Dietary riboflavin deficiency promotes N-nitrosomethylbenzylamine-induced esophageal tumorigenesis in rats by inducing chronic inflammation

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Abstract: Epidemiological studies in high-incidence areas of esophageal cancer in China suggest that environmental carcinogen N-nitrosomethylbenzylamine (NMBA) and riboflavin (RBF) deficiency may be the main risk factors for esophageal cancer. However, it is not clear that the combination induces cancer. Here, experiment (Exp) 1 evaluated the effects of NMBA and RBF deficiency individually or in combination on esophageal tumorigenesis. Male F344 rats were randomly assigned to 4 groups into a 2 (no NMBA vs. NMBA) × 2 (normal RBF vs. RBF-deficient) factorial design, including normal RBF (6 mg/kg, R6), RBF-deficient (0 mg/kg, R0), normal RBF combined with NMBA (R6N), and RBF-deficient combined with NMBA (R0N) groups. The Exp 2 explored the effects of RBF deficiency at different doses combined with NMBA (0.6 mg/kg, R6N; 0.06 mg/kg, R0.06N) on esophageal tumorigenesis. Results showed that R0N enhanced the incidence of esophageal intraepithelial neoplasia (EIN, 53.3%, P = 0.06), including carcinoma in situ, whereas R6N mainly induced the occurrence of esophageal benign hyperplasia (38.9%) and EIN (16.7%). RBF deficiency promotes EIN in a dose-dependent manner, and R0.06N significantly increases the incidence of EIN (57.9%, P < 0.05). Gene expression profiling demonstrated that inflammatory cytokines were highly expressed in R6N EIN tissues, whereas R0N EIN tissues had a proliferation and differentiation gene signature (fold-change > 1.5). Furthermore, RBF deficiency aggravated oxidative DNA damage (8-OHdG) and double-strand breaks (γH2AX) (P < 0.05). Our results suggest that RBF deficiency causes chronic inflammation-associated genomic instability contributes to NMBA-induced esophageal tumorigenesis.

Keywords: Esophageal cancer, rat model, esophageal intraepithelial neoplasia, inflammatory cytokines, 8-OHdG, γH2AX

Introduction

Esophageal cancer is the eighth most common and sixth most lethal cancer worldwide, having a 5-year survival rate between 15-25% [1]. Esophageal squamous cell carcinoma (ESCC) is the predominant histological subtype of esophageal cancer and accounts for approximately 90% of esophageal cancer cases [2, 3]. Development and progression of ESCC involves multiple stages, individually known as low-grade intraepithelial neoplasia, high-grade intraepithelial neoplasia, subsequent invasive ESCC, and final metastasis [4]. Esophageal intraepithelial neoplasia is graded according to the proportion of epithelial dysplasia involved, with the lower third to two-thirds being referred to as low-grade intraepithelial neoplasia, and above two-thirds or carcinoma in situ being referred to as high-grade intraepithelial neoplasia [5-7]. At the initial stage, squamous epithelial cells exhibit nuclear atypia and abnormal maturation. This stage is known as dysplasia, and is believed to precede ESCC [6]. From a genomic perspective, little is known about the evolution from dysplasia to ESCC, especially
how and at which stage the key carcinogenic events are acquired.

Epidemiological and etiological studies have shown that ESCC has a striking geographic distribution worldwide, suggesting that environmental factors play crucial roles in esophageal carcinogenesis [8]. The major risk factors for ESCC are chronic alcohol consumption, tobacco use, nutritional deficiency, and exposure to environmental carcinogens, such as N-nitrosomethylbenzylamine (NMBA) [9, 10]. NMBA has been widely used to induce esophageal tumors in rodents [11-13]. A typical esophageal tumorigenesis chemoprevention study showed that weekly administration of low doses of NMBA for 15 weeks (cumulative dose = 7.5 mg/kg) produces a 100% incidence of squamous papilloma, but without ESCC development [14]. Combination with other risk factors, such as nutritional deficiency (zinc deficiency, vitamin E deficiency), alcohol, and arecoline, can greatly increase the incidence of ESCC [12, 15-17]. Multiple risk factors are involved in the development of ESCC.

