HHS Public Access

Author manuscript

Mol Carcinog. Author manuscript; available in PMC 2022 March 01.

Published in final edited form as:

Mol Carcinog. 2021 March ; 60(3): 213–223. doi:10.1002/mc.23285.

Prevention of mammary carcinogenesis in MMTV-*neu* mice by targeting RLIP

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Abstract

The overexpression and amplification of the protooncogene *neu* (ERBB2) play an important role in the development of aggressive breast cancer (BC) in humans. Ral-interacting protein (RLIP), a modular stress-response protein with pleiotropic functions, is overexpressed in several types of cancer, including BC. Here, we show that blocking RLIP attenuates the deleterious effects caused by the loss of the tumor suppressor p53 and inhibits the growth of human BC both *in vitro* and *in vivo* in MMTV-*neu* mice. In addition, we show that treatment with the diet-derived, RLIP-targeting chemotherapeutic 2'-hydroxyflavanone (2HF), alone or in combination with RLIP-specific antisense RNA or antibodies, significantly reduced the cumulative incidence and/or burden of mammary hyperplasia and carcinoma in MMTV-*neu* mice. 2HF treatment correlated with reduced tumor cell proliferation and increased apoptosis, and the average number of Ki67-positive (proliferating) cells was significantly lower in the tumors of 2HF-treated mice than in the tumors of control mice. Furthermore, targeting RLIP also resulted in the overexpression of E-cadherin and the infiltration of $CD3^+$ T cells into mammary tumors. Taken together, these results

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Authors' contributions:

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Conflicts of interest: The authors declare no conflicts of interest.

Declarations

Ethics approval and consent to participate: No human subjects were involved in the present study. All animal studies were conducted according to a protocol approved by the City of Hope Institutional Animal Care and Use Committee (IACUC protocol #12024).

Availability of data and material: All data generated and analyzed during the current study are available from the corresponding author upon request.

Competing interests: The authors declare that they have no competing interests.

underscore the translational potential of RLIP-targeting agents and provide a strong rationale to validate them in the clinic.

One-sentence summary:

Combination treatments incorporating 2HF and RLIP-inhibiting or depleting agents are more effective in suppressing spontaneous mammary carcinogenesis in MMTV-*neu* mice than either treatment strategy alone.

Keywords

RLIP; *RalBP1*; breast cancer; MMTV-*neu* mice; prevention

1. Introduction

Breast cancer (BC), the most common cancer among women in developed countries, is one of the most prominent causes of cancer-related deaths worldwide. Unfortunately, despite advances in the early diagnosis of BC, as well as improvements to treatment in the adjuvant, neoadjuvant, and post-neoadjuvant settings, the disease remains a major health issue (1). Like most cancers, BC is a heterogeneous disease with multiple subtypes, each with a unique biology, prognosis, clinical characteristics, and treatment response (2). The interplay between estrogen receptor (ER), cellular proliferation, and apoptotic networks influences disease subtype, incidence, and response to therapy. The amplification of epidermal growth factor receptor 2 (ERBB2, or HER2/*neu*) is another major factor influencing response to clinical interventions and risk of recurrence. The protooncogene *neu* encodes a 185-kDa transmembrane protein in the epidermal growth factor receptor family (3, 4). The oncogenic activation of *neu* can occur due to a point mutation in the transmembrane domain (5), deletion of the extracellular domain, or amplification and overexpression (6). ERBB2 amplification and overexpression have been observed in many primary BC tumors and are inversely correlated with patient survival (7, 8).

Selective ER modulators, such as tamoxifen, have shown promise in preventing BC tumorigenesis but are ineffective against ER-negative BCs (9). Furthermore, patients treated with selective ER modulators may suffer severe side effects, including increased risk of uterine cancer, thromboembolism, cataracts, and perimenopausal symptoms (10). Therefore, there is a need for relatively safe, novel agents that can prevent the onset and/or progression of BC, irrespective of hormone receptor status. As a result, increased attention has recently been directed toward using natural products as novel chemopreventative and/or cancer therapeutic agents (11).

Numerous phytochemicals from edible sources have been shown to have antineoplastic properties and are “generally regarded as safe” by the United States Food and Drug Administration (12). We have demonstrated the antineoplastic effects of 2’-hydroxyflavanone (2HF), a nontoxic flavonoid phytochemical abundant in orange peel, on breast, renal, and lung carcinoma (13–15). 2HF exerts its effects by impairing the mercapturic acid pathway (MAP), which defends cancer cells against apoptosis caused by

exogenous toxins, such as chemotherapy drugs, and endogenous pro-apoptotic metabolites, such as 4-hydroxynonenal (4HNE) derived from the oxidation of ω−6 fatty acids.

Mechanistically, 2HF reduces the activity and expression of two key MAP enzymes: glutathione S-transferase (GST) and RLIP (Ral-interacting protein), a stress-response protein (13–15). GST catalyzes the conjugation of glutathione (GSH) to exogenous and endogenous electrophilic (alkylating) compounds. The resulting GSH-electrophile conjugates (GS-Es) are removed from cells via ATP-dependent efflux through RLIP. ATPase couples this ATP-dependent efflux to the clathrin-dependent endocytosis (CDE) of ligand-receptor complexes in the plasma membrane (16).

The anti-apoptotic function of RLIP is important for the survival of cancer cells, as supported by multiple *in vivo* studies demonstrating that the inhibition/depletion of RLIP causes regression of various cancers (17–21). Furthermore, as the administration of toxic electrophiles, as well as radiation, can cause apoptosis through the generation of 4HNE, $RLIP^{-/-}$ mice are highly sensitive to stress caused by these treatments (17, 18). However, $RLIP^{-/-}$ mice also have impaired CDE and thus exhibit deficient ligand-receptor signaling mechanisms important for carcinogenesis, diabetes, and obesity, which gives rise to their remarkable resistance to chemical carcinogenesis (16), diabetes, metabolic syndromes (22, 23), and obesity (24). Moreover, we recently showed that RLIP haploinsufficiency prevents spontaneous carcinogenesis in $p53^{-/-}$ mice, which typically develop malignancy before six months of age (25). This astounding effect had not yet been achieved by any pharmacological or genetic interventions previously studied in $p53^{-/-}$ mice.

