Published OnlineFirst October 3, 2012; DOI: 10.1158/1055-9965.EPI-12-0745

Cancer Epidemiology, Biomarkers & Prevention

Research Article

Influence of Quercetin-Rich Food Intake on microRNA Expression in Lung Cancer Tissues

Tram K. Lam[1], Stephanie Shao[4], Yingdong Zhao[2], Francesco Marincola[3], Angela Pesatori[5], Pier Alberto Bertazzi[5], Neil E. Caporaso[1], Ena Wang[3], and Maria Teresa Landi[1]

Abstract

Background: Epidemiologic studies have reported that frequent consumption of quercetin-rich foods is inversely associated with lung cancer incidence. A quercetin-rich diet might modulate microRNA (miR) expression; however, this mechanism has not been fully examined.

Methods: miR expression data were measured by a custom-made array in formalin-fixed paraffin-embedded tissue samples from 264 lung cancer cases (144 adenocarcinomas and 120 squamous cell carcinomas). Intake of quercetin-rich foods was derived from a food-frequency questionnaire. In individual-miR–based analyses, we compared the expression of miRs ($n = 198$) between lung cancer cases consuming high versus low quercetin-rich food intake using multivariate ANOVA tests. In family-miR–based analyses, we used Functional Class Scoring (FCS) to assess differential effect on biologically functional miR families. We accounted for multiple testing using 10,000 global permutations (significance at $P_{global} < 0.10$). All multivariate analyses were conducted separately by histology and by smoking status (former and current smokers).

Results: Family-based analyses showed that a quercetin-rich diet differentiated miR expression profiles of the tumor suppressor let-7 family among adenocarcinomas ($P_{FCS} < 0.001$). Other significantly differentiated miR families included carcinogenesis-related *miR-146*, *miR-26*, and *miR-17* ($P_{FCS} < 0.05$). In individual-based analyses, we found that among former and current smokers with adenocarcinoma, 33 miRs were observed to be differentiated between highest and lowest quercetin-rich food consumers (23 expected by chance; $P_{global} = 0.047$).

Conclusions: We observed differential expression of key biologically functional miRs between high versus low consumers of quercetin-rich foods in adenocarcinoma cases.

Impact: Our findings provide preliminary evidence on the mechanism underlying quercetin-related lung carcinogenesis. *Cancer Epidemiol Biomarkers Prev; 21(12); 2176–84. ©2012 AACR.*

Introduction

Quercetin is a polyphenol ubiquitously present in certain fruits (e.g., apples and grapes) and vegetables (e.g., onions, kale, broccoli, lettuce, and tomatoes) and has been found to possess anticarcinogenic properties (1). We (2) and others (3–5) previously observed that a quercetin-rich diet was associated with lower risk of lung cancer in epidemiologic studies. Quercetin and quercetin-rich foods may prevent carcinogenesis via several mechanisms, including free radical scavenging, proapoptotic and antiproliferation pathway mediation, modification of anti-inflammatory responses, and activation of detoxifying Phase II enzymes (6–9).

New findings suggested that polyphenol compounds such as quercetin are more likely to interact with cellular signaling cascades that regulate transcription factors (10). More specifically, *in vivo* and *in vitro* studies showed that they modulate a wide range of miR expressions and may consequently influence carcinogenesis (11–13). MiRs are short, noncoding, single-stranded RNAs involved in gene expression of multiple target mRNAs (14). Mis-regulated miRs have been implicated in many cancers where they act to promote overexpression of oncogenes and underexpression of tumor suppressor genes (14, 15). For example, the *let-7* class of miRs function as tumor suppressors by repressing cell proliferation and regulating both RAS and *c-myc* oncogenes (16). In lung cancer, we previously showed that the *let-7* family is differentially expressed by

Authors' Affiliations: Divisions of [1]Cancer Epidemiology and Genetics, and [2]Cancer Treatment and Diagnosis, NCI/NIH; [3]Department of Transfusion Medicine, Clinical Center and Center for Human Immunology, NIH, Bethesda, Maryland; [4]School of Public Health, Yale University, New Haven, Connecticut; and [5]Unit of Epidemiology, Fondazione IRCCS Ospedale Maggiore Policlinico and Department of Occupational and Environmental Health, Università degli Studi di Milano, Milan, Italy

Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (http://cebp.aacrjournals.org/).

T.K. Lam and S. Shao contributed equally to the work.

Corresponding Author: Maria Teresa Landi, Senior Investigator, Genetic Epidemiology Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, NIH, Bethesda, MD 20892. Phone: 301-402-9519; Fax: 301-402-4489; E-mail: landim@mail.nih.gov

doi: 10.1158/1055-9965.EPI-12-0745

Published OnlineFirst October 3, 2012; DOI: 10.1158/1055-9965.EPI-12-0745

histology and is associated with survival in the Environment And Genetics in Lung Cancer Etiology (EAGLE) study (17).

Polyphenolic compounds, including quercetin, have been shown in experimental studies to alter expression of several cancer-related miRs, including the cancer-associated *let-7* family (18–23). Quercetin metabolites were observed to modulate *miR-155* in murine macrophages (18) and *miR-146a* in colon cancer cells (21). Another polyphenol, epigallocatechin, has been shown to upregulate *miR-16* in human hepatocellular cells (23). In addition, differential expression of the *let-7* family and other miRs was observed in human hepatocellular cancer cells exposed to ellagitannin (22).

The emerging evidence from *in vitro* and *in vivo* investigations provides biologic rationale to examine the influence of quercetin on miR expression in lung carcinogenesis in the present epidemiologic study. As a follow-up study to our observation that a quercetin-rich diet was inversely associated with lung cancer in EAGLE participants (2), we investigated the influence of quercetin-rich food consumption on miR expression signatures in lung tissues of EAGLE lung cancer patients. Given the importance of *let-7* in lung carcinogenesis (24, 25) and their association with polyphenols (20), we specifically focused on several members of the *let-7* family as *a priori* candidates for quercetin modification. To our knowledge, this is the first mechanistic investigation of this nature using human tissues in relation to dietary quercetin-rich food consumption.

