolat[6,8]

Abstract

We investigated the effect of daily dietary curcumin intake on the development and progression of spontaneous ovarian cancer in a galline (hen) model, as the chicken is the only nonhuman animal in which ovarian cancer spontaneously develops with a high prevalence. At the end of 12 months, ovarian cancer had spontaneously developed in 39% (35/90) of control hens not fed curcumin ($n = 90$). In comparison, it spontaneously developed in 27% (24/90) and 17% (15/90) of hens given curcumin at 25.8 ($n = 90$) and 53.0 mg/day ($n = 90$), respectively ($P = 0.004$). This represented significant dose-dependent reductions in overall ovarian cancer incidence in the 25.8 and 53.0 mg/day curcumin-fed groups (31% and 57%, respectively). Daily curcumin intake also reduced ovarian tumor sizes ($P = 0.04$) and number of tumors ($P = 0.006$). Evaluation of the molecular mechanisms underlying the chemopreventive and antitumor effects of curcumin revealed that NF-κB and STAT3 signaling pathways were significantly inhibited but that the nuclear factor erythroid 2/heme oxygenase 1 antioxidant pathway was induced by curcumin intake in a dose-dependent manner in ovarian tissues ($P < 0.05$). Sequencing of the Ras family genes (*KRAS, NRAS,* and *HRAS)* revealed less frequent *KRAS* and *HRAS* mutations in ovarian tumors in the curcumin-fed animals. In conclusion, our results demonstrated for the first time that daily curcumin intake leads to a significant and dose-dependent reduction in spontaneous ovarian cancer incidence and tumor growth, indicating a tremendous role for curcumin as a chemopreventive strategy for ovarian cancer. *Cancer Prev Res; 11(1); 59–67. ©2017 AACR.*

Introduction

Despite therapeutic advancements, ovarian cancer continues to be the most lethal gynecological cancer, having a 30% 5-year survival rate and causing more than 14,000 deaths per year in the United States (1). The poor survival is directly attributable to a lack of early diagnostic tests and effective treatment strategies owing to significant intratumoral heterogeneity, the aggressive nature of the disease, and early metastatic spread (2, 3). Therefore, identification of effective chemopreventive strategies, understanding of molecular mechanisms driving malignant transformation and tumorigenesis, and development of therapeutic strategies are urgently needed to reduce ovarian cancer incidence and improve its clinical outcome, survival, and prognosis.

Curcumin (diferuloylmethane) is a nonflavonoid polyphenolic compound found in turmeric (*Curcuma longa* Linn) known for its antioxidant and anti-inflammatory properties (4, 5). Moreover, quite a few studies, including studies performed by our group, have demonstrated that curcumin exhibits activity against various types of cancer, including breast, pancreatic, and colon cancer by modulating multiple oncogenic signaling pathways (6, 7). Curcumin also has exerted antiproliferative and antitumor effects *in vitro* and *in vivo* in ovarian cancer models (8–11). Furthermore, people who consume curcumin-rich diets, such as Indian populations, are known to have significantly reduced incidence of some cancers, including ovarian cancer (12, 13). Curcumin consumption has demonstrated promising results in studies of cancer prevention and treatment using various rodent models of carcinogen-induced mammary cancer, hepatocellular carcinoma, adenomatous polyposis, esophageal cancer, and colon cancer (4, 8, 14–16). Curcumin is reported to have multiple mechanisms of action that are involved in the regulation of programmed cell death and survival pathways by modulating the expression and the activity of transcription factors, growth factors, inflammatory cytokines, receptors, and

[1]Department of Animal Nutrition, Faculty of Veterinary Medicine, Firat University, Elazig, Turkey. [2]Department of Biology, Firat University, Elazig, Turkey. [3]Department of Genetics Biology, Faculty of Science, Firat University, Elazig, Turkey. [4]Department of Pathology, Faculty of Medicine, Firat University, Elazig, Turkey. [5]Department of Pharmacology, Veterinary Control Research and Institute, Firat University, Elazig, Turkey. [6]Department of Experimental Therapeutics, the University of Texas MD Anderson Cancer Center, Houston, Texas. [7]Winship Cancer Institute of Emory University, Atlanta, Georgia. [8]Center for RNA Interference and Non-Coding RNA, the University of Texas MD Anderson Cancer Center, Houston, Texas.

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Corresponding Authors: Bulent Ozpolat, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: 713-563-0166; Fax: 713-792-0362; E-mail: Bozpolat@mdanderson.org; and Kazim Sahin, Department of Animal Nutrition, School of Veterinary Medicine and Science, Firat University, Elazig, Turkey. Phone: 90-424-2370000; Fax: 90-424-23881; E-mail: ksahin@firat.edu.tr

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enzymes (4, 6, 17). In addition to anti-inflammatory actions and reactive oxygen stress scavenging, curcumin modulates NF-κB and p53 signaling and the transcription of genes such as p21 and Bax, leading to cell-cycle arrest and apoptosis in a variety of cancer cells (18).

Recent studies demonstrated that the laying hen (*Gallus gallus domesticus*) is the only nonhuman animal that experiences spontaneous development of ovarian cancer, occurring at a high prevalence rate of up to 35%. Thus, it is an emerging experimental model for studying the development and progression of this cancer (19, 20). More importantly, ovarian cancer in hens has histology and morphology similar to those of the human disease and shares many clinical and pathologic features with the frequently occurring epithelial subtypes of human ovarian cancer, such as metastasis and production of ascites (21). The common histopathologic subtypes of ovarian cancer in hens include high-grade serous cancer, and similar molecular pathways and markers, such as CA-125 expression and mutation frequency. More recent molecular characterizations of galline ovarian cancer demonstrated naturally occurring genetic mutations, such as K-Ras and Her2/neu (22–24). In addition to CA-125, expression of mesothelin, cyclooxygenase-1, selenium-binding protein 1, E-cadherin, and VEGF is similarly altered in human and galline ovarian tumors (21, 25, 26).