These ground-breaking studies indicate that spontaneous carcinogenesis caused by the loss of the tumor-suppressive functions of p53 can be bypassed by RLIP deficiency and establish the importance of the stress resistance and CDE functions of RLIP for cancer cell survival. Because 2HF inhibits RLIP and targeted RLIP inhibition regresses primary BC tumors in orthotopic mouse models (26, 27), we posited that 2HF, alone or in combination with other RLIP-targeting agents, would inhibit the incidence and/or burden of BC in MMTV-*neu* mice. Here, we show that, indeed, the inhibition of RLIP using 2HF reduced both the incidence and burden of mammary hyperplasia and carcinoma in MMTV-*neu* mice.

Material and Methods

2.1. Reagents

2HF (purity ~99%), horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit secondary antibodies, and MTT were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies against pAKT (S^{473}), ERBB2, CD3, CD31, Ki67, CDK4, Bax, Bcl2, survivin, vimentin, and E-cadherin were purchased from Santa Cruz Biotechnology (Columbus, OH) and Cell Signaling Technologies (Danvers, MA). CellTiter-Glo was procured from Promega (Madison, WI). The Universal Avidin/Biotin Complex (ABC) Detection Kit was procured from Vector (Burlingame, CA). RLIP antibodies were obtained from the same source as previously described (19, 20).

2.2. RLIP76 antisense preparation

Chemically synthesized phosphorothioate DNA in desalted form was purchased from Biosynthesis, Inc. (Lewisville, TX). A 21 nucleotide-long scrambled phosphorothioate DNA was used as a control (17–20).

2.3. Chemoprevention protocol

Six-week-old female transgenic MMTV-*neu* mice were purchased from the Jackson Laboratory (FVB/N-Tg(MMTVneu)202Mul/J; stock # 002376). The spontaneous development of BC in these mice is driven by the expression of the rat *Erbb2* oncogene in the mammary epithelium under the control of the MMTV promoter (28). After a one-week acclimation period, the mice were randomized into seven groups (n = 10 per group) for 28 weeks of control or experimental treatment. The control treatments consisted of **1)** corn oil; **2)** scrambled antisense (CAS); and **3)** pre-immune serum (PIS). The experimental treatments consisted of **4)** 2HF (50 mg/kg b.w. by oral gavage on alternate days); **5)** RLIP antisense (RAS; 5 mg/kg b.w., *i.p.* weekly); **6)** RLIP antibody (Rab; 5 mg/kg b.w., *i.p.* weekly); and **7)** a combination of 2HF, RAS, and Rab (2HF+RAS+Rab). The bodyweights of all mice were recorded thrice weekly, and all mice were monitored on alternate days for signs of distress, including impaired movement or posture, indigestion, and areas of redness or swelling. Any mice showing signs of distress, pain, or suffering due to tumor burden were humanely euthanized. When the mice reached 35 weeks of age, they were euthanized and their mammary tissues and vital organs (heart, lung, liver, spleen, brain, bone marrow, and kidney) were collected for morphological, immunohistological, and Western blot analyses. Paraffin-embedded sections of the mammary glands and vital organs (5-μm thick) were also examined by whole-mount and hematoxylin and eosin (H&E) staining, as described below, to monitor tumor initiation and progression. The use and treatment of the MMTV-*neu* mice were approved by the Institutional Animal Care and Use Committee (IACUC) of City of Hope National Medical Center, and the experiments were conducted in strict compliance with IACUC regulations.

2.4. Whole breast mounts

The entire intact lower abdominal mammary gland (#4) was dissected and processed as described previously (29), then placed in 70% ethanol until photographed using a Leica MZ 10F Stereomicroscope (Chicago, IL). Slides were placed in methyl salicylate for long-term storage (30).

2.5. Histopathological examination of tissues for markers of differentiation, proliferation, and angiogenesis

Complete autopsies were performed, including gross and microscopic examinations. Mice were euthanized by CO_2 asphyxiation, and their mammary glands and other tissues were evaluated for carcinogenicity. Tissues from all mice were fixed in 10% buffered formalin for 12 h. H&E staining to assess hyperplasia and carcinoma was performed on paraffin-embedded mammary gland, spleen, bone marrow, brain, heart, kidney, liver, and lung sections (5-μm thick). Mammary carcinogenicity was classified histologically according to the criteria outlined by Russo and Russo (31). The histopathological criteria used to

determine malignancy were loss of the tubular-alveolar pattern of the normal mammary gland; presence of large epithelial cells with elevated nuclear-cytoplasmic ratios and rates of mitosis; stromal response (fibrosis and inflammatory cell infiltration); necrosis and hemorrhage; and evidence of infiltration of surrounding tissues and metastasis. Hyperplasia was characterized by thickened and multilayered mammary ducts and cells with large nuclei. Carcinoma was characterized by the loss of ductal structure, uncontrolled expansion of enlarged cells, and invasion of healthy breast tissue.

Tumor and mammary gland sections were also immunostained and analyzed using the ABC detection kit to measure the expression of proteins involved in the epithelial-mesenchymal transition (EMT), such as E-cadherin and vimentin; CD31 to visualize blood vessels; and Ki67 and RLIP to assess cell proliferation. A dark brown stain indicated immunoreactivity, in contrast to the non-reactive areas displaying only the background color. For CD3 staining to determine infiltration of T cells, a characteristic pink color was developed by incubation with Vulcan red. Photomicrographs at 40x magnification were acquired using an Olympus DP72 microscope. The percentage of positive staining was determined by measuring positive immunoreactivity per unit area. The intensity of antigen staining was quantified by digital image analysis using DP2-BSW software. Bars represent the mean ± S.E. (n = 5); * $p < 0.003$ compared to controls.

2.6. Western blot analysis

Tumor tissues were homogenized, and lysates were prepared as we previously described (13). Total lysates containing 60 μg protein were resolved by SDS-PAGE, transferred onto nitrocellulose membranes, and analyzed by Western blot. Blots were incubated with primary and secondary antibodies conjugated to HRP and developed using enhanced chemiluminescence reagents (Amersham Biosciences, Piscataway, NJ). Protein expression was quantified by densitometric scanning of the immunoreactive bands. Prior to re-probing with a different antibody, the membranes were stripped, washed five times in TBST, and blocked. β-actin was used as a loading control.