Materials and Methods

Study population

The present study is based on 144 adenocarcinoma (AD) and 120 squamous cell carcinoma (SQ) subjects from the EAGLE case–control study. The EAGLE study design has been previously described (26). Briefly, EAGLE is a population-based case–control study of lung cancer conducted in the Lombardy region of Italy between 2002 and 2005. Primary, incident, lung cancer cases ($N = 2{,}100$) were recruited from 13 hospitals that examined approximately 80% of all cases within the catchment area, which included 5 cities (Milan, Monza, Brescia, Pavia, and Varese), surrounding towns, and villages. The majority of cases (95%) were confirmed by pathology reports and the remaining cases by imaging and documentation of clinical history. Histologic type was recorded for all cases.

miR expression data

We previously described the miR expression data from EAGLE (17). Briefly, the miR expression data were derived from formalin-fixed paraffin-embedded tissue samples in 144 lung AD and 120 SQ cases from EAGLE. The 264 individuals included in the current study were a subgroup with both dietary quercetin information and miR expression data. These individuals did not differ markedly in relevant characteristics (e.g., age, sex, body mass index, smoking, and alcohol consumption, Supplementary Table S1) from EAGLE lung cancer cases that were excluded because of lack of data on quercetin and/or miR expression data.

The miRs were analyzed using a custom-made, 2-channel oligo-array using 1 Epstein-Barr virus (EBV) cell line as the reference sample. The array included a total of 713 human, mammalian, and viral mature antisense miRs plus 2 internal controls with 7 serial dilutions. Intensities for duplicate spots of each miR were averaged. Individual miRs with low overall signal intensity (<100) in both channels and/or low signal/noise ratio were excluded. A spot size smaller than 25 pixels and miRs with more than 50% missing data were additionally filtered out. Global median normalization was used as the most robust method with median-normalization calculated by subtracting out the median log-ratio for each array. Of the 440 human miRs, a total of 198 miRs were retained in the final analysis and are reported in Supplementary Table S2. We validated 5 miRs (*let-7g, let-7f, miR-26a, miR-638*, and *miR-107*) by quantitative real-time PCR using Taqman miR assays (Applied Biosystems) in 49 EAGLE samples normalized to Endogenous Control RNU6B that had sufficient tumor miR expression remaining after array analysis (17).

Epidemiologic and quercetin-rich food data

At baseline, epidemiologic data were collected using both a computer-assisted personal interview and a self-administered questionnaire to address potential risk factors associated with lung cancer, including comprehensive data on smoking exposure and dietary intake specific to this Italian population (26). Dietary intake in the previous year was obtained from a self-administered 58 item food frequency questionnaire (FFQ) where frequency of consumption was designated using 11 possible response categories that ranged from "never" to "2 or more times a day." Quercetin-rich food items (apples, grapes, onions, artichoke/fennel/celery, beans/chick peas, plum, turnips, peppers, strawberries, tomatoes, and broccoli) in the FFQ were identified based on data published in the United States Department of Agriculture on food-specific quercetin content (>0.50 mg/100g; ref. 27). We created a summary measure of quercetin-rich foods by adding the seasonal frequency of intake reported for the individual food item (2).

Statistical analysis

Quercetin-rich food intake was divided into sex-specific tertiles based on the distribution of the controls from the EAGLE study (2). We further defined highest and lowest consumers of quercetin-rich foods to be those in the third and first tertile, respectively. We previously showed that miR levels differed by histology in this population (17) and miR expression might be associated with smoking status (28). To address potential residual confounding by smoking and differential effect by histology, all our analyses were conducted for

Published OnlineFirst October 3, 2012; DOI: 10.1158/1055-9965.EPI-12-0745

Table 1. Selected characteristics by sex-specific tertile (T1-T3) of quercetin-rich food[a] intake in EAGLE, separately for histologic subtypes

Subject characteristics	AD				SQ			
	T1 ($n = 57$)	T2 ($n = 47$)	T3 ($n = 40$)	*P*	T1 ($n = 48$)	T2 ($n = 42$)	T3 ($n = 30$)	*P*
Quercetin-rich food intake[b], median (IQR)	0.79 (0.42)	1.56 (0.46)	2.45 (0.53)		0.71 (0.37)	1.47 (0.44)	2.53 (0.67)	
Age, mean (SD)	62.58 + 9.03	64.58 + 8.21	65.57 + 8.55	0.22[c]	68.48 + 7.09	69.02 + 6.30	66.37+ 8.62	0.29[c]
Male, n, %	38 (66.67)	25 (53.19)	20 (50.0)	0.20[d]	46 (95.83)	42 (100)	30 (100)	0.22[d]
BMI, mean (SD)	24.42 + 3.76	25.59 + 3.98	24.42 + 3.17	0.21[c]	25.42 + 3.10	26.73 + 3.71	27.74 + 3.92	**0.02[c]**
Smoking status, %				**0.01[d]**				0.08[d]
Never	4 (7.02)	16 (34.04)	7 (17.50)		0	1 (2.38)	0	
Former	22 (38.60)	12 (25.53)	22 (55.0)		18 (37.50)	20 (47.62)	20 (66.67)	
Current	31 (54.39)	19 (40.43)	11 (27.50)		30 (62.50)	21 (50.0)	10 (33.33)	
Pack years, median (IQR) intake	40.0 (24.0)	34.5 (32.0)	33.0 (28.0)	0.63[e]	54.0 (28.25)	46.25 (21.70)	42.50 (33.0)	0.22[e]
Vegetables[b,f]median (IQR)	1.01 (0.43)	2.03 (0.79)	2.46 (1.93)	<**0.01[e]**	0.95 (0.51)	1.68 (0.94)	2.61 (1.83)	<**0.01[e]**
Fruits[b,g], median (IQR)	0.96 (0.82)	1.78 (1.28)	2.80 (1.40)	<**0.01[e]**	1.03 (0.89)	1.55 (0.69)	3.00 (1.37)	<**0.01[e]**
Meats[b,h], median (IQR)	0.70 (0.68)	1.07 (0.79)	0.94 (0.98)	**0.01[e]**	1.07 (0.94)	1.23 (1.27)	1.33 (0.62)	0.06[e]
Lifetime alcohol[b], median (IQR)	23.13 (25.17)	14.79 (34.01)	7.41 (19.58)	**0.01[e]**	30.69 (24.69)	36.23 (20.30)	31.18 (31.97)	0.66[e]