The factors described above demonstrate that the chicken model of spontaneous ovarian cancer is a relevant experimental model for the study of ovarian cancer chemoprevention, carcinogenesis, development, and progression and is an invaluable tool for translational research. Therefore, in the present study, we evaluated the chemopreventive and antitumor effects of curcumin in a chicken model of spontaneous ovarian cancer. We found that a daily diet enriched in curcumin significantly reduced the incidence of spontaneous ovarian cancer in a dose-dependent manner. We also found significant inhibition of the size and growth of ovarian tumors in curcumin-fed chickens and that is associated with reduced NF-κB and STAT3 signaling and induced nuclear factor erythroid 2 (Nrf2)/heme oxygenase 1 (HO-1) antioxidant pathway by curcumin intake in a dose-dependent manner and reduction in mutations in the *KRAS* and *NRAS* genes, suggesting a potential role for curcumin as a chemopreventive strategy for ovarian cancer.

Materials and Methods

Animals and experimental design

A total of 270 brown laying hens (104 weeks old; White leghorn, strain W96) were used in accordance with animal welfare regulations and under the Guide for the Care and Use of Laboratory Animals of the Institute at the Veterinary Control and Research Institute (Elazig, Turkey). The protocol was approved by the Institutional Animal Care and Use Committee at the Veterinary Control and Research Institute. Hens were assigned randomly to one of three groups given basal diets containing (1) 0 (control), (2) 200, or (3) 400 mg of curcumin per kilogram of diet over 12 months. Curcumin (CurcuWIN), which was tested in a human clinical trial (27), was obtained from OmniActive Health Technologies. The chickens in the 200 and 400 mg/kg curcumin groups were given standard diets including 25.8 and 53.0 mg of curcumin per day, respectively. Briefly, depending on the dose, small amount of curcumin was mixed with a larger amount of the basal diet until the total amounts of the respective diets were homogenously mixed. Chickens typically consumed about 130 g standard diet with or without curcumin in a day. Diets were stored in black plastic containers at 4°C to protect against oxidation. The nutrient composition of the standard diet is listed in Supplementary Table S1. The hens had free access to water and food *ad libitum* and were housed using a 14-hour:10-hour light:dark cycle.

Sample collection

At the end of the study, blood samples were collected from the hens via the axillary vein and centrifuged at 3,000 × *g* for 10 minutes. The hens were then euthanized, and their ovaries and surrounding tissues and the morphologies and histologies were evaluated and compared. The size and the number of tumors were measured, and tumor types were determined using hematoxylin and eosin staining of tissue sections as described previously (28). The presence or absence and sizes of ovarian tumors were recorded. Normal tissue and serum samples and tumor tissues were immediately frozen and stored at −80°C until analysis. Tissue samples were fixed in 10% neutral-buffered formalin, routinely processed for histology, and embedded in paraffin. Tissue samples were then stained with hematoxylin and eosin. Sections (6 μm) were cut from each tissue block and examined under a light microscope to determine whether they were normal ovarian tissue or ovarian cancer and for other pathologic findings and microscopic features.

Analysis of serum levels of curcumin using high-performance liquid chromatography

Curcumin levels in serum samples obtained from the hens ($n = 12$) were measured using high-performance liquid chromatography (HPLC) with a Shimadzu ultraviolet-visible spectroscopy detector at C18-ODS-3V and 5 μm with a 4.6 × 250 mm column (Inertsil ODS-3V; GL Sciences) as described previously with minor modifications (29, 30). Specifically, 200 μL of serum was mixed with 200 μL of 0.1 mol/L sodium phosphate buffer (pH 6.8) containing 0.1% ethylenediaminetetraacetic acid, 200 μL of distilled water, and 600 μL of methanol. The mixture was vortexed for 3 minutes, and 4 mL of hexane was added to it. The mixture was then shaken vigorously and centrifuged at 1,000 × *g* for 10 minutes at 4°C. After discarding the hexane layer, 1.2 mL of distilled water and 3 mL of ethyl acetate were added to the mixture (aqueous-methanol layer). This mixture was shaken vigorously and centrifuged at 1,000 × *g* for 15 minutes at 4°C, and the ethyl acetate layer was collected. This ethyl acetate extraction was repeated 3 times. The combined ethyl acetate layers were evaporated under nitrogen in the dark, and the residues were dissolved in 100 μL of methanol. All extracts were kept on ice and protected from light. An aliquot of this solution was injected onto a column kept at −35°C. The mobile phase was an acetonitrile: methanol:water mixture (2% acetic acid; 40:20:40, v/v/v, pH 3.0), and the flow rate was 1.3 mL/min. The eluent was monitored using an ultraviolet-visible spectroscopy detector at 425 nm.

Detection of serum malondialdehyde concentrations using HPLC

Levels of malondialdehyde (MDA), a marker for oxidative stress, in serum samples ($n = 12$) were measured using HPLC with an LC-20AD pump, SIL-20A autosampler, SPD-20A ultraviolet-visible spectroscopy detector (at C18-ODS-3V and 5 μm

with a 4.6 × 250 mm column), and CTO-10ASVP column oven (Shimadzu) as described previously by us (28). Tissue samples (300 μL) were homogenized in a mixture of 200 μL of $HClO_4$ (0.5 mol/L) and 100 μL of 500-ppm 2[6]-di-tert-butyl-p-cresol. Next, the samples were centrifuged, and supernatants were injected (injection volume, 20 μL) into an HPLC system. The mobile phase was 30 mmol/L KH_2PO_4-methanol (82.5 + 17.5, v/v%, pH 3.6), the flow rate was 1.2 mL/min, and detection at 250 nm.