2.7. Statistical Analysis

Unless otherwise specified, data are expressed as the mean ± SD and were evaluated using two-tailed, unpaired Student's *t*-tests. Changes in tumor size and bodyweight over the course of the experiments were visualized by scatter plot. Statistical significance of difference in tumor incidence between control and experimental treatment groups was determined by one-tailed Fisher's exact test. $p < 0.05$ was considered statistically significant.

2.8. Ethics statement

No human subjects were involved in this study. All animal studies were conducted according to a protocol approved by the City of Hope IACUC (protocol #12024).

3. Results

3.1. RLIP protein overexpression in human BC cell lines

RLIP protein levels are higher in various cancer cell lines and tissues than in their normal counterparts (21). Recently, we investigated the protein expression of RLIP in immortalized non-tumorigenic mammary epithelial cells (MCF10a) and BC cells (MCF7, T47D, SKBR3, TMD231, and MDA-MB231). Consistent with previous findings, RLIP protein expression was higher in all BC cell lines than in MCF10a cells (13). These observations suggest that RLIP may be an attractive target for inhibiting BC cell growth.

3.2. Inhibition of mammary cancer by 2HF and RLIP-targeting agents in MMTV-*neu* mice

The intake of flavonoids has been inversely associated with the risk of various neoplasms. For example, oranges provide a rich dietary source of many antioxidant compounds with anticancer properties (32). However, limited literature exists on the effects of flavonoids in BC. We previously observed that the anticancer effects of the citrus flavonoid 2HF against lung, breast, and renal carcinogenesis were associated with the inhibition of GSTs, the key enzymes in the first step of the MAP. We observed that 2HF treatment reduced MAP activity by reducing the expression and transport activity of RLIP (13–15).

Because MAP enzymes are upregulated in BC, we evaluated the anti-carcinogenic activity of 2HF, as well as its effects on spontaneous breast carcinogenesis in MMTV-*neu* mice, a well-characterized mouse model of mammary cancer (28). The doses of 2HF and the RLIP-targeting agents (RAS and Rab) were selected based on previous chemoprevention studies on chemically-induced tumor models (16, 29). At the end of the experiment, mice were anesthetized using isoflurane, and tissues were harvested for morphological, immunohistological, and Western blot analyses. Mammary glands were also examined by whole-mount staining and by sectioning and H&E staining of paraffin-embedded tissues to monitor tumor initiation and progression. Representative photographs from the complete gross necropsies of MMTV-*neu* mice that received control and experimental treatments are presented (Fig 1A). Tumors were counted and reported as percent incidence (Fig 1B). As expected, several mice in the control groups developed large mammary tumors, whereas few in the experimental groups showed palpable tumor growth by the end of the study, leading to a significant difference in mammary tumor frequency between groups ($p < 0.04$).

2HF and the RLIP-targeting agents were well tolerated by the mice and did not cause any significant weight loss or histological changes in vital organs. Three mice in the control groups, one in the RAS group, and two in the Rab group died before the termination of the study at 35 weeks; however, necropsies were inconclusive with respect to the cause of death. Mice in the experimental treatment groups had lower average bodyweights than mice in the control treatment groups at the onset of the study and for the duration of the experiment (Figs 1C–E). Average food consumption (grams/day/mouse) was modestly but significantly higher for mice in the experimental treatment groups than for mice in the control groups (data not shown).

3.3. Effects of 2HF and RLIP-targeting agents on normal organ development and function

Female MMTV-*neu* mice were treated with 2HF, RAS, and/or Rab for 28 weeks and monitored for spontaneous mammary carcinogenesis. Whole-mount staining was performed to determine if the treatments had any discernable influence on normal mouse breast development (Fig 1F). In all groups, the lymph nodes associated with the fat pads were present; however, compared to mice in the experimental treatment groups, mice in the control groups exhibited markedly attenuated mammary ductal growth. Specifically, there were fewer sectioned duct profiles in fat pads collected from control-treated mice than in fat pads collected from mice in any of the experimental groups. However, the number of sectioned duct profiles was similar across all experimental treatment groups (Fig.S1).

We also assessed the amount of fat and size of the adipocytes in the fat pads of the mice. Whereas these features were similar in the fat pads of mice that received the experimental treatments, the fat pads of the mice in the control groups had less fat and smaller adipocytes. Upon scoring the whole-mount preparations and H&E-stained sections of the mammary glands for the presence of hyperplasia and neoplasia, we observed that the incidence of mammary hyperplasia was significantly lower in the experimental groups than in the control groups ($p = 0.03$ by Fisher's test). Moreover, the overall incidence of abnormal structures (hyperplasia and carcinoma combined) was significantly lower in the experimental treatment groups than in the control treatment groups ($p = 0.01$ by Fisher's test). Collectively, these results suggest that the administration of dietary 2HF and/or RLIP-targeting agents can reduce the incidence and burden of mammary hyperplasia in MMTV-*neu* mice without causing side effects.

Next, we examined the organs that are typically affected by the toxic effects of anticancer treatments. Consistent with observations in the whole-mount preparations of mammary gland fat pads (Fig 1F), the amount of lymphoid tissue in the white pulp of the spleen was much lower for mice in the control treatment groups than for mice in the experimental treatment groups. Additionally, the number of megakaryocytes and the amount of hemosiderin (yellow-brown, granular material) were greater in the control groups than in the experimental groups. However, it is important to note that these apparent differences may be relative rather than actual, reflecting the reduced size of spleens in mice receiving the control treatments.