NOTE: Column percent totals may not sum to 100% because of rounding; bolded *P* values indicated statistical significance.
Abbreviations: T1–T3 = first tertile through third tertile; IQR, interquartile range; SD, standard deviation.
[a]Quercetin-rich foods: summary measure of apples, grapes, onions, artichoke/fennel/celery, beans, apricots, plums, turnips, peppers, strawberries, tomatoes, and broccoli.
[b]Frequency (food groups, servingsper day; alcohol, grams per day).
[c]ANOVA test.
[d]χ^2 test.
[e]Nonparametric Kruskal–Wallis test.
[f]Total vegetables intake: summary measure of tomatoes, peppers, carrots, salad, peas, beans/chickpeas, mushrooms, broccoli, turnips, savoy, black cabbage, onions, cooked spinach/Swiss.
[g]Total fruits intake: summary measure of apples, pears, bananas, kiwis, oranges/grapefruits, mandarins/clementines, grapes, peaches/clingstones, apricots, plums, strawberries, melons, and fruit cocktails.
[h]Total meat intake: summary measure of cooked ham (prosciutto cotto), smoked ham (prosciutto crudo), cured ham (speck), salami, baloney (mortadella), wurstel, salted sliced beef, coppa, pancetta, and other types of processed meats.

smoking-specific (former and current smokers) and lung cancer subtype (AD and SQ). Although we examined the influence of quercetin-rich food intake on miR expression in never smokers, the results were unstable and not reported because of too few individuals ($n = 28$).

Individual-miR–based analyses

We first compared the expression levels of 198 miRs between highest (T3) and lowest (T1) quercetin-rich food consumers using multivariate ANOVA tests. Models were adjusted for age (continuous), sex, body mass index (continuous), pack years of smoking (continuous), consumption of non–quercetin-rich fruits and vegetables (continuous), red/processed meats (continuous), and lifetime alcohol consumption (continuous). In the larger EAGLE study, frequency of quercetin-rich intake was correlated with frequency of non–quercetin-rich fruits and vegetables ($r = 0.64$) and consumption of red/processed meat ($r = 0.07$). Selection of other covariates was based on factors that have been associated with either miR expression or lung cancer risk. Individual food items comprised within the individual food groups are described in Supplementary Table S3.

To further address the issue of multiple comparisons we calculated a global *P* value (P_{global}). For this calculation, we randomly permuted the highest and lowest classes of quercetin intake $\times 10{,}000$ where the number of significant miRs from ANOVA testing was recorded (n^P). The global *P* value was then defined as one plus

Published OnlineFirst October 3, 2012; DOI: 10.1158/1055-9965.EPI-12-0745

the number of times in which n^P was at least as large as the number of original significant miRs divided by 10,000.

There is a lack of data on quercetin-associated effects on miR expression in human tissues in the current literature, particularly at habitual consumable level. To minimize false negative findings at the initial analyses, we considered a permuted $P_{global} < 0.10$ to be statistically significant.

Family-miR–based analyses

We grouped each miR into known biologic functional families using MiRBase release 17.0. We assembled miRs into families based on unique "seed sequence" (nucleotides 2–7 at the 5′end) and identified 21 miR families (Supplementary Table S4). *A piori*, we restricted our family-miR–based analyses to 9 miR families with at least 1 miR identified at a P value less than 0.05. We used Functional Class Scoring (FCS) to compare the expression profile of each miR family between high versus low consumers of a quercetin-rich diet.

FCS computes P values by assigning all miRs within a particular group (or family) an aggregate raw score (the arithmetic mean of the negative natural logarithm of each P value obtained from miR analyses; ref. 29). This raw score was then compared with the score of randomly derived groups of the same size through q repeated samplings ($q = 1{,}000$). Each score was ordered in ascending order to build an empirically derived score distribution. The FCS P value was determined as the fraction of randomly sampled groups having a higher score than the group score of interest. For these analyses, we defined statistical significance at a $P_{FCS} < 0.05$. We further evaluated the statistical significance using the more conservative Bonferroni P value ($0.05/9 = 0.006$).

Permutations were conducted in the statistical package R. All other analyses were conducted using SAS, version 9.2.

Results

In the present study, lung cancer cases had a mean age at diagnosis of 65 years. Table 1 presents the distribution of selected characteristics by tertiles of quercetin intake, separately for AD and SQ. Among ever smokers, highest (T3) consumers of quercetin-rich foods smoked less and were more likely to be AD cases compared with low consumers. Former smokers on average consumed more servings of quercetin-rich foods per day than current smokers (1.65 ± 0.88 vs. 1.29 ± 0.79). Compared with AD cases, SQ cases tended to be males, smoked more, and consumed more alcohol and meat across tertiles of quercetin intake. Never smokers ($n = 29$) were all AD except for one individual.

Individual-miR–based expression

We identified 16 miRs that were differentially expressed for AD (4 miRs) and SQ (12 miRs) cases ($P < 0.05$) between high versus low consumers of quercetin-rich

Table 2. MiRs that significantly (at $P < 0.05$) differentiate highest (T3) versus lowest (T1) consumers of quercetin-rich food intake, separately by histology

	T1 mean ± SD	T3 mean ± SD	Fold change[a]	P[b]
AD ($n = 97$)				
hsa-miR-502	0.085 ± 0.353	0.202 ± 0.350	1.124	0.017
hsa-mir-564	0.565 ± 0.258	0.449 ± 0.273	0.890	0.030
hsa-miR-124a	0.232 ± 0.447	0.072 ± 0.423	0.852	0.044
hsa-miR-125a	0.625 ± 0.723	1.034 ± 0.715	1.505	0.045
SQ ($n = 78$)				
hsa-miR-510	0.283 ± 0.361	0.147 ± 0.295	0.872	0.003
hsa-mir-605	2.118 ± 0.815	1.279 ± 0.765	0.432	0.004
hsa-miR-155	−5.113 ± 1.071	−4.777 ± 0.784	1.399	0.012
hsa-miR-373	−0.005 ± 0.420	−0.091 ± 0.330	0.917	0.014
hsa-miR-453	0.597 ± 0.324	0.491 ± 0.211	0.899	0.017
hsa-miR-502	0.318 ± 0.309	0.095 ± 0.223	0.801	0.017
hsa-miR-18b	−2.621 ± 0.735	−2.227 ± 0.724	1.483	0.020
hsa-miR-183	1.160 ± 0.495	0.779 ± 0.470	0.683	0.022
hsa-mir-573	0.267 ± 0.355	0.126 ± 0.406	0.869	0.024
hsa-miR-524[a]	0.074 ± 0.259	−0.082 ± 0.341	0.855	0.036
hsa-mir-612	−0.171 ± 0.851	−0.104 ± 0.719	1.069	0.042
hsa-miR-363[a]	−0.076 ± 0.778	0.124 ± 0.703	1.222	0.046

[a]Fold change is the ratio (T3/T1) of geometric means (>1.0 indicates upregulation and < 1.0 downregulation).