Western blot analysis

Proteins were extracted from ovarian tumor samples for Western blot analysis as described previously (28, 31). Tumor samples were homogenized at 1:10 (w/v) in 10 mmol/L Tris-HCl buffer at pH 7.4 containing 0.1 mmol/L NaCl, 0.1 mmol/L phenylmethylsulfonyl fluoride, and 5 μmol/L soluble soybean powder (Sigma) as a trypsin inhibitor. After centrifugation at 15,000 × *g* at 4°C for 30 minutes, the supernatant was transferred into fresh tubes for immediate assay. Supernatants were mixed with Laemmli sample buffer and boiled for 5 minutes. Aliquots containing 20 μg of protein were subjected to 10% SDS–PAGE and subsequently transferred to nitrocellulose membranes (Schleicher & Schuell BioScience). Nitrocellulose blots were washed twice for 5 minutes in PBS and blocked with 1% BSA in PBS for 1 hour prior to application of primary antibodies. Antibodies against NF-κB, IκB, STAT3, Pias-3, Nrf2, and HO-1 (Abcam) were diluted at 1:1,000 in the buffer containing 0.05% Tween-20. The nitrocellulose membrane was incubated at 4°C with antibodies overnight. Western blots were washed and incubated with horseradish peroxidase–conjugated goat anti-mouse IgG (Abcam). Specific binding was detected using diaminobenzidine and hydrogen peroxide as substrates. Protein loading was controlled using an anti–β-actin antibody (Sigma). Samples were analyzed in quadruplicate under each experimental condition, and protein levels were measured densitometrically using the image analysis software program ImageJ (National Institutes of Health).

Gene mutation analysis

Nucleotide alterations in the *KRAS*, *HRAS*, and *NRAS* genes were evaluated using DNA extraction and PCR, restriction fragment length polymorphism (RFLP), and sequence analysis of the genes from ovarian tumors resected from control and curcumin-fed animals (Table 1). DNA isolated from galline blood samples was analyzed using a DNA Miniprep kit (845-KS-1020010; Biometra). Specific primers (reverse and forward) for the *KRAS*, *HRAS*, and *NRAS* genes were used in the PCR analysis. Sequence and RFLP analyses were performed to examine variations in homologous DNA sequences. PCR products were exposed to restriction enzymes for RFLP analysis, and DNA fragments were separated via electrophoresis using 2% agarose gels. The DNA fragments were also visualized using a transilluminator and photographed (32).

Differences in the tumor incidences and numbers in the groups were evaluated statistically using the χ^2 test. Data were analyzed via analysis of variance using the general linear model with the SAS program (2002; SAS Institute Inc.) to determine the effects of curcumin supplementation on tumor diameter, protein staining intensities, and serum metabolites. When a significant *F* statistic ($P \leq 0.05$) in the analysis of variance was noted, the least squares mean procedure was performed to separate means that were significantly different ($P < 0.05$). Linear and quadratic polynomial contrasts of the responses were used to evaluate the effects of the three dosages of curcumin (0, 25.8, and 53.0 mg/day) administered to the animals for serum metabolites. The χ^2 test was used for analysis of gene mutations and allelic frequency and Hardy–Weinberg equilibrium for the genotypic frequency, and odd ratio tests for ratio of changes were used.

Results

Curcumin intake leads to a dose-dependent reduction in overall ovarian cancer incidence

First, we investigated its effect on ovarian cancer incidence in the chicken, the only nonhuman animal in which ovarian cancers develop spontaneously at high prevalence rates, ranging from 20% to 35% (19, 20). For this purpose, we fed hens different dosages of curcumin (0, 25.8, and 53.0 mg/day; 90 hens/per group) as part of their daily diets for about 12 months and examined the ovarian cancer incidence and various endpoints in them (Fig. 1). At the end of 12 months, whereas ovarian cancer spontaneously developed in 35 (39%) of the control hens not given curcumin, it did so in 24 (27%) and 15 (17%) of the hens in the 25.8 and 53.0 mg/day groups, respectively ($P = 0.004$; Table 2). This indicated marked dose-dependent reductions in the overall ovarian cancer incidence in these two groups of curcumin-fed animals (31% and 57%, respectively). To determine the effect of curcumin consumption on the incidence of different ovarian tumor types, we analyzed the tumors according to histologic type. In the 0, 25.8, and 53.0 mg/day groups, we found serous adenocarcinoma incidences of 22/90, 16/90, and 10/90, respectively, and mucinous adenocarcinoma incidences of 13/90, 8/90, and 5/90, respectively (Supplementary Table S2). Hens fed curcumin at 25.8 and 53.0 mg/day also had smaller tumor size [1.92 mm ± 0.45; mean ± standard error; 29.4% reduction and 1.02 mm ± 0.31 (63.1% reduction), respectively] than did the control group (2.72 mm ± 0.63; $P = 0.04$). Also, the mean number of tumors found in each animal was lower in the

Table 1. *RAS* gene family mutation analysis

Gene	Codon	Primers	PCR conditions	PCR product (bp)	Analysis
KRAS	12	5-CTGAATATAAAC TTGTGGTAGTTGGACCT-3 5-TCAAAGAATGGT CCTGGACC-3	94°C for 55 seconds, 58°C for 45 seconds, and 72°C for 45 seconds	157	Sequence analysis
HRAS	12	5-AGACCCTGTAGG AGGACCC-3 5-GGTGCTGAGACG AGGGACT-3	94°C for 55 seconds, 62°C for 50 seconds, and 72°C for 50 seconds	312	Sequence analysis + RFLP (AVA II)
NRAS	12	5-AACTGGTGGTGG T TGGACCA-3 5-ATATTCATCTAC AAAGTGGT-3	94°C for 55 seconds, 57°C for 50 seconds, and 72°C for 40 seconds	83	Sequence analysis