Interestingly, whereas the spleens of all mice that received the experimental treatments were similar (Fig.S1), the cellularity of bone marrow was markedly lower in mice that received control treatments. Similarly, although the glomeruli in the kidneys were morphologically unremarkable in mice that received the experimental treatments (Fig.S1), those of the control animals exhibited irregular cellularity and mesangial deposits of eosinophilic material. Additionally, the cells of Bowman's capsule were prominent in the glomeruli of the mice treated with PIS, and a rim of fibrous tissue surrounded the glomeruli. Moreover, dilated, protein-filled renal tubules lined with flattened epithelium were seen in the kidneys of mice that received CAS and PIS (Fig.S1). Histopathologic analyses of brain, lung, liver, and heart tissues performed to evaluate toxic effects did not uncover any obvious differences between groups. Taken together, these data demonstrate that 2HF and RLIP-targeting agents significantly reduced the incidence of mammary carcinogenesis in spontaneous MMTV-*neu*

mice, highlighting the autonomous role of RLIP in the regulation of mammary ductal growth in the mammary epithelium and providing strong evidence that the inhibition or depletion of RLIP selectively protects the mammary glands in MMTV-*neu* mice.

3.4. Effects of RLIP inhibition/depletion on Blc2 family proteins and Akt/Cdk1 signaling in MMTV-*neu* mice

The *Bcl2* family of genes modulates the interplay between cell survival and death factors to regulate tissue development and homeostasis, and members of this family are characterized as anti- or pro-apoptotic, depending on the cellular context. Bcl2 exerts anti-apoptotic effects and inhibits cellular progression through the cell cycle. Several relatives of Bcl2, including Bcl-X, Bax, Bak, Bad, Bcl-W, Bfl-1, and the Bcl2-binding protein Bag1 are expressed in mammary tissue. In human BC, Bcl2 expression has been linked to good prognosis, and low Bax expression has been associated with poor clinical outcomes (33). Therefore, we assessed the mammary gland tissue of MMTV-*neu* mice in the control and experimental treatment groups for markers of proliferation, apoptosis, and cell cycle progression, including those in the Bcl2 family. Because the Akt signaling pathway plays a critical role in BC initiation and progression (34), we also examined whether any components of this signaling pathway were dysregulated.

Compared to the mammary tumor extracts of mice in the experimental treatment groups, the mammary tumor extracts of control-treated mice had lower levels of the pro-apoptotic protein Bax and much higher levels of the anti-apoptotic protein Bcl2. Although total Akt protein expression was similar across groups, we observed higher levels of the activated form of Akt, pAkt S^{473}, in control-treated mice than in mice that received the experimental treatments, suggesting that RLIP inhibition can suppress Bcl2 upregulation and inhibit Akt activation in MMTV-*neu* mammary tumor tissues (Fig 2). Furthermore, the protein expression of Cdk1 was higher in the mammary tumor tissues of control-treated MMTV-*neu* mice than that in that of the mice that received the experimental treatments. Interestingly, the effects of the 2HF+RAS+Rab combination treatment were more pronounced than the effects of any single agent (Fig 2). These findings indicate that RLIP is required for Akt signaling pathway-stimulated upregulation of Cdk1 during the initiation and progression of the disease in MMTV-*neu* mice.

3.5. Histological effects of 2HF and RLIP-targeting agents on the mammary tissues of MMTV-*neu* mice

Mammary gland and tumor tissues were fixed in 4% paraformaldehyde, blocked in paraffin, sectioned at 5 μm, routinely H&E stained, and examined as indicated in the legends of Figures 3 and 4. Immunohistochemical analysis of the mammary gland and tumor tissues sections of MMTV-*neu* mice revealed that, compared to mice that received control treatments, mice that received 2HF and RLIP-targeting agents had lower levels of RLIP, the proliferation marker Ki67, and CD31 (also known as platelet cell endothelial adhesion molecule), which marks the formation of new blood vessels and is critical for tumor growth. Tissues from the mice in the experimental treatment groups also had higher levels of the epithelial differentiation marker E-cadherin and lower levels of the mesenchymal marker vimentin (Figs 3 and 4). Together, these results indicate that 2HF exerts strong anticancer

effects against BC by targeting RLIP and the inhibition/depletion of RLIP in MMTV-*neu* mice can significantly extend the latency and reduce the frequency of mammary tumor incidence (35, 36). Mechanistically, RLIP deficiency appears to suppress spontaneous mammary carcinogenesis in these mice by attenuating the upregulation of pro-apoptotic proteins and the downregulation of anti-apoptotic proteins.

3.6. Effects of 2HF and RLIP-targeting agents on the tumor infiltration by T cells

T cells are implicated in the surveillance of tumors (37). Thus, to examine the presence of infiltrating T cells in the mammary tumors of MMTV-*neu* mice, we conducted an immunohistochemical analysis of the pan T cell marker CD3. Infiltrating T cells were visible in the tumors of mice in both the control groups and the experimental groups (Fig 3); however, the fraction of infiltrating CD3+ T cells was ~65% higher in the tumors of mice in the experimental treatment groups ($p = 0.04$ by two-tailed Student's *t*-test), suggesting that the anticancer effects of 2HF and RLIP-targeting agents are accompanied by increased T cell surveillance.

3.7. Effects of 2HF and RLIP-targeting agents on *neu* (ERBB2) protein expression

Because the overexpression of *neu* (ERBB2) has been implicated as an important step in human BC tumor progression, we compared the levels of ERBB2 expression in the tumor and adjacent mammary epithelial tissue of MMTV-*neu* mice. As expected, ERBB2 protein was detected at high levels in the mammary hyperplasia and carcinoma of mice in all groups and was not significantly altered by any treatments (Fig 4B). Collectively, these observations are consistent with prior studies showing that elevated expression of ERBB2 is important for tumorigenesis and indicate that the 2HF-mediated prevention of mammary carcinogenesis in MMTV-*neu* mice was not due to the suppressed expression of the transgene.

4. Discussion

In an effort to develop safe, novel agents to prevent the onset and/or progression of BC, we have conducted several studies investigating the effects of dietary 2HF in BC. We have demonstrated that 2HF enhances the inhibitory effects of RLIP depletion and inhibits RLIP-mediated doxorubicin transport in BC cells (38). In addition, using RNA sequencing, we showed that 2HF treatment has minimal effects on MCF10A immortalized breast epithelial cells but strongly reverses the expression patterns of numerous genes in ER^+ MCF7, $ERBB2/HER2^+$ SKBR3, and triple-negative MDA-MB-231 BC cells (38).