[b]Coefficient P value from ANOVA model adjusted for age, sex, BMI, smoking status, non–quercetin-rich fruits and vegetables, red/processed meat, alcohol, and cigarette pack years.

Published OnlineFirst October 3, 2012; DOI: 10.1158/1055-9965.EPI-12-0745

Table 3. Influence of quercetin-rich food intake (T3 vs. T1) on individual miR, stratified by histology and smoking status

AD

Former smokers ($n = 44$)					Current smokers ($n = 42$)				
miR name	T1 mean ± SD	T3 mean ± SD	Fold change[a]	P[b]	miR name	T1 mean ± SD	T3 mean ± SD	Fold change[a]	P[b]
hsa-mir-641	−0.048 ± 0.548	−0.237 ± 0.879	0.828	0.003	hsa-mir-580	0.492 ± 0.340	0.226 ± 0.286	0.767	0.003
hsa-miR-29b	−1.110 ± 1.390	−1.021 ± 1.158	1.092	0.003	hsa-miR-215	−0.665 ± 0.403	−0.932 ± 0.456	0.766	0.004
hsa-miR-146a	−4.714 ± 0.805	−4.830 ± 1.334	0.890	0.006	hsa-miR-194	−0.726 ± 0.662	−1.159 ± 0.883	0.648	0.011
hsa-miR-500a	0.646 ± 0.360	0.471 ± 0.539	0.839	0.008	hsa-mir-598	−0.119 ± 0.498	−0.674 ± 0.538	0.574	0.016
hsa-let-7e	−1.121 ± 0.808	−0.882 ± 0.804	1.270	0.018	hsa-miR-518a-2[a]	0.077 ± 0.343	0.004 ± 0.228	0.929	0.020
hsa-miR-134	0.404 ± 0.304	0.354 ± 0.322	0.952	0.020	hsa-miR-503	0.147 ± 0.682	−0.244 ± 0.451	0.677	0.037
hsa-miR-26b	−1.624 ± 1.514	−0.930 ± 1.157	2.003	0.021	hsa-miR-146b	−4.682 ± 1.234	−4.278 ± 1.388	1.497	0.043
hsa-miR-302c[a]	0.107 ± 0.333	0.244 ± 0.305	1.147	0.023	hsa-miR-381	0.044 ± 0.432	−0.127 ± 0.269	0.843	0.047
hsa-miR-98	−1.804 ± 1.199	−1.798 ± 1.527	1.006	0.024					
hsa-let-7c	−1.634 ± 1.559	−1.265 ± 1.326	1.446	0.024					
hsa-miR-27a	−1.351 ± 1.218	−1.097 ± 1.002	1.290	0.025					
hsa-let-7a	−2.044 ± 1.400	−1.663 ± 1.288	1.464	0.026					
hsa-let-7g	−2.283 ± 1.344	−2.396 ± 1.469	0.893	0.026					
hsa-let-7i	−2.153 ± 1.492	−1.810 ± 1.300	1.409	0.028					
hsa-let-7f	−2.512 ± 1.587	−2.191 ± 1.433	1.377	0.030					
hsa-miR-195	−2.106 ± 1.343	−2.141 ± 1.111	0.966	0.031					
hsa-miR-16	−2.852 ± 1.482	−2.495 ± 1.081	1.429	0.032					
hsa-miR-146b	−4.292 ± 1.012	−4.254 ± 1.336	1.039	0.034					
hsa-miR-26a	−0.943 ± 1.723	−0.364 ± 1.552	1.783	0.034					
hsa-miR-19b	−3.679 ± 1.048	−3.947 ± 1.108	0.764	0.036					
hsa-mir-564	0.556 ± 0.263	0.495 ± 0.220	0.941	0.037					
hsa-miR-20a	−4.321 ± 1.456	−4.262 ± 1.075	1.061	0.041					
hsa-miR-106a	−3.747 ± 1.533	−3.702 ± 0.988	1.047	0.044					
hsa-miR-34a	−0.896 ± 0.743	−0.779 ± 0.530	1.124	0.046					
hsa-miR-92a	−3.729 ± 1.292	−3.614 ± 1.051	1.121	0.048					

SQ

Former smokers ($n = 38$)					Current smokers ($n = 40$)				
miR name	T1 mean ± SD	T3 mean ± SD	Fold change[a]	P[b]	miR name	T1 mean ± SD	T3 mean ± SD	Fold change[a]	P[b]
hsa-miR-492	−1.383 ± 0.540	−1.262 ± 0.394	1.129	0.012	hsa-miR-502	0.354 ± 0.222	0.108 ± 0.220	0.782	0.010
hsa-miR-510	0.408 ± 0.374	0.166 ± 0.307	0.785	0.021	hsa-mir-605	2.198 ± 0.861	1.105 ± 0.701	0.335	0.013
hsa-miR-491	−0.274 ± 0.798	−0.121 ± 0.638	1.165	0.023	hsa-miR-506	0.245 ± 0.222	−0.195 ± 0.615	0.644	0.017
hsa-mir-612	−0.264 ± 1.072	−0.109 ± 0.789	1.168	0.025	hsa-miR-183	1.282 ± 0.518	0.357 ± 0.390	0.397	0.028
hsa-miR-500a	0.387 ± 0.278	0.291 ± 0.385	0.908	0.028	hsa-miR-524[a]	0.094 ± 0.294	−0.170 ± 0.151	0.768	0.029
hsa-mir-663	−0.169 ± 0.551	−0.117 ± 0.495	1.054	0.034					
hsa-miR-503	−0.069 ± 0.710	−0.149 ± 0.417	0.923	0.034					
hsa-miR-453	0.584 ± 0.324	0.482 ± 0.226	0.903	0.035					
hsa-mir-654	−1.239 ± 0.422	−1.111 ± 0.366	1.137	0.041					
hsa-mir-658	0.211 ± 0.793	0.123 ± 0.372	0.916	0.047					

NOTE: miRs are ordered by P value within strata.