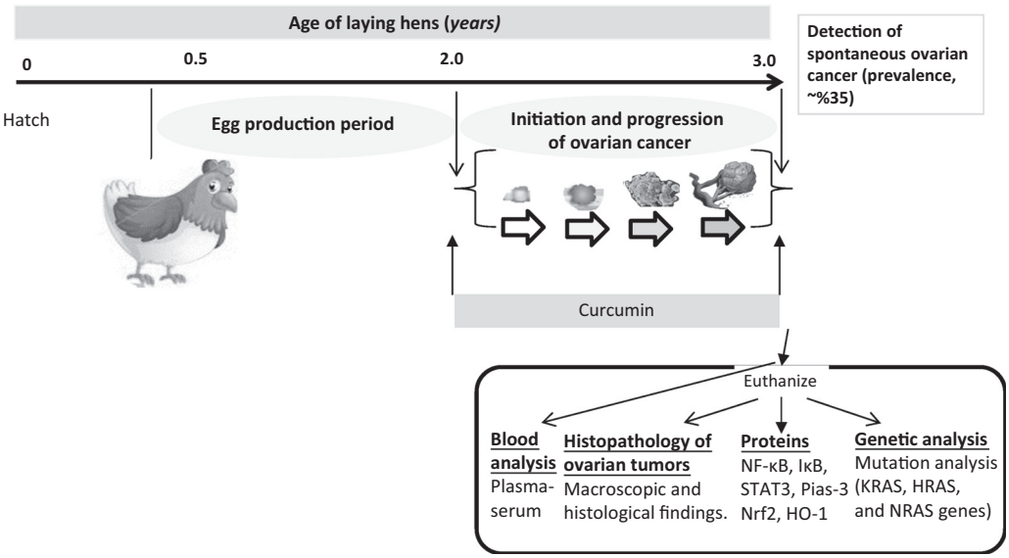

Figure 1.
The curcumin-based chemoprevention schedule and study design.

curcumin-fed groups than in the control group (0.39 ± 0.06, 0.27 ± 0.05, and 0.16 ± 0.04 in the 0, 25.8, and 53.0 mg/day groups, respectively; $P = 0.006$).

Macroscopic and histologic changes in normal ovarian tissue and ovarian tumors

In hens with ovarian cancer, the affected ovaries contained solid nodular masses with no yolky preovulatory follicles (Fig. 2A and B). Tumors metastasized to peritoneal organs, accompanied by profuse reddish brown ascites in some hens. We observed more developing follicles in ovarian tissue sections obtained from hens with normal ovaries than in those from hens with ovarian tumors. Also, atresia of follicles containing fat globules with ruptured theca and granulosa layers and cysts occurred frequently in the ovaries of hens with nontumor abnormalities. Furthermore, we observed carcinomas with glandular structures resembling endometrial glands having linings of columnar, cuboidal, or oval epithelial cells (Fig. 2Ac and d). We analyzed the tumors according to histologic type in tumors from the 0, 25.8, and 53.0 mg/day groups (Fig. 2Ab). In the tumors from non–curcumin-fed animals, larger and more solid tumors were observed and tumors composed of atypical tumor cells with marked overlaying infiltrative pattern. In addition, occasionally poorly differentiated or mixed ovarian carcinomas were observed. The tumors from the 25.8 mg/day group contained less infiltrative pattern and some glandular structures with atypical cells. In the 53 mg group, tumors contained atypical cells in the glandular structures with much less infiltrative pattern.

Table 2. Effect of curcumin intake on the development of spontaneous ovarian cancers in hens after 12 months

	Curcumin dosage (mg/day)			
Parameter	**0**	**25.8**	**53.0**	***P***
Tumor incidence (%)	35/90 (39)	24/90 (27)	15/90 (17)	0.004
0	55/90 (61)	62/90 (69)	73/90 (81)	
1	29/90 (32)	24/90 (27)	15/90 (17)	
>1	6/90 (7)	0/90 (0)	0/90 (0)	
Number of tumors	0.39 ± 0.06	0.27 ± 0.05	0.16 ± 0.04	0.006
Tumor size (mm)	2.72 ± 0.63	1.92 ± 0.45	1.02 ± 0.31	0.044

Serum curcumin levels increase in hens fed with curcumin

We measured curcumin levels in serum samples obtained from 12 randomly selected hens in each group to determine whether they increased in a dose-dependent manner with the administered dietary curcumin dosages. Curcumin levels were not detectable in serum of control hens but increased in a dose-dependent manner in the curcumin-fed animals, with levels of 0.071 and 0.116 μmol/L in the 25.8 and 53.0 mg/day groups, respectively ($P < 0.001$; Fig. 3A; Supplementary Fig. S1).

Curcumin-containing diet reduces oxidative stress and lipid peroxidation in serum and tissues and ovarian tissue

To investigate the alterations of oxidative stress induced by treatment with curcumin, we collected serum and ovarian tissue samples from hens at the end of the 12-month study period and measured their levels of MDA, a product of lipid peroxidation, using HPLC. MDA is widely used as a reliable biomarker for lipid peroxidation (33, 34). We found the MDA levels to be significantly lower in curcumin-fed animals than in control group (Fig. 3B and C). Specifically, the mean MDA levels in serum samples were 0.809, 0.634, and 0.549 nmol/mL in the 0, 25.8, and 53.0 mg/day groups, respectively, whereas those in ovarian tissue samples were 2,338, 1,342, and 1,168 nmol/mg protein in the 0, 25.8, and 53.0 mg/day groups, respectively ($P < 0.001$).