The overexpression of ERBB2 due to amplification of the *neu* gene has been linked to the development and progression of BC (39). $ERBB2/HER2^+$ tumors, which account for ~20% of all BCs, tend to be more aggressive and are associated with poorer prognoses than ERBB2/HER2-negative BC (40). Using a mouse model with mammary epithelium-specific expression of the *neu* oncogene (MMTV-*neu*), we found that the administration of 2HF and RLIP-targeting agents suppressed the incidence and/or burden of mammary hyperplasia and carcinoma without causing weight loss or any other adverse effects. Additionally, upon examination, whole breast tissues from mice treated with 2HF and RLIP-targeting agents exhibited greater differentiation than whole breast tissues from mice in the control groups;

the mammary ducts of mice in the control groups had a significantly lower density of terminal end buds.

We previously showed that treatment with 2HF, as well as RAS and Rab, suppressed the growth of human BC cells (MCF7, SKBR3, and MDA-MB231) and induced apoptosis, regardless of ER responsiveness or p53 status (13). Cancer cell lines and cell-based models are invaluable tools for rapidly screening potential chemotherapeutic agents and elucidating their mechanisms of action. However, *in vivo* studies to validate the cellular findings can provide additional insights and are essential in preclinical drug development. The concordance between our cell-based findings (13) and our studies in the MMTV-*neu* mice, demonstrating that the administration of 2HF and RLIP-targeting agents elicits growth inhibitory and pro-apoptotic responses *in vivo*, lend further credence to these anticancer agents as promising therapeutics in BC. We also observed that expression of the proliferation marker Ki67 was significantly reduced in the mammary tumors of MMTV-*neu* mice upon administration of 2HF and RLIP-targeting agents. Likewise, compared to tumors from mice in the control groups, the spontaneous tumors from mice treated with 2HF and/or RLIP-targeting agents exhibited significantly lower expression of pAkt, RLIP, Bcl2, and Cdk1. Based on these observations, we conclude that cell proliferation and apoptosis are valid biomarkers to assess response to RLIP inhibition in future clinical trials.

E-cadherin plays an important role in various physiological processes, including those necessary for maintaining proper development, cell polarity, and tissue morphology (41). E-cadherin also acts as a tumor suppressor through its role in the inhibition of EMT (42). Indeed, the frequent downregulation of E-cadherin during cancer progression correlates with poor prognosis, whereas its expression has been shown to reduce BC tumor progression and invasiveness (43). In the present study, the administration of dietary 2HF and RLIP-targeting agents caused a marked increase in the expression of E-cadherin in the tumors of MMTV-*neu* mice. Likewise, treatment with these experimental agents, compared to control treatments, significantly reduced the expression of vimentin in the spontaneous tumors of MMTV-*neu* mice (Fig 3). Thus, we conjecture that the overexpression of E-cadherin likely contributes to anti-mammary cancer effects of dietary 2HF and RLIP-targeting agents.

T cells are involved in the cell-mediated immune response and play a critical role in the control of tumor development (44). We observed greater T cell infiltration in the tumors of mice treated with 2HF and/or the RLIP-targeting agents than in the tumors of mice in the control groups, suggesting that the RLIP inhibition/depletion-mediated prevention of mammary carcinogenesis involves cytotoxic T cell infiltration.

It is well established that intrinsic apoptotic signaling mediated by Bcl2 family members, including Bax and Bcl2, is important in the development of normal mammary glands, as well as BC. Whereas higher Bax:Bcl2 ratios indicate enhanced pro-apoptotic signaling and/or reduced anti-apoptotic processes, lower ratios, such as those observed in BC, suggest low pro-apoptotic rates (45). We observed that Bax expression was lower and Bcl2 expression was higher in control-treated mice, indicating that 2HF and RLIP-targeting agents downregulated Bcl2 expression and upregulated Bax levels in the MMTV-*neu* mice. Several human cancers also exhibit increased expression and activity of Cdk1, which plays

an important role in cell cycle regulation (46, 47). We observed that treating MMTV-*neu* mice with 2HF and/or RLIP-targeting agents downregulated Cdk1 expression (Fig 2), further underscoring the efficacy of this treatment modality.

Lastly, in the present study, along with previous studies on various other types of cancer (14, 15, 18–20), we have shown that 2HF administration, as well as RLIP depletion/inhibition, exerts antineoplastic effects in both wild type and p53-mutated cancers. The significance of targeting RLIP using 2HF is significant and impactful, particularly in light of our recent studies establishing the existential requirement of RLIP in p53-null malignancies (25). We discovered that RLIP haploinsufficiency completely prevented spontaneous malignancy in $p53^{-/-}$ mice, which otherwise always develop malignancies, and almost completely normalized their epigenomes and transcriptomes (25).

5. Significance

The development of RLIP-targeting therapies for BC is strongly justified based on the apparent sensitivity of BC to RLIP knockdown and a lack of significant toxicity in preclinical studies, as well as a reasonably well-defined mechanism of action involving several key cancer-related signaling pathways in BC. The evidence presented in this report supports a model in which 2HF exerts its anticancer effects by targeting RLIP, thus inhibiting CDE and attenuating multiple pathways that promote BC growth and resistance. Considering the lack of toxicity observed in this study, 2HF appears to be an attractive option for treating the intractable problem of metastases in BC and perhaps other malignancies. The synergy between the anticancer activities of 2HF and RLIP antisense/antibodies supports the importance of RLIP and the MAP in the mechanisms of action of 2HF. This study also suggests that *neu* acts as a potent oncogene in the mammary epithelium and provides a strong rationale for developing RLIP-targeting agent(s) for the prevention of mammary carcinogenesis.