Riboflavin (RBF) is the precursor of the coenzymes flavin mononucleotide and flavin adenine dinucleotide, which participate in a wide range of biological oxidation-reduction reaction. RBF deficiency may result in disease, such as cancer [18]. The blood concentration of RBF in Kazakh ESCC patients is lower than that in healthy controls [19]. Our early studies demonstrated that plasma RBF levels of ESCC patients are significantly lower than that of healthy controls in the Chaoshan area of Guangdong Province [20]. Moreover, we observed that enhanced subcutaneous tumorigenicity in NU/NU mice following injection of RBF-depleted HEK293T cells [21].

In this study, we established a rat models to evaluate the effects of NMBA and RBF deficiency individually or in combination on esophageal tumorigenesis. Furthermore, we reveal the potential molecular mechanisms of RBF deficiency combined with NMBA inducing esophageal tumors.

Materials and methods

Animals and diets

Five-week-old male F344 rats were obtained from the Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Rats were housed in a controlled environment and were allowed access to water ad libitum. The normal RBF diets were AIN-93M purified diet, which contains RBF 6 mg/kg (R\(_{6}\)). RBF-deficient diets were made with the same formula, but contained RBF 0 mg/kg (R\(_{0}\)), 0.6 mg/kg (R\(_{0.6}\)), or 0.06 mg/kg (R\(_{0.06}\)) (Trophic Animal Feed HighTech Co., Ltd., Nantong, China) [22, 23]. The concentration of RBF in the diets was analyzed by reversed-phase high-performance liquid chromatography (HPLC, Agilent 1200 system). N-nitrosomethylbenzylamine (NMBA, CAS NO. 937-40-6) was purchased from Shi Ruikai Technology Co., Ltd. (Wuhan, China).

Experimental design

This study was approved by the Institutional Animal Care and Use Committee of Shantou University. The Exp 1 design is shown in Figure 1A. Sixty-nine male F344 rats were randomly assigned to 4 groups (n = 15-18/group) into a 2 (no NMBA vs. NMBA) × 2 (normal RBF vs. RBF-deficient) factorial design, including normal RBF (R\(_{6}\)), RBF-deficient (R\(_{0}\)), normal RBF combined with NMBA (R\(_{6}\)N) and RBF-deficient combined with NMBA groups (R\(_{0}\)N). For NMBA treatment, the rats received subcutaneous 0.1 mL injections of 20% dimethyl sulfoxide (DMSO) containing NMBA (0.35 mg/kg body weight) 3 times per week for 5 weeks. Control rats received an equivalent volume of 20% DMSO (vehicle control). All rats were euthanized by general anesthesia with an overdose of diethyl ether at week 25 to assess the development of esophageal lesions.

The Exp 2 design is shown in Figure 1B. Forty-three male F344 rats were randomized into 3 groups (n = 12-19/group), including R\(_{6}\)N, R\(_{0.6}\)N and R\(_{0.06}\)N groups. All rats were euthanized at 35 week to assess the development of esophageal lesions.

Serum free RBF

Analysis of serum free RBF was performed using HPLC as described previously [24]. Briefly, proteins were removed from plasma by acid precipitation, and an aliquot of the resulting supernatant was analyzed by reversed-phase HPLC. Impurities were separated from RBF isocratically, and the target material was detected fluorometrically (excitation 450 nm; emission 520 nm).
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Histopathological analysis

Tissue was fixed in 4% paraformaldehyde for 6 hours. After paraffin embedding, serial 4-μm tissue sections were cut for staining. Hematoxylin and eosin (HE)-stained sections were used for histological diagnosis.

Immunohistochemical staining and quantitative analysis

Immunohistochemistry (IHC) was performed using a 2-step protocol (PV-9000 Polymer Detection System; ZSGB-BIO, China) according to the manufacturer’s instructions and has been described in our previous studies [25]. Then, the esophageal sections were incubated with primary antibody for pan-Cytokeratin (1:250, Abcam, ab7753, UK), Ki67 (prediluted, Abcam, ab21700, UK), 8-hydroxy-2’-deoxyguanosine (8-OHdG) (1:16,000, Abcam, ab62623, UK), γH2AX (1:2,000, Novus Biologicals, NB-100-384, USA) overnight at 4°C. Cells with a brown reaction product were defined as positive. Stain-positive cells were analyzed by a PerkinElmer Vectra system (PerkinElmer, PerkinElmer Vectra, USA). The percentage of positive cells of each group was calculated as the number of positive cells divided by the total number of basal cells.