Effect of curcumin consumption on NF-κB, STAT3, Nrf2, and HO-1 protein expression in ovarian tumors

To identify the underlying molecular mechanism by which dietary curcumin mediates its effects in ovarian tumors, we collected ovarian tumor samples obtained from study hens in the three groups (0, 25.8, and 53.0 mg/day) and analyzed the

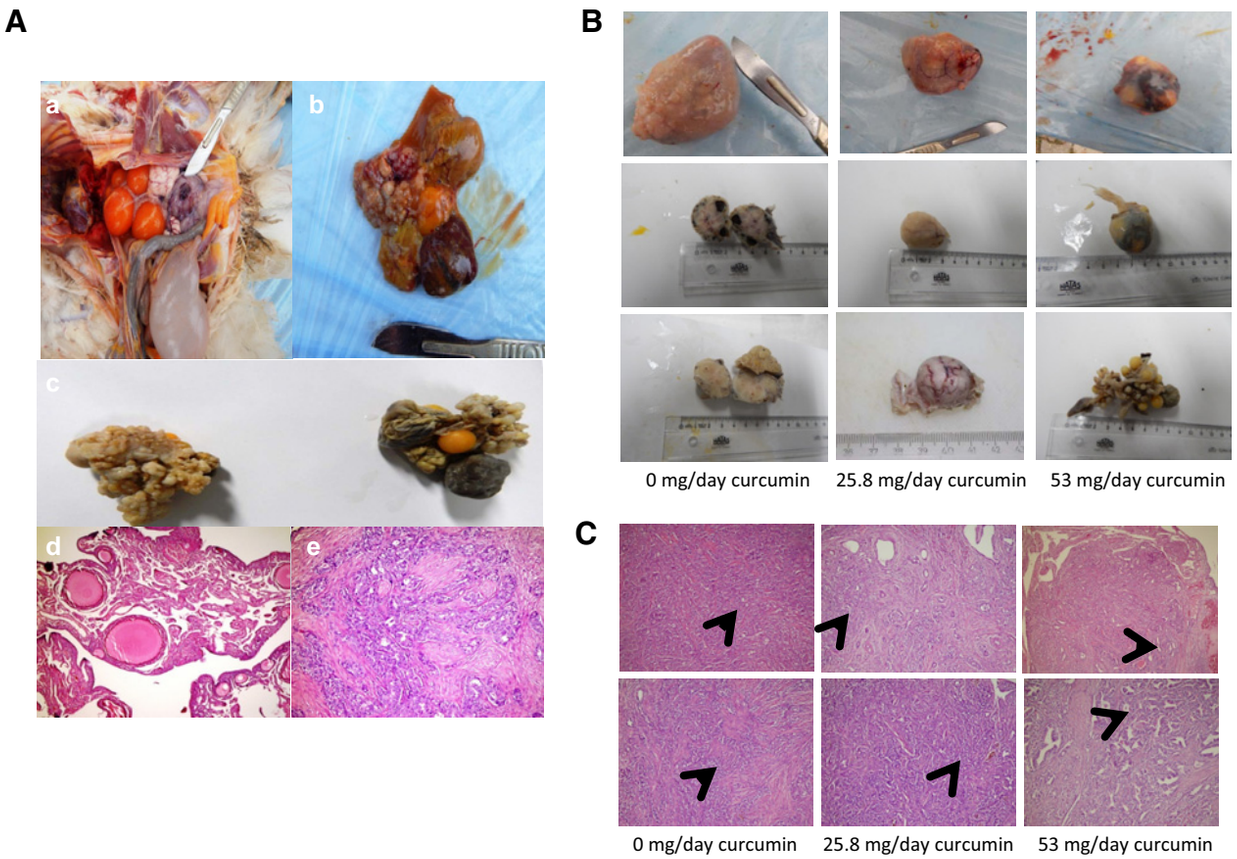

Figure 2.

A, Gross morphology of ovaries of egg-laying hens in normal tissues (**a**) with primary malignant ovarian tumors (**b**, **c**) in hens. Histopathology sections of normal ovarian tissue (**d**) and an ovarian adenocarcinoma (**e**) in laying hens stained with hematoxylin and eosin (magnification, ×100). **B,** The effects of curcumin supplementation on morphology of tumors (**A**) and histopathologic appearance (**C**) of biopsy samples from chickens treated with 0, 28.5, and 53 mg/kg of curcumin (magnification, ×100). Arrows indicate atypical cells overlying the infiltrative patterns in tumors.

protein expression in them using Western blotting. We first investigated expression of the transcription factor NF-κB, which is known to play a key role in the development and progression of cancer (34) and considered one of the most important targets for cancer prevention and therapy (4, 35). NF-κB induces the expression of genes that promote cell proliferation and survival and help cells evade apoptosis, one of the hallmarks of cancer (36, 37). We found that curcumin consumption led to inhibition of NF-κB protein expression by about 42% in the Western blot analysis (Fig. 4A and G), in which we examined the mean values from three independent analyses ($P < 0.05$). Also, we evaluated IκBα that binds to NF-κB dimers and sterically blocks the function of the nuclear localization sequence, thereby preventing NF-κB translocation from the cytoplasm to the nucleus. We found that curcumin intake resulted in a dose-dependent induction of IκBα (Fig. 4B and G).

STATs are transcription factors activated in response to cytokines and growth factors. Constitutively active STAT3 has led to oncogenic transformation. Moreover, STAT3 is highly overexpressed in ovarian cancer cells (38). The activated STAT3 protein is inhibited by inhibitory proteins such as Pias-1 and Pias-3. As shown in Fig. 4C, D, and G, STAT3 protein expression was inhibited by up to 36% by daily curcumin intake, whereas Pias-3 expression was significantly increased.

The transcription factor Nrf2 is one of the major defense mechanisms against oxidants and induces the expression of genes encoding for antioxidant proteins, including HO-1, by binding to the antioxidant response element (39). Activation of Nrf2 antioxidant pathway signaling is reported to play an important role in cancer prevention (39). Also, increased HO-1 activity decreases the formation of oxidants such as superoxide anion. We found that expression of Nrf2 (Fig. 4E and G) and HO-1 (Fig. 4F and G) protein increased in a dose-dependent manner in ovarian tumor samples obtained from curcumin-fed animals.