6. Conclusions

It is well-established that the bioactive polyphenols present in edible plants can act as chemopreventive agents, interfering with cancer initiation, promotion, and progression (48–50). 2HF is cytotoxic to cancer cells and inhibits angiogenesis, which is critical for cancer progression and metastasis. Based on the observations reported here, we conclude that cell proliferation and apoptosis can serve as suitable biomarkers to assay the efficacy of 2HF in clinical trials. Furthermore, our findings suggest that flavanones may explain the inverse association between fruit and vegetable consumption and BC risk. Taken together, the chemotherapeutic and chemopreventive actions of 2HF represent a novel strategy to combat BC. In conclusion, the present study demonstrates that RLIP inhibition/depletion using 2HF and/or RLIP-targeting agents inhibits mammary carcinogenesis in MMTV-*neu* mice without causing weight loss or any other noticeable side effects. Furthermore, we showed that the RLIP inhibition/depletion-mediated prevention of mammary cancer correlates with reduced cell proliferation, increased apoptosis, CD3+ T cell infiltration, and overexpression of E-cadherin. These preclinical observations call for clinical studies to discern the efficacy of RLIP inhibition/depletion in human BC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

We thank the personnel in the Animal Research Center and Pathology Cores for their invaluable assistance. The authors are grateful to Dr. Jianying Zhang, Ph.D., Department of Bioinformatics at City of Hope, for assistance with statistical analyses. Research reported in this publication included work performed in the Pathology and Biostatistics Cores at City of Hope, which is supported by the National Cancer Institute of the National Institutes of Health under award number P30CA33572. We sincerely thank Dr. Ravi Salgia, MD, PhD, Professor and Chair, Department of Medical Oncology at City of Hope, for providing research space and support.

Funding:

This work was supported in part by grants from the United States Department of Defense (W81XWH-16–1–0641 and W81XWH-20–1–0362) and the National Cancer Institute of the National Institutes of Health (P30CA33572). Funding from the Beckman Research Institute of City of Hope is also acknowledged.

Abbreviations:

BC	breast cancer
EMT	epithelial-mesenchymal transition
ER	estrogen receptor
GSH	glutathione
GS-E	glutathione-electrophile conjugate
2HF	2'-hydroxyflavanone
MAP	mercapturic acid pathway
MMTV	mouse mammary tumor virus
PR	progesterone receptor
RLIP	Ral-interacting protein
TNBC	triple-negative breast cancer

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7.

Highlights

- Elevated levels of *neu* are sufficient for mammary tumorigenesis.
- RLIP plays an essential role in mammary carcinogenesis.
- RLIP plays a mammary epithelium-autonomous role in the regulation of mammary ductal growth and carcinogenesis.
- RLIP is a promising therapeutic target for preventing the initiation and progression of human BC.
- In the MMTV-*neu* mouse model of spontaneous BC, depletion of RLIP protects the mammary glands from carcinogenesis.

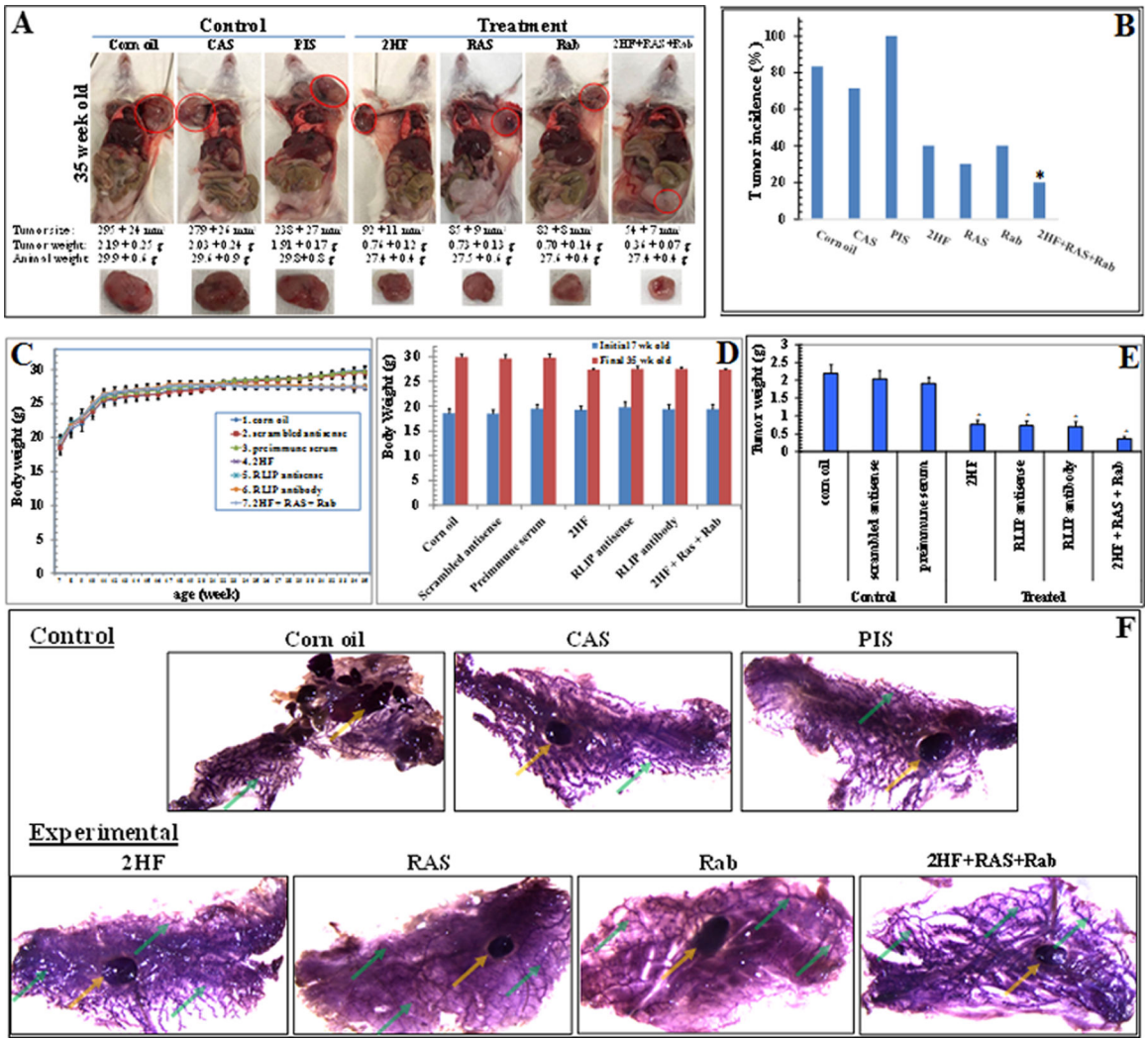

Figure 1. Effects of 2HF, RAS, Rab, and their combination on spontaneous breast carcinogenesis in MMTV-*neu* mice.