RNA extraction and gene chip analysis

Comparative transcriptomic analysis of esophageal intraepithelial neoplasia (EIN) tissues vs. adjacent normal tissues in the $R_6N$ and $R_0N$ groups ($n = 2$ rats/group) was performed using GeneChip® Rat Transcriptome Array 1.0 (also known as the Clarion™ D Assay; Thermo Fisher Scientific Inc., 902634). (GEO accession numbers: GSE111207). HE-stained tissue was observed under a microscope, and the tissue of interest was isolated with a manual tissue arrayer and placed in a 1.5-mL microcentrifuge tube. The RNA extraction and gene chip analysis were performed by Shanghai Biotechnology Corporation (Shanghai, China) and in accordance with relevant protocols.

Expression data analysis

Raw data was normalized using the quantile normalization of the robust multi-array average (RMA) method in Expression Console (software provided by Affymetrix). Fold change was used to identify differentially-expressed mRNAs. Significantly up-regulated genes were defined by a fold change $> 1.5$, and significantly down-regulated genes were defined by a fold change $< 0.66$.

Gene ontology and pathway analysis

To explore the biological functions of the identified differentially-expressed genes, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were con-
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ducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID) [26, 27]. Significant GO terms and KEGG pathways were selected as the enriched terms based on values with \( P < 0.05 \).

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

All primer sequences are shown in Supplementary Table 1. PCR amplifications were performed using SYBR Green Premix (TaKaRa Bio Inc., RR420B) according to the manufacturer’s instructions, and qRT-PCR analysis and data collection were carried out on an ABI 7500 qPCR system. For each mRNA, qRT-PCR reactions were performed in triplicate, with \( \beta \)-actin as the internal control gene for normalization. The relative mRNA expression was calculated using the 2\(^{-\Delta\Delta CT} \) method [28].

Enzyme-linked immunosorbent assay (ELISA)

The level of serum inflammatory cytokines S100A8, S100A9, and tumor necrosis factor alpha (TNF-\( \alpha \)) were assayed using an ELISA kit (CSB-EL020641RA, CSB-EL020642RA and CSB-E11987r; Cusabio, China). ELISAs were performed according to the manufacturer’s instructions. The optical density value of each well was detected using a microplate reader set to 450 nm.

Routine blood tests

Collect rat post-glomus venous plexus blood and send it to the Department of Clinical Laboratory of the Cancer Hospital of Shantou University Medical College. Red blood cells, white blood cells, platelets and other indicators were detected by automatic biochemical analyzer (LH780, BECKMAN-COULTER).

Statistical analysis

Statistical analysis was performed using SPSS 22.0 software (SPSS, Chicago, IL). Data are presented as the means \( \pm \) SD. Exp 1 data were tested by two-way analysis of variance (ANOVA) followed by Least Significant Difference (LSD) post hoc test. Exp 2 data were tested by one-way ANOVA followed by LSD post hoc test. Data having unequal variances were log-transformed to achieve equal variances. Differences in tumor incidence were assessed by Fisher’s exact test. Differential expression of mRNA between esophageal intraepithelial neoplasia and normal tissue were tested by Student’s \( t \)-test. Statistical tests were two-sided and were considered significant at \( P < 0.05 \).

Results

RBF deficiency combined with NMBA increases the incidence of esophageal tumors

In Exp 1, serum free RBF levels in RBF-deficient diet-fed rats (\( R_0 \) and \( R_6N \) groups) were significantly lower than that in normal RBF diet-fed rats (\( R_6 \) and \( R_6N \) groups) at 25 weeks (\( P < 0.001 \), Figure 1A). There was no interaction between diet and NMBA on the reduction of serum free RBF levels. RBF-deficient (\( R_0 \)) diet causes weight loss in rats, a representative picture is shown in Supplementary Figure 1A. At week 5, \( R_0 \) diet significantly reduced the rat body weight (Supplementary Figure 1B, Exp 1, \( P < 0.001 \)) and no interaction between diet and NMBA was observed. In Exp 2, the serum free RBF concentration was significant differences among the three groups at 35 weeks (\( P < 0.05 \), Figure 1B), resulting in the \( R_6N \) group > \( R_0N \) group > \( R_0 \) group. Similarly, there were significant differences among the three groups in rat body weight at week 10 (Supplementary Figure 1B, Exp 2, \( P < 0.05 \)). The above results indicate that a RBF-deficient diet establishes RBF deficiency in our model.