[a]Fold change is the ratio (T3/T1) of geometric means (>1.0 indicates upregulation and < 1.0 downregulation).

[b]Coefficient P value from ANOVA model adjusted for age, sex, BMI, non–quercetin-rich fruits and vegetables, red/processed meat, alcohol, and cigarette packyears.

foods (Table 2). Likewise, 19 miRs were differentially expressed for former (12 miRs) and current (7 miRs) smokers (see Supplementary Table S5).

Table 3 presents analyses examining the influence of quercetin-rich diet on miR expression within histologic subtypes for former and current smokers separately. Considering the 4 subgroups, we identified overall 48 unique miRs that were differentially expressed between highest vs. lowest quercetin-rich consumers ($P < 0.05$, Table 3).

Published OnlineFirst October 3, 2012; DOI: 10.1158/1055-9965.EPI-12-0745

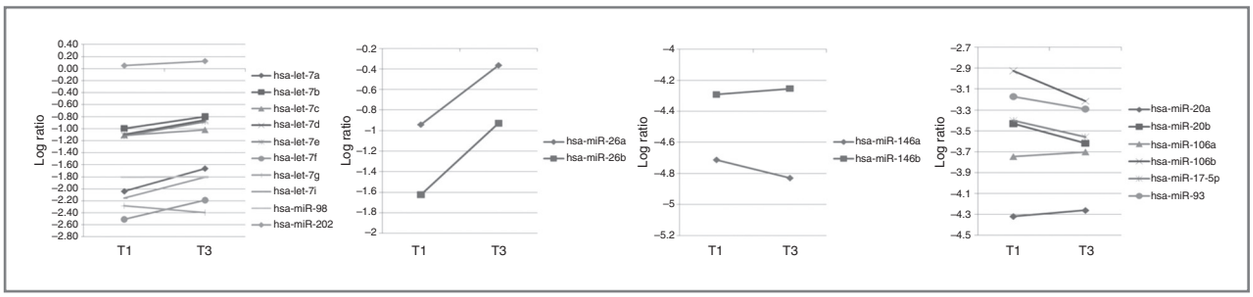

Figure 1. Mean expression levels for significant miR groups comparing the highest versus lowest tertile of quercetin-rich food intake in former smokers with adenocarcinoma.

Among former and current smokers with AD, 33 miRs were observed to be differentiated between highest and lowest quercetin-rich consumers (23 expected by chance; $P_{global} = 0.047$, Table 3). For SQ cases, we identified 15 miRs ($P_{global} = 0.15$, Table 3).

Quercetin-mediated miR expression profiles appeared to be more prevalent among former smokers with AD compared with current smokers. In this group, we identified 25 miRs with a P value less than 0.05 (22 expected by chance; $P_{global} = 0.076$; see Supplementary Fig. S1 for heat map). The largest fold change was observed for miR-26b, a proapoptotic miR (fold change = 2.00; $P = 0.020$). Notably, among the identified significant miRs, all of the *let-7* family members (*let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, let-7i*, and *miR-98*) were associated with a quercetin-rich diet. Moreover, the majority of let-7 family members were upregulated with increasing frequency of quercetin intake (Fig. 1).

In comparison to AD cases, far fewer miRs were identified at a P value less than 0.05 among SQ cases. None of the SQ subgroups was statistically significant ($P_{global} > 0.10$).

Family-miR–based expression

Table 4 presents the results examining the association of quercetin-rich foods with families of miRs in smoking-specific analyses for AD and SQ cases. A quercetin-rich diet appears to significantly differentiate miR expressions in former smokers with AD. Among this group, our data showed that the *let-7* family was strongly differentiated by a quercetin-rich diet ($P_{FCS} < 0.001$, Table 4) followed by *miR-146* ($P_{FCS} = 0.002$), miR-26 ($P_{FCS} = 0.010$), and *miR-17* ($P_{FCS} = 0.031$). Both the let-7 and the *miR-146* families remained significant after Bonferroni correction for multiple comparisons ($P_{bonferonni} = 0.05/9 = 0.006$). We observed no significant difference in miR expression for SQ cases and current smokers regardless of histology.

Figure 1 graphically depicts the directionality of quercetin-associated miR expression of *let-7, miR-146, miR-26*, and *miR-17* families with a quercetin-rich diet for formers smokers with AD. In general, members of the let-7 family, miR-26 as well as *miR-146b* were upregulated. In contrast, the expression of *miR-146a* was downregulated.

Discussion

We previously observed that higher consumption of quercetin-rich foods was associated with lower risk of lung cancer in a large population-based case–control study in Italy (2). The present study tests the hypothesis that a quercetin-rich diet modulates the expression of miRs in human lung tissues. In individual-miR–based analyses, we identified significant quercetin-mediated miR expression signatures for 48 unique miRs. These identified miRs have been shown to decrease tumor metastasis and invasion (*miR-146a/b, 503*, and *194*), decrease cell proliferation (*miR-125a, 155, let-7* family, *302c, 195, 26a, 503*, and *215*), increase apoptosis (*miR-125a, 605, 26b, let-7g, 34a, 491*, and *16*), and target tumor suppressors (*let-7* family, *miR-125a, 183, 146a, 98, 19b, 106a*, and *381*). In family-miR–based analyses, we found that the large majority of members of the let-7 family was strongly upregulated among former smokers with AD who consumed a higher intake of quercetin compared with low consumers ($P_{FCS} < 0.001$). We also observed similar family-based results for *miR-146*, *miR-26*, and *miR-17* families ($P_{FCS} < 0.05$) in this group.