Representative photographs from the complete gross necropsies of MMTV-*neu* mice in the control and experimental treatment groups **(A)**. Percent incidence of tumors in all treatment groups. *$p < 0.035$ compared to corn-oil controls, and $p < 0.002$ compared to PIS controls by Fisher's test **(B)**. Weights of all mice over the course of the experiment, as a measure of possible treatment toxicity **(C and D)**. Mammary tissues and tumors and other vital organs, such as liver, lung, heart, brain, kidney, spleen, and bone marrow, were collected for immunohistochemical analyses. Tumors were measured by calipers and weighed **(A and E)**. *$p < 0.02$, compared to respective controls. **Whole-mount preparations of the mammary gland fat pads of MMTV mice:** Mayer's hematoxylin-stained mammary gland fat pads, excised from MMTV-*neu* mice that received 28 weeks of control treatment (corn oil; CAS; PIS) or experimental treatment (2HF; RAS; Rab; 2HF+RAS+Rab). Mammary gland fat pads from the control treatment groups were smaller and edematous, making it difficult to achieve adequate spread on the glass slides. Lymph nodes associated with the mammary gland fat pads were present in all groups (yellow arrows). The development of the duct system (green

arrows) was markedly attenuated in mice from the control treatment groups. The fat pads were similar among mice in all of the experimental treatment groups **(F)**. Abbreviations: **2HF**, 2'-hydroxyflavanone; **CAS,** scrambled antisense; **RAS,** RLIP antisense; **PIS**, pre-immune serum; **Rab**, RLIP antibody.

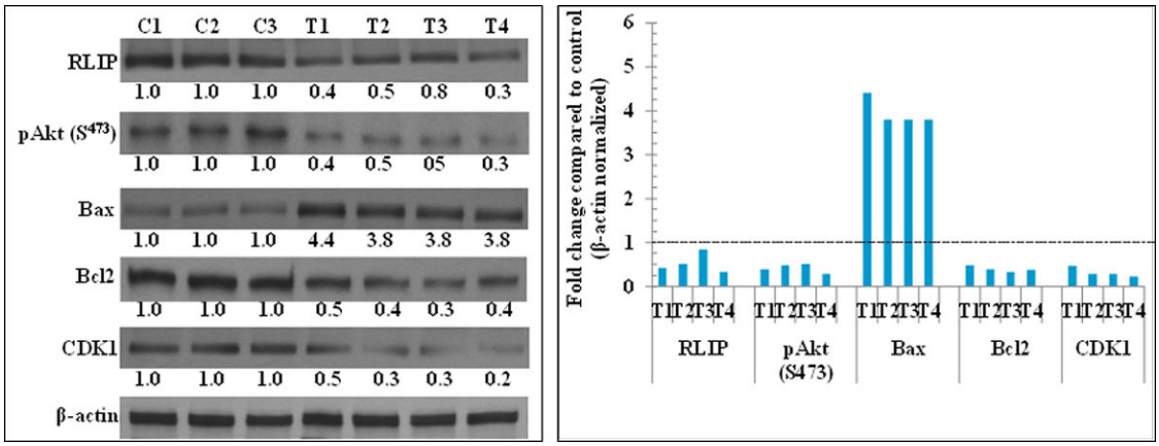

Figure 2. Effects of 2HF and RLIP-targeting agents on protein expression in the tumor tissues of MMTV-*neu* mice.

Sixty microgram protein of tumor tissues excised from MMTV-*neu* mice that received 28 weeks of control treatments (corn oil [**C1**], CAS [**C2**], and PIS [**C3]**) or experimental treatments (2HF [**T1**], RAS [**T2**], Rab [**T3**], and 2HF+RAS+Rab [**T4]**) were analyzed for changes in RLIP, pAkt, Bax, Bcl2, and CDK1 expression. β-actin was used as a loading control. The experiment was performed three times and the image shown is representative of one experiment. The numbers below the blots represent the fold-change in protein expression levels in the tumor tissues of mice in the experimental treatment groups compared to the control treatment groups, as determined by densitometry. Bar diagram shows the quantification of respective Western blots. Dotted line represents no significant change as observed with respective controls.