Representative images of esophageal papilloma (\( R_6 \)) and carcinoma in situ (\( R_0 \)) are shown in Figure 2A. In Exp 1, no benign hyperplasia (including hyperplasia and papilloma) occurred in either \( R_6 \) or \( R_0 \) group (0/18, 0%, respectively). \( R_0 \) group induced a case of esophageal intraepithelial neoplasia (1/18, 5.6%), while \( R_6 \) group did not induce. There was no significant difference between the \( R_6 \) group and \( R_0 \) group in esophageal tumors (\( P > 0.05 \)). The results indicate that RBF deficiency alone is not sufficient to cause esophageal tumors.

The incidence of pathological damage in the esophagus is summarized in Figure 2B (Exp 1). In combination with NMBA treatment, \( R_6N \) mainly elicited an incidence of 38.9% benign hyperplasia and 16.7% esophageal intraepithelial neoplasia. \( R_0N \) elicited an incidence of 53.3% esophageal intraepithelial neoplasia,
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including carcinoma in situ. The incidence of esophageal intraepithelial neoplasia in the R\textsubscript{0}N group tends to be higher than that in the R\textsubscript{6}N group (P = 0.06). Three rats in R\textsubscript{0}N group died before the end of study and were not included in the statistics. It may die from esophageal tumors. Other important organs, such as the heart, lung, liver, kidney, stomach, duodenum, jejunoleum, and colon tissues showed no histopathological lesions. Representative pictures are shown in Supplementary Figure 1C. Taken together, RBF deficiency promotes NMBA-induced esophageal tumorigenesis.

RBF deficiency promotes the incidence of esophageal tumors in a dose-dependent manner

In Exp 2, the incidence of pathological damage in the esophagus is summarized in Figure 2B (Exp 2). The results show that R\textsubscript{6}N treatment elicited a 33.3% incidence of benign hyperplasia and 16.7% incidence of esophageal intraepithelial neoplasia. Mild RBF deficiency, when combined with NMBA (R\textsubscript{0.6}N), elicited an incidence of 75.0% benign hyperplasia and 8.3% esophageal intraepithelial neoplasia. The inci-

**Figure 2.** RBF deficiency combined with NMBA induces esophageal tumors in rats. A. HE staining and IHC for cytokeratin were performed on rat esophageal tissues. Representative images of esophageal papilloma (R\textsubscript{6}N) and carcinoma in situ (R\textsubscript{0}N). Scale bars, 50 μm. B. Incidence of benign hyperplasia and esophageal intraepithelial neoplasia in Exp 1 (n = 15-18/group) and Exp 2 (n = 12-19/group). Difference in incidence were assessed by Fisher’s exact test (P < 0.05). *Means differ, P < 0.05.
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an incidence of 26.3% benign hyperplasia and 57.9% esophageal intraepithelial neoplasia, including a case of carcinoma in situ. The incidence of esophageal intraepithelial neoplasia in the R_{0.06}N group was increased above that in the R_{0.6}N group (P < 0.05). Therefore, RBF deficiency promotes esophageal tumorigenesis in a dose-dependent manner.

RBF deficiency induces an inflammatory gene expression pattern in rat esophageal tumors

Hierarchical clustering analyses revealed that R_{N} esophageal intraepithelial neoplasia (EIN) tissues had distinct gene expression patterns when compared with R_{N} EIN tissues, although the expression profile exhibited a 2-branch partition with the 4 EIN samples clustered together and well separated from their matched normal controls (Figure 3A, 3B). Overall, a total of 163 genes (128 up- and 35 downregulated) were identified to be specifically differentially expressed in the R_{0.6}N group; a total of 101 genes (36 up- and 65 downregulated) were identified to be specifically differentially expressed in the R_{0.06}N group. The 2 sets of genes shared 8 common genes (Figure 3B).