Because of its association with lung cancer (25) and possibly with polyphenols (20), we specifically focused on the *let-7* class of miRs in relation to a quercetin-rich diet. In addition to being the most statistically significant result based on FCS in the family-based analyses, the *let-7* family remained significant after Bonferroni correction at $P < 0.006$. Members of the *let-7* family are known to function as tumor suppressors in lung carcinoma by repressing non–small cell lung carcinoma cell proliferation (16, 30) and by negatively regulating the *RAS* oncogene (31). Among the *let-7* miRs in the present study, let-7a, a known suppressor of *k-RAS* and *c-Myc* oncogenes (32), exhibited the largest fold change (fold change = 1.46). Our data suggest a possible mechanism of quercetin-related tumor protection through the increased expression of these key tumor suppressors.

We also observed differential quercetin-mediated expression of the *miR-17* family (*miR-20a, 20b, 106a, 106b, 17*, and *93*) in former smokers with AD. *MiR-17* family belongs to the oncogenic *miR-17–92* cluster (33).

Published OnlineFirst October 3, 2012; DOI: 10.1158/1055-9965.EPI-12-0745

Table 4. Influence of quercetin-rich food intake (T3 vs. T1) on family of functional[a] miR, stratified by histology and smoking status

		AD		SQs	
Family Function	**miRNA members**	**Former P^a**	**Current P^a**	**Former P^a**	**Current P^a**
Let-7 family	hsa-miR-let-7a	***P* < 0.001**	$P = 0.426$	$P = 0.366$	$P = 0.988$
Tumor suppressor	hsa-miR-let-7b				
	hsa-miR-let-7c				
	hsa-miR-let-7d				
	hsa-miR-let-7e				
	hsa-miR-let-7f				
	hsa-miR-let-7g				
	hsa-miR-let-7i				
	hsa-miR-98				
	hsa-miR-202				
miR-146 family	hsa-miR-146a	***P* = 0.002**	$P = 0.092$	$P = 0.753$	$P = 0.222$
Tumor growth and invasion	hsa-miR-146b				
miR-26 family	hsa-miR-26a	$P = 0.010$	$P = 0.623$	$P = 0.588$	$P = 0.664$
Apoptosis	hsa-miR-26b				
miR-17 family	hsa-miR-20a	$P = 0.031$	$P = 0.943$	$P = 0.766$	$P = 0.283$
Tumor progression	hsa-miR-20b				
	hsa-miR-106a				
	hsa-miR-106b				
	hsa-miR-17-5p				
	hsa-miR-93				
miR-29 family	hsa-miR-29a	$P = 0.064$	$P = 0.373$	$P = 0.886$	$P = 0.137$
DNA methylation	hsa-miR-29b				
	hsa-miR-29c				
miR-18 family	hsa-miR-18a	$P = 0.705$	$P = 0.220$	$P = 0.156$	$P = 0.392$
Tumor progression	hsa-miR-18b				
miR-34 family	hsa-miR-34a	$P = 0.142$	$P = 0.649$	$P = 0.275$	$P = 0.568$
Tumor suppressor	hsa-miR-34c				
miR-19 family	hsa-miR-19a	$P = 0.072$	$P = 0.991$	$P = 0.608$	$P = 0.103$
Tumor progression	hsa-miR-19b				
miR-15/16 family	hsa-miR-503	$P = 0.286$	$P = 0.073$	$P = 0.307$	$P = 0.763$
Apoptosis	hsa-miR-15a				
	hsa-miR-16				
	hsa-miR-195				
	hsa-miR-424				

NOTE: Only results of miR families that had at least 1 miR that were significant at $P < 0.05$ from individual-based miR analyses (Table 2); bolded *P* values indicated results that remained significant after Bonferroni correction for multiple comparisons. Models adjusted for age, sex, BMI, non–quercetin-rich fruits and vegetables, red/processed meat, alcohol, and cigarette pack years.

[a]Refer to Supplementary Table S5 for more detailed functions.

*P value based on FCS as described in the Materials and Methods section.

Investigators have shown that the *miR-17–92* cluster is frequently overexpressed in lung cancer (25, 34). Expression of *miR-17* is associated with poorer prognosis and cellular proliferation (35), whereas *miR-106b* targets p21 and subsequently promotes cell cycle progression (36). In addition, suppression of *miR-20a* induces apoptosis in lung cancer (37). In our study, the majority of the miRs (67%) in *miR-17* family were downregulated in frequent consumers of quercetin-rich food among former smokers with AD.

Quercetin-rich food consumption also significantly differentiated *miR-146* and *miR-26* families in our study. Neither of these miR families has been extensively studied with respect to lung carcinogenesis; however, both families have been associated with tumor development. In lung alveolar epithelial cells, *miR-146*

Published OnlineFirst October 3, 2012; DOI: 10.1158/1055-9965.EPI-12-0745

was observed to negatively regulate proinflammatory chemokines (38). In addition, *miR-146a* is one of 2 known miRs (*miR-146a* and *155*) involved in inflammatory signaling pathways and has been observed to be upregulated by quercetin in experimental study (21). We corroborated this upregulation of *miR-146a* among higher consumers of quercetin in the present study. One study showed that *miR-155*, was downregulated with quercetin in murine cells (18). In lung carcinoma, *miR-155* has often been seen to be upregulated and to have prognostic impact (39). However, it has also been suggested to function as a tumor suppressor by repressing cell proliferation (40). In the present study, however, miR-155 was not significant in the individual-miR–based analyses by histology and smoking status.

Both *miR-26a* and *miR-26b* exhibited the greatest fold change in our individual-miR–based analyses (*miR-26a*, FC = 1.78) and (*miR-26b*, FC = 2.00). Our data suggest that a quercetin-rich diet increases the expression of the miR-26 family, which has been shown to suppress cell proliferation in nasopharyngeal carcinoma through G_1 phase arrest and repression of *c-Myc* (41) as well as to induce apoptosis in breast cancer cells (42). Proapoptotic characteristics of *miR-26* make this particular group of miRs an important candidate for study in future research investigating quercetin-mediated miR targets.