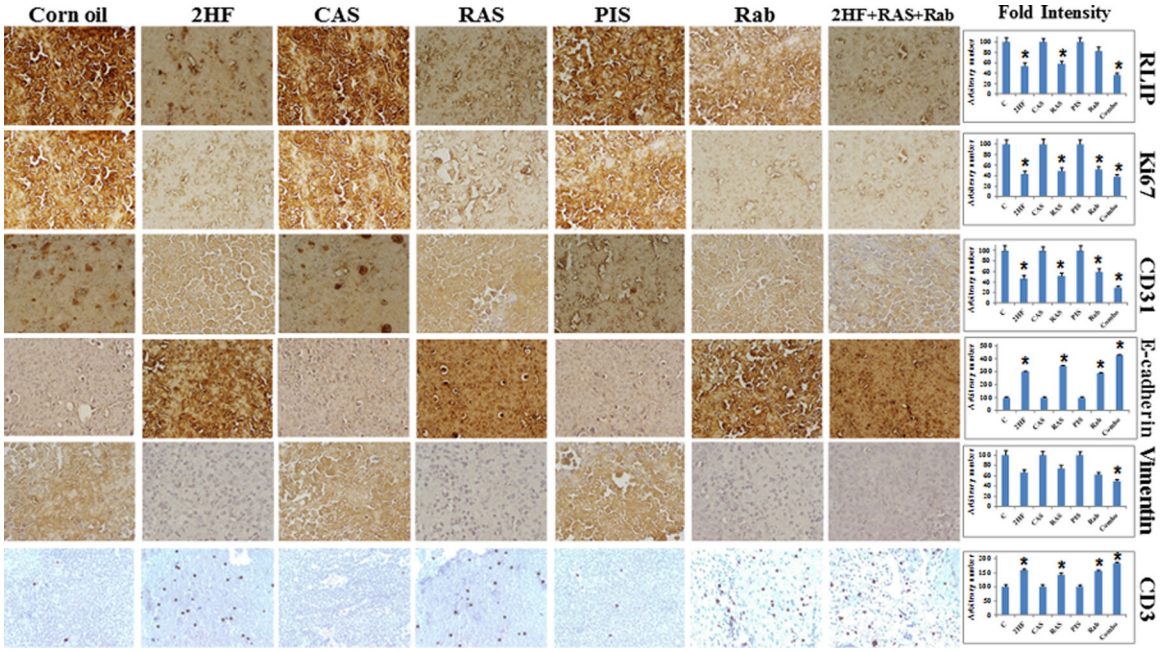

Figure 3. Immunohistochemical analysis of the tumor tissue of MMTV-*neu* mice

Immunohistochemical analysis was performed to detect RLIP, Ki67, CD31, E-cadherin, vimentin, and CD3+ T cells in tumor tissue isolated from MMTV-*neu* mice that received control treatments (corn oil, CAS, or PIS) or experimental treatments (2HF, RAS, Rab, and 2HF+RAS+Rab) for 28 weeks. The intensity of antigen staining was quantified by digital image analysis using Pro Plus software. Bars represent mean ± S.E. (n = 5). One representative image for each treatment group is shown. ***p* < 0.02, compared to respective control tissues by two-tailed Student's *t*-test.

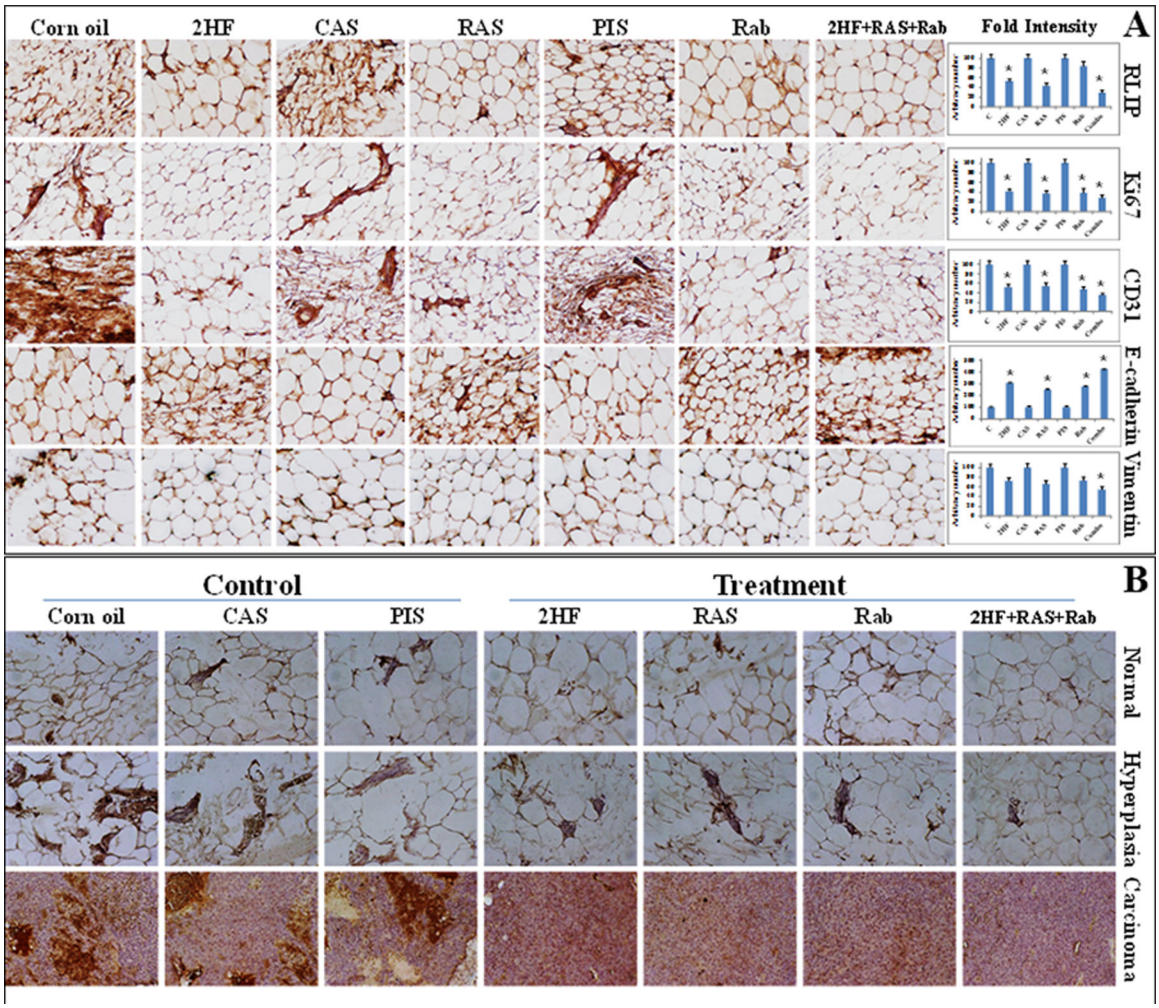

Figure 4. Immunohistochemical analysis of mammary gland tissue from MMTV-*neu* mice

Immunohistochemical analysis was performed to detect RLIP, Ki67, CD31, E-cadherin, and vimentin in mammary gland tissue isolated from MMTV-*neu* mice that received control treatments (corn oil, CAS, or PIS) or experimental treatments (2HF, RAS, Rab, or 2HF +RAS+Rab) for 28 weeks. The intensity of antigen staining was quantified by digital image analysis. Bars represent mean ± S.E. (n = 5). One representative image for each treatment group is shown. *****$p < 0.05$, compared to respective control tissues by two-tailed Student's *t*-tests (**A**). Immunohistochemical analysis of ERBB2 protein expression in the normal breast, hyperplasia, and carcinoma tissue of mice in the control and experimental treatment groups. Representative images at x400 magnification are shown (**B**). Abbreviations: **2HF**, 2'-hydroxyflavanone; **CAS,** scrambled antisense; **RAS,** RLIP antisense; **PIS**, pre-immune serum; **Rab**, RLIP antibody.