Among them, S100a8, Il36g, and Olr459 were upregulated, and Cav3 was downregulated in the 2 groups; Akr1b8, Fgfbp1, Rad23b, and Gspt1 were upregulated in the R_{0.6}N group, but downregulated in the R_{0.06}N group (Figure 3C, 3D).
Figure 4. Effect of RBF deficiency on the levels of inflammatory markers in rat esophageal neoplasia tissues and peripheral blood. A. Validation of microarray data by qRT-PCR (EIN vs. Normal tissues, normalized to β-actin, n = 3/group). Data were analyzed by Student’s t-test. B. Detection of inflammatory marker expression levels in rat serum.
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by ELISA (n = 6/group). C. At 15 weeks, the percentage of peripheral blood neutrophils, and monocytes in the total white blood cells (n = 15-18/group). Values are means ± SD. Exp 1 different letters (a and b for diet effect; x and y for NMBA effect) are significantly different by two-way ANOVA and LSD test (P < 0.05). Exp 2 different letters (a and b) are significantly different from each other by one-way ANOVA and LSD test (P < 0.05). *Means differ, P < 0.05. **Means differ, P < 0.01.

Discussion

Several epidemiological studies have shown that RBF deficiency is a risk factor for esophageal cancer. Supplementation of RBF through nutritional intervention or RBF-fortified salt can effectively reduce the incidence of esophageal cancer in high-risk areas of China [33-35]. In animal studies, supplementing marginally deficient corn or wheat diets with various combinations of nicotinic acid, RBF, zinc, magnesium, molybdenum, and selenium significantly reduces NMBA-induced esophageal tumors [36, 37]. In this study, our data demonstrate that RBF deficiency promotes NMBA-induced esophageal tumorigenesis. Mechanistically, RBF-deficient diet increases inflammatory markers, forming an inflammatory microenvironment in esophageal tumor tissue. Chronic inflammation-related DNA damage (as demonstrated by accumulation of DNA oxidative damage and DNA double-strand breaks) may trigger esophageal tumorigenesis.

The proposed mechanism of NMBA carcinogenicity is through microsomal activation to form benzaldehyde and an electrophilic metabolite that methylates DNA, producing O⁶- and 7-methylguanine adducts [38]. The occurrence of O⁶-methylguanine is thought to play a primary role in inducing DNA mutations [39, 40]. Tp53 and Ha-Ras gene mutations are present in the majority of NMBA-induced rat esophageal pap-

RBF deficiency combined with NMBA promotes DNA damage in esophageal epithelium

Representative images are shown in Figure 5A. The percentage of Ki67-positive cells in the NMBA treatment group (R₇₅ and R₇₅ groups) were significantly higher than that in the NMBA untreated group (R₅ and R₅ groups) (Figure 5B, P < 0.001). There was no interaction between diet and NMBA in the elevation of Ki67-positive cells. Considering that prolonged inflammation may lead to DNA damage, we detected oxidative DNA damage biomarker 8-OHdG and double-strand breaks biomarker γH2AX [29-32]. Both diet and NMBA elevated the percentage of nuclear staining of 8-OHdG and γH2AX (Figure 5C, 5D, P < 0.05). However, we did not find any interaction between diet and NMBA in the elevation of 8-OHdG and γH2AX positive cells. These results revealed that NMBA, not RBF deficiency, is the main cause of esophageal cell proliferation. Both NMBA and RBF deficiency promote DNA damage.
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Figure 5. RBF deficiency combined with NMBA promotes DNA oxidative damage and double-strand breaks in the rat esophagus. A. Representative pictures of Ki67, 8-OHdG, and γH2AX immunohistochemistry in esophageal epithelium. Scale bars, 50 μm. B-D. Expression level of Ki67, 8-OHdG, and γH2AX was quantified by immunohistochemical staining, respectively (n = 15-18/group). Values are means ± SD. Different letters (a and b for diet effect; x and y for NMBA effect) are significantly different by two-way ANOVA and LSD test (P < 0.05).
illomas [41, 42]. In addition, dysregulation of TGF-alpha and EGFR expression play an important role in NMBA-induced rat esophageal tumorigenesis [43]. Our studies show that RNF esophageal intraepithelial neoplasia has a proliferation, differentiation, and development gene signature. The overall Ki67 (a cell proliferation marker) staining in esophagus of NMBA treatment group were significantly higher than that in NMBA untreated group. In summary, the primary carcinogenic mechanism of NMBA is to cause gene mutations, cell proliferation, and abnormal differentiation. DNA damage and double-strand breaks are not severe in epithelial cells of the esophagus, which may be responsible for the low tumorigenicity of NMBA.