Molecular alterations of the *KRAS* and *HRAS* proto-oncogenes in ovarian tumors

RAS genes encode for the 21-kDa Ras family of oncoproteins, including H-Ras, K-Ras, and N-Ras. KRAS mutations are among the most frequently observed abnormalities in human cancers, including ovarian cancer (2, 40). Activating mutations of *RAS* genes are most often point mutations detected in various cancers that cause p21 GTPase activity to stop, thus constitutively

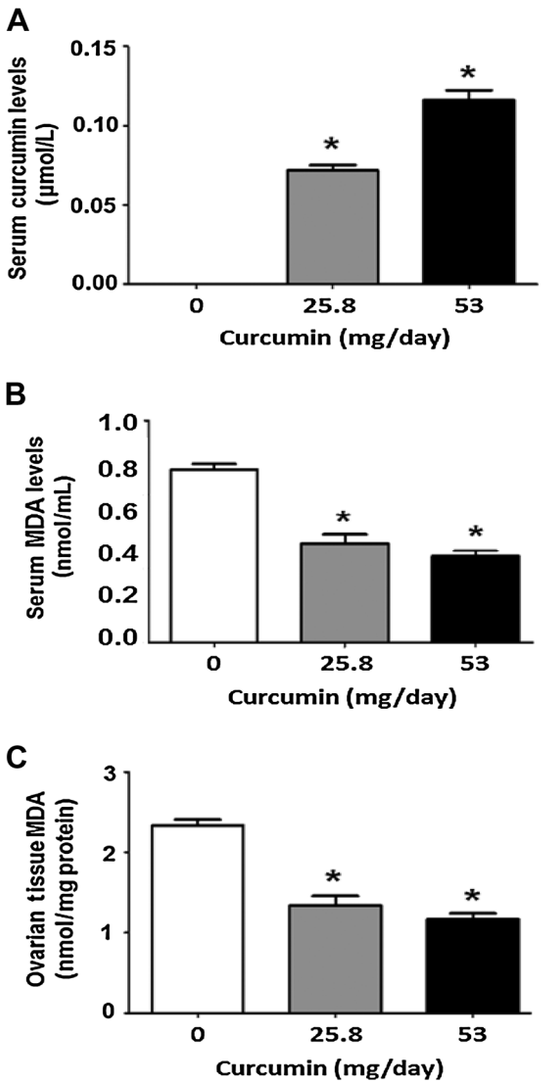

Figure 3.

The effects of curcumin supplementation on serum curcumin and MDA levels (**B** and **C**) in laying hens. **A,** Serum curcumin levels were measured as mention in the Material and Methods. Comparisons were made using the Fisher exact test. Means values differ significantly ($P < 0.05$). The effects of curcumin supplementation on MDA levels in serum (**B**) and (**C**) ovarian tissue samples obtained from laying hens. Mean values differ significantly between groups and higher ($P < 0.05$).

activating Ras/Raf/mitogen-activated protein kinase kinase (MAPK)/ERK and NF-κB pathways and providing oncogenic signaling by promoting cell proliferation, invasion, and apoptosis evasion (41). To identify alterations and mutations of the *KRAS* gene, we sequenced and analyzed the gene in ovarian tumor samples resected from control and curcumin-fed hens using the primers listed in Table 1. Because most of the common mutations of these genes are found in codon 12 (>90%), we first evaluated this and other parts of the genes for mutations via direct sequencing using tumor samples obtained from control- and curcumin-fed animals ($n = 26$/group). Sequencing of the *KRAS* genes revealed frequent alterations, such as c.-40T>C, in the tumors from the control group but significantly fewer of them in tumors from curcumin-fed hens ($P = 0.001$). *KRAS* gene sequencing analysis shows T>C changes occur as in no-curcumin (26; 1.0) compared with curcumin-fed animals, including 25.8 mg/day (12; 0.46) and 53 mg/day groups (3; 0.12), indicating a dose-dependent reduction in the frequency of alterations (Supplementary Table S3).

We also commonly observed SNPs, including IVS (intervening sequence/intron) 1+86 C>T, IVS 1+148 G>A, IVS 1+152 C>T, IVS 1+178 C>T, IVS 1+183 G>A, and IVS 1+227 C>T, as homozygous alterations ($P < 0.001$). However, the number of these mutations was significantly reduced in ovarian tumors obtained from curcumin-fed animals ($P = 0.0005$). In a small number of samples, we observed heterozygous alterations (IVS 1+148 G>A, IVS 1+152 C>T, IVS 1+178 C>T, and IVS 1+183 G>A) at a higher frequency ($P < 0.001$; Supplementary Table S3). In the *HRAS* gene, SNPs, including the homozygous alterations IVS 1+20 da C>T, IVS 1+147 G>T, and IVS 1+159 T>C, in most tumor samples were detected ($P = 0.001$). The numbers of these alterations were significantly lower in the curcumin-fed groups than in the control group ($P = 0.014$). However, we did not find any previously detected alterations of the *HRAS* gene, such as c.-23C>T (rs315505027 SNP) and c.105A>C (rs316850941 SNP). We detected no alterations in the *NRAS* gene in the tumor samples.

Discussion

Curcumin has been shown to exert chemopreventive effects in various high-dose carcinogen-induced rodent models of cancer, including those of mammary cancer, hepatocellular carcinoma, adenomatous polyposis, esophageal cancer, and colon cancer (14, 15, 42–44). However, a direct link between curcumin intake and reduced cancer incidence has yet to be established. In the current study, we demonstrated for the first time that daily curcumin intake significantly reduced the development of spontaneous ovarian cancer. We found that 12 months of dietary curcumin intake at 25.8 or 53.0 mg/day led to marked dose-dependent reductions (about 31% and 57%, respectively) in ovarian cancer (serous and mucinous) incidence in hens, which have a naturally high incidence of ovarian cancer. Daily curcumin intake also resulted in tumors that were markedly smaller than those in the controls, overall indicating that curcumin even given orally as a dietary supplement has a significant impact on chemoprevention and growth of ovarian cancer.