We previously identified a miR expression profile that strongly differentiated AD from SQ with prognostic implications in EAGLE (17). Results from this present study showed consumption of quercetin-rich foods is associated with differential miR expression by histology. In general, our data suggest that quercetin-rich foods influenced miR expression in tumor tissues of former smokers with AD, but not for SQ and current smokers. The modest fold-change effect of dietary quercetin on miR expression might only be detectable in a milieu that is less saturated by smoking exposure, as in former smokers and in AD that is less associated with smoking than SQ (43). Furthermore, AD cases included in the present study on average smoked less intensely than SQ cases. The anticarcinogenic capabilities associated with dietary quercetin may be weakened in SQ cases by competing tobacco-related carcinogens.

To our knowledge, the present study is the only investigation examining the association between dietary quercetin, at a habitual consumable level, and miR expression in lung tissues. In addition to having both dietary information and miR expression data, this study included several variables that allowed tight control for potential confounders. This richness of epidemiologic data coupled with epigenetic data from human tissues permitted an integrative approach—making it possible to explore underlying mechanisms that may explain the protective effect of quercetin and lung cancer risk seen in observational studies.

Despite its uniqueness, the study had a limited sample size, which reduces statistical precision, and explored a limited number of human miRs. Although the EAGLE questionnaire assessed food consumption a year before the study, we cannot exclude that the lung cancer diagnosis had influenced the patients' responses. However, this potential recall bias should not have differentially affected the AD or SQ cases, and could not explain the different association with quercetin by histology type. Secondly, the northern Italian population comprised of the EAGLE study consumed very high intake of meat products (44) and in contrast substantially lower intake of fruits and vegetables; therefore, the FFQ captured a limited variety of food sources of quercetin as reflective of the consumption in this population. While our exposure measure of quercetin-rich diet is likely subject to measurement errors, the errors however would be nondifferential errors and would not alter our findings and conclusion. Moreover, we did not measured quercetin content directly; thus, we cannot rule out the contribution of other flavonoids or nutrients that are found in those foods.

In conclusion, we observed that a quercetin-rich diet is associated with differential expression of key miRs in lung tissue within smoking specific histology groups. Notably, expression of miRs in the *let-7* family, a known tumor suppressor, was strongly associated with frequent quercetin-rich food intake in the present study. Our findings provide suggestive insights into a possible mechanism to explain the inverse association between quercetin-rich food consumption and lung cancer risk observed in epidemiologic studies. Confirmation in a larger prospective study with both dietary quercetin information, miR expression from lung tissue, and clinical data is warranted.

Disclosure of Potential Conflicts of Interests

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: T.K. Lam, N.E. Caporaso, E. Wang, M.T. Landi
Development of methodology: T.K. Lam, F.M. Marincola, N.E. Caporaso, E. Wang, M.T. Landi
Acquisition of data: T.K. Lam, F.M. Marincola, A.C. Pesatori, P.A. Bertazzi, N.E. Caporaso, E. Wang, M.T. Landi
Analysis and interpretation of data: T.K. Lam, S. Shao, Y. Zhao, N.E. Caporaso
Writing, review, and/or revision of the manuscript: T.K. Lam, S. Shao, A. C. Pesatori, P.A. Bertazzi, N.E. Caporaso, M.T. Landi
Administrative, technical, or material support: T.K. Lam, N.E. Caporaso, E. Wang
Study supervision: T.K. Lam, A.C. Pesatori, P.A. Bertazzi, N.E. Caporaso, M.T. Landi

Acknowledgments

The authors thank the participants of the Environment and Genetics in Lung Cancer Etiology study and the investigators who made the study possible (listed on the EAGLE website http://eagle.cancer.gov).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 18, 2012; revised September 7, 2012; accepted September 25, 2012; published OnlineFirst October 3, 2012.