Flavins play a central role in whole-body energy metabolism. RBF deficiency leads to low efficiency of growth energy utilization in rats [44, 45]. Recently, multiple studies highlighted the pro-inflammatory effects of RBF-deficient. The expression and release of inflammatory cytokines are elevated in RBF-deficient adipocytes [46, 47]. Chronic inflammation and low efficiency of growth energy utilization may be the main cause of weight loss in RBF-deficient rats. RBF deficiency elicits a pro-inflammatory gene expression profile in T lymphoma Jurkat cells [48]. Accordingly, our research shows that RBF deficiency induces an inflammatory gene expression pattern in esophageal tumors. The elevated inflammatory cytokines cause activation of NF-κB signaling pathway in ESCC cells [28]. NF-κB family members modulate the transcription of genes that regulate inflammation, proliferation, and immune regulation [49, 50]. We speculate that RBF deficiency increases inflammatory cytokines and activates NF-κB, resulting in the release of cytokines, which in turn causes the activation of NF-κB. The positive feedback regulatory mechanism maintains chronic inflammation.

In humans, esophageal intraepithelial neoplasia and ESCC share high frequency of mutations and large-scale chromosome aberrations, indicating that the genetic stability of esophageal intraepithelial neoplasia has collapsed [51, 52]. Chronic inflammation-related DNA damage (genomic instability) may trigger the initiation of esophageal squamous dysplasia [53]. In this study, RBF deficiency is a trigger of chronic inflammation that provides a microenvironment conducive to tumor initiation. Normal RBF combined with NMBA mainly induces the occurrence of benign esophageal hyperplasia. RBF deficiency in combination with NMBA accumulates DNA oxidative damage and DNA double-strand breaks, resulting in chronic inflammation-associated DNA damage, and promotes NMBA-induced esophageal tumorigenesis. The underlying molecular mechanisms still need to be revealed.

In conclusion, the present study demonstrates that RBF deficiency causes chronic inflammation-associated genomic instability, which may contribute to NMBA-induced esophageal tumorigenesis in rats. Our study suggests that RBF deficiency in the diet is a likely etiologic agent of chronic inflammation in the esophagus that contributes to ESCC development in humans. RBF supplementation in high incidence area of esophageal cancer has important clinical significance in reducing the incidence of esophageal cancer.

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Disclosure of conflict of interest

None.

Abbreviations

DAVID, the Database for Annotation, Visualization and Integrated Discovery; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; ESCC, esophageal squamous cell carcinoma; EIN, esophageal intraepithelial neoplasia; Exp, experiment; GO, Gene Ontology; γH2AX, the specific phosphorylation at serine 139 of the histone H2AX; HE, hematoxylin and eosin; 8-OHdG, 8-hydroxy-2’-deoxyguanosine; HPLC, high performance liquid chromatogra-
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phy; IHC, immunohistochemistry; KEGG, Kyoto Encyclopedia of Genes and Genomes; NMBA or N, N-nitrosomethylbenzylamine; RBF or R, riboflavin; wk, week.

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References

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[43] Wang QS, Sabourin CL, Bijur GN, Robertson FM and Stoner GD. Alterations in transforming


Supplementary Table 1. Primer sequences for qRT-PCR

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A

At 5 weeks:
Diet: P < 0.001(a,b)
NMBA: P = 0.73
Diet x NMBA: P = 0.83

B

Exp 1

Exp 2
Supplementary Figure 1. Effect of RBF deficiency combined with NMBA on body weight and histopathological changes of organs in rats. A. Representative pictures of rat body weight. B. Rat body weight increase. Exp 1, \( n = 15-18 \)/group; Exp 2, \( n = 12-19 \)/group. Values are means ± SD. Exp 1 different letters (a and b for diet effect) are significantly different by two-way ANOVA and LSD test (\( P < 0.05 \)). Exp 2 different letters (a, b and c) are significantly different from each other by one-way ANOVA and LSD test (\( P < 0.05 \)). C. HE staining of rat heart, lung, liver, kidney, stomach, duodenum, jejunoileum, and colon. Scale bars, 50 μm.

Supplementary Figure 2. Effect of RBF deficiency combined with NMBA on the total number and percentage of white blood cells. A. Number of various cells in peripheral blood. B. The percentage of lymphocytes, eosinophils, and basophils in the total white blood cells, respectively. \( n = 15-18 \)/group. Values are means ± SD. Different letters (a and b for diet effect) are significantly different by two-way ANOVA and LSD test (\( P < 0.05 \)).