Previous studies demonstrated that curcumin is well tolerated at up to 8 g/day in humans, with no toxic and adverse effects. The highest dose of curcumin used in our study was 53.0 mg/day, which corresponds to 1.4 g/day in humans, which is easily achievable (45). In fact, the Joint Food and Agriculture Organization of the United Nations/World Health Organization Expert Committee on Food Additives recommended an adequate daily intake of curcumin of 0 to 3 mg/kg body weight/day (EFSA panel, 20100). In a previous study, administration of 4, 6, and 8 g of curcumin per day for 3 months in human subjects resulted in mean curcumin plasma concentrations of 0.51, 0.63, and 1.77 μmol/L, respectively, 1 to 2 hours after consumption (46). In our study, we found dose-dependent increases in serum curcumin levels up to 0.12 μmol/L with daily intake of 53 mg of curcumin, indicating that the serum concentrations of curcumin were comparable with those in

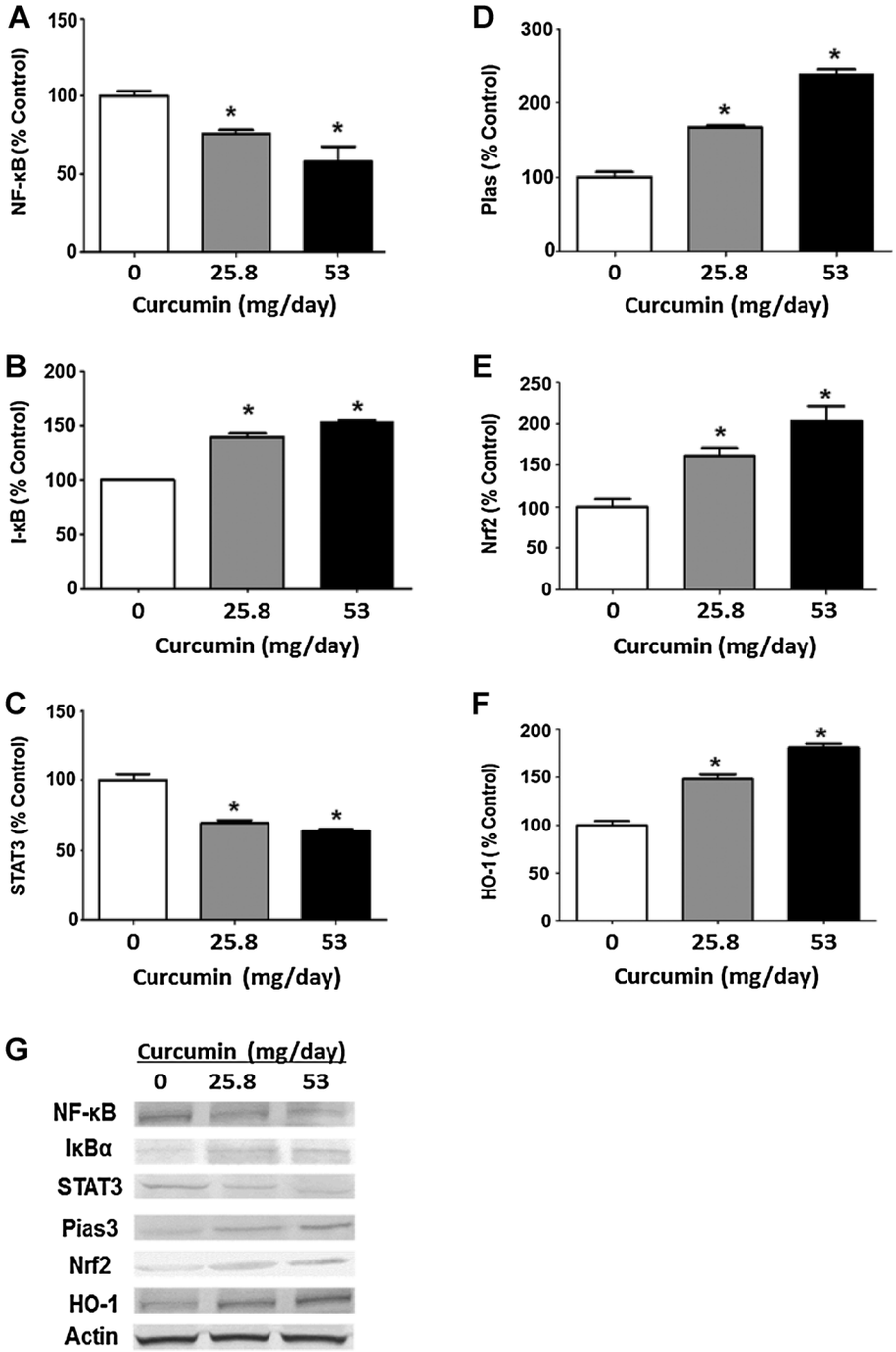

Figure 4.
The effects of curcumin supplementation on (**A**) NF-κB, (**B**) IκB, (**C**) STAT3, (**D**) PIAS3, (**E**) Nrf2, and (**F**) HO-1 expression in ovarian tumor tissues from chickens treated with 0, 28.5, and 53 mg/kg of curcumin. Western blot analysis (**G**) was performed with actin included to ensure equal protein loading (bottom). Blots were repeated at least 3 times; representative blots for each are shown. The values are presented as the least squares means ± standard error. The data are percentages of the control. Mean values differ significantly ($P < 0.05$).

human subjects and that our results therefore can be easily translated to chemopreventive studies in humans. Previous studies have conducted a phase II clinical trial of curcumin to evaluate it in the prevention of colorectal neoplasia (47). The chemopreventive role of curcumin for ovarian cancer should also be investigated in human trials.

One of the major mechanisms by which curcumin exerts chemopreventive and antitumor effects is thought to be potent inhibition of NF-κB signaling, which induces the expression of genes that promote cell proliferation and survival (4, 10, 34, 37). Thus, inhibition of NF-κB is considered one of the most important targets for cancer prevention and therapy (37, 48, 49). We found that daily dietary curcumin intake dose-dependently inhibited NF-κB expression in ovarian tissue samples collected from the hens. This effect was mediated by IκB, which binds to the nuclear localization sequence of NF-κB and blocks its function by preventing NF-κB translocation from the cytoplasm to the nucleus. Previous studies demonstrated that curcumin-based treatment

leads to inhibition of NF-κB signaling, supporting our present findings (8, 10).

Of the various STAT family members, STAT3 is frequently overexpressed in tumor cells, regulates the expression of numerous oncogenic genes controlling the growth and metastasis, and exerts chemopreventive and therapeutic effects (38). Constitutively active STAT3 has resulted in oncogenic transformation (50). Also, STAT3 is highly overexpressed in ovarian cancer cells (38). Our study demonstrated that daily dietary curcumin intake inhibits STAT3 expression in ovarian cancer cells by inducing the expression of STAT3 inhibitors such as Pias-1 and Pias-3. Curcumin can exert other chemopreventive and antitumor effects via carcinogen-detoxifying enzymes such as glutathione *S*-transferase and by suppression of expression of the isoenzyme cyclooxygenase-2, inhibition of protein kinase C, EGFR, and activation and the expression of c-Jun, c-Fos, c-Myc, and inducible nitric oxide synthase. Thus, these important mediators may be evaluated in future studies after curcumin intake for comprehensive analysis of the effects.

Another important mechanism that contributes to the chemopreventive actions of curcumin is upregulation of antioxidation-related proteins. We found that expression of the proteins Nrf2 and HO-1 increased in a dose-dependent manner in the hens' ovarian tumors with curcumin intake, indicating that this antioxidant mechanism may underlie reduced incidence of mutations in ovarian cancer tissues. The transcription factor Nrf2 is one of the major antioxidant defense mechanisms that protect cells and tissues from a variety of oxidative stresses (31, 39, 49). Specifically, Nrf2 induces the expression of genes encoding for the antioxidant proteins, including HO-1, by binding to the antioxidant response element (31, 49). HO-1 also is one of the phase II detoxifying enzymes and exerts a strong antioxidant effect, and it is regulated by the redox-sensitive transcription factors. Currently, several Nrf2 activators are being tested as chemopreventive compounds in clinical trials. Furthermore, lipid peroxidation is an important indicator of oxidant damage induced by free radicals or nonradical molecules in unsaturated lipids, producing such as MDA. We found that MDA levels in serum and ovarian tumor samples obtained from hens fed curcumin daily were markedly and dose-dependently reduced. MDA is considered the most mutagenic product of lipid peroxidation (33). Thus, reduced ovarian cancer incidence may result from blockage of oxidant attack by curcumin. In fact, we previously reported that in response to supplemental curcumin MDA levels decreased linearly in muscle tissues (28). The present study confirmed our previous findings that curcumin supplementation reduces free radical-induced damage to cells.

Ras genes are implicated in a wide range of human tumors, including colon, lung, breast, uterine, kidney, and stomach tumors. Mutations of these genes are most often point mutations and seen in 10% to 35% of ovarian cancer patients. Hakim and colleagues reported that an SNP was more common in the K-Ras than in the *HRAS* gene in a hen model of ovarian cancer (23). Overall, our study demonstrates for the first time that the incidence of mutations of the *KRAS* and *HRAS* oncogenes in galline ovarian tumors is significantly reduced by curcumin intake. This can be explained in part by curcumin-induced activation of a DNA repair mechanism by upregulating expression of the protein excision repair cross-complementary-1. Also, increased DNA methylation in histone proteins owing to increased activity of histone acetylase may lead to reduced expression of oncogenic transcription factors and, thus, cancer incidence. Recently, a comprehensive study by the TCGA Network demonstrated that p53 mutations are common genetic alterations in ovarian carcinomas, especially human high-grade serous tumors (51). Thus, the frequency of p53 mutations in ovarian tumors in subjects fed curcumin should be analyzed in the future studies.

Overall, in addition to an antioxidant mechanism, multiple molecular mechanisms may underlie the curcumin-induced reduction of ovarian cancer incidence. These mechanisms must be analyzed in future studies. The results of the present study warrant testing of curcumin-based chemopreventive strategies in humans. Currently, researchers are conducting at least 14 phase II clinical trials of curcumin-based therapy along with standard therapies in patients with various cancers. These studies will soon shed light on the potential benefits of curcumin consumption by ovarian cancer patients, who have poor prognoses with standard treatment regimens.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: K. Sahin, M. Tuzcu, N. Sahin, O. Güler, O. Kucuk, B. Ozpolat

Development of methodology: K. Sahin, C. Orhan, M. Tuzcu, N. Sahin, O. Kucuk, B. Ozpolat

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Sahin, C. Orhan, M. Tuzcu, N. Sahin, O. Güler, B. Ozpolat

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Sahin, C. Orhan, M. Tuzcu, N. Sahin, B. Ozpolat

Writing, review, and/or revision of the manuscript: K. Sahin, O. Kucuk, B. Ozpolat

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Sahin, H. Tastan, I.H. Özercan, N. Kahraman

Study supervision: K. Sahin, B. Ozpolat

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