Published OnlineFirst October 3, 2012; DOI: 10.1158/1055-9965.EPI-12-0745

References

1. Murakami A, Ashida H, Terao J. Multitargeted cancer prevention by quercetin. Cancer Lett 2008;269:315–25.
2. Lam TK, Rotunno M, Lubin JH, Wacholder S, Consonni D, Pesatori AC, et al. Dietary quercetin, quercetin-gene interaction, metabolic gene expression in lung tissue and lung cancer risk. Carcinogenesis 2010;31:634–42.
3. Cui Y, Morgenstern H, Greenland S, Tashkin DP, Mao JT, Cai L, et al. Dietary flavonoid intake and lung cancer-a population-based case-control study. Cancer 2008;112:2241–8.
4. Hirvonen T, Virtamo J, Korhonen P, Albanes D, Pietinen P. Flavonol and flavone intake and the risk of cancer in male smokers (Finland). Cancer Causes Control 2001;12:789–96.
5. Knekt P, Kumpulainen J, Jarvinen R, Rissanen H, Heliovaara M, Reunanen A, et al. Flavonoid intake and risk of chronic diseases. Am J Clin Nutr 2002;76:560–8.
6. Murakami A, Ashida H, Terao J. Multitargeted cancer prevention by quercetin. Cancer Lett 2008;269:315–25.
7. Lotito SB, Zhang WJ, Yang CS, Crozier A, Frei B. Metabolic conversion of dietary flavonoids alters their anti-inflammatory and antioxidant properties. Free Radic Biol Med 2011;51:454–63.
8. Yeh SL, Yeh CL, Chan ST, Chuang CH. Plasma rich in quercetin metabolites induces G2/M arrest by upregulating PPAR-γ expression in human A549 lung cancer cells. Planta Med 2011;77:992–8.
9. Khanduja KL, Gandhi RK, Pathania V, Syal N. Prevention of N-nitrosodiethylamine-induced lung tumorigenesis by ellagic acid and quercetin in mice. Food Chem Toxicol 1999;37:313–8.
10. Spencer JP. Beyond antioxidants: the cellular and molecular interactions of flavonoids and how these underpin their actions on the brain. Proc Nutr Soc 2010;69:244–60.
11. Parasramka MA, Ho E, Williams DE, Dashwood RH. MicroRNAs, diet, and cancer: new mechanistic insights on the epigenetic actions of phytochemicals. Mol Carcinog 2012;51:213–30.
12. Chen J, Xu X. Diet, epigenetic, and cancer prevention. Adv Genet 2010;71:237–55.
13. Milenkovic D, Deval C, Gouranton E, Landrier JF, Scalbert A, Morand C, et al. Modulation of miRNA expression by dietary polyphenols in apoE deficient mice: a new mechanism of the action of polyphenols. PLoS One 2012;7:e29837.
14. Lovat F, Valeri N, Croce CM. MicroRNAs in the pathogenesis of cancer. Semin Oncol 2011;38:724–33.
15. Calin GA, Sevignani C, Dumitru CD, Hyslop T, Noch E, Yendamuri S, et al. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. Proc Natl Acad Sci U S A 2004;101:2999–3004.
16. Kumar MS, Erkeland SJ, Pester RE, Chen CY, Ebert MS, Sharp PA, et al. Suppression of non-small cell lung tumor development by the let-7 microRNA family. Proc Natl Acad Sci U S A 2008;105:3903–8.
17. Landi MT, Zhao Y, Rotunno M, Koshiol J, Liu H, Bergen AW, et al. MicroRNA expression differentiates histology and predicts survival of lung cancer. Clin Cancer Res 2010;16:430–41.
18. Boesch-Saadatmandi C, Loboda A, Wagner AE, Stachurska A, Jozkowicz A, Dulak J, et al. Effect of quercetin and its metabolites isorhamnetin and quercetin-3-glucuronide on inflammatory gene expression: role of miR-155. J Nutr Biochem 2011;22:293–9.
19. Arola-Arnal A, Blade C. Proanthocyanidins modulate microRNA expression in human HepG2 cells. PLoS One 2011;6:e25982.
20. Li Y, VandenBoom TG II, Kong D, Wang Z, Ali S, Philip PA, et al. Up-regulation of miR-200 and let-7 by natural agents leads to the reversal of epithelial-to-mesenchymal transition in gemcitabine-resistant pancreatic cancer cells. Cancer Res 2009;69:6704–12.
21. Noratto GD, Kim Y, Talcott ST, Mertens-Talcott SU. Flavonol-rich fractions of yaupon holly leaves (Ilex vomitoria, Aquifoliaceae) induce microRNA-146a and have anti-inflammatory and chemopreventive effects in intestinal myofibroblast CCD-18Co cells. Fitoterapia 2011;82:557–69.
22. Wen XY, Wu SY, Li ZQ, Liu ZQ, Zhang JJ, Wang GF, et al. Ellagitannin (BJA3121), an anti-proliferative natural polyphenol compound, can regulate the expression of MiRNAs in HepG2 cancer cells. Phytother Res 2009;23:778–84.
23. Tsang WP, Kwok TT. Epigallocatechin gallate up-regulation of miR-16 and induction of apoptosis in human cancer cells. J Nutr Biochem 2010;21:140–6.
24. Ortholan C, Puissegur MP, Ilie M, Barbry P, Mari B, Hofman P. MicroRNAs and lung cancer: new oncogenes and tumor suppressors, new prognostic factors and potential therapeutic targets. Curr Med Chem 2009;16:1047–61.
25. Osada H, Takahashi T. let-7 and miR-17–92: small-sized major players in lung cancer development. Cancer Sci 2011;102:9–17.
26. Landi MT, Consonni D, Rotunno M, Bergen AW, Goldstein AM, Lubin JH, et al. Environment And Genetics in Lung cancer Etiology (EAGLE) study: an integrative population-based case-control study of lung cancer. BMC Public Health 2008;8:203.
27. Nutrient Data Laboratory. In: United States Department of Agriculture ARS, editor. Database for the flavonoid content of selected foods. Beltsville, MD: Beltsville Human Nutrition Research Center, Nutrient Data Laboratory. 2003.
28. Izzotti A, Calin GA, Arrigo P, Steele VE, Croce CM, De Flora S. Downregulation of microRNA expression in the lungs of rats exposed to cigarette smoke. FASEB J 2009;23:806–12.
29. Pavlidis P, Lewis DP, Noble WS. Exploring gene expression data with class scores. Pac Symp Biocomput 2002:474–85.
30. Roush S, Slack FJ. The let-7 family of microRNAs. Trends Cell Biol 2008;18:505–16.
31. Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, et al. RAS is regulated by the let-7 microRNA family. Cell 2005;120:635–47.
32. He XY, Chen JX, Zhang Z, Li CL, Peng QL, Peng HM. The let-7a microRNA protects from growth of lung carcinoma by suppression of k-Ras and c-Myc in nude mice. J Cancer Res Clin Oncol 2010;136:1023–8.
33. Mendell JT. miRiad roles for the miR-17-92 cluster in development and disease. Cell 2008;133:217–22.
34. Osada H, Takahashi T. let-7 and miR-17–92: small-sized major players in lung cancer development. Cancer Sci 2011;102:9–17.
35. Yu J, Ohuchida K, Mizumoto K, Fujita H, Nakata K, Tanaka M. MicroRNA miR-17–5p is overexpressed in pancreatic cancer, associated with a poor prognosis, and involved in cancer cell proliferation and invasion. Cancer Biol Ther 2010;10:748–57.
36. Ivanovska I, Ball AS, Diaz RL, Magnus JF, Kibukawa M, Schelter JM, et al. MicroRNAs in the miR-106b family regulate p21/CDKN1A and promote cell cycle progression. Mol Cell Biol 2008;28:2167–74.
37. Matsubara H, Takeuchi T, Nishikawa E, Yanagisawa K, Hayashita Y, Ebi H, et al. Apoptosis induction by antisense oligonucleotides against miR-17–5p and miR-20a in lung cancers overexpressing miR-17–92. Oncogene 2007;26:6099–105.
38. Perry MM, Moschos SA, Williams AE, Shepherd NJ, Larner-Svensson HM, Lindsay MA. Rapid changes in microRNA-146a expression negatively regulate the IL-1beta-induced inflammatory response in human lung alveolar epithelial cells. J Immunol 2008;180:5689–98.
39. Raponi M, Dossey L, Jatkoe T, Wu X, Chen G, Fan H, et al. MicroRNA classifiers for predicting prognosis of squamous cell lung cancer. Cancer Res 2009;69:5776–83.
40. Dorsett Y, McBride KM, Jankovic M, Gazumyan A, Thai TH, Robbiani DF, et al. MicroRNA-155 suppresses activation-induced cytidine deaminase-mediated Myc-Igh translocation. Immunity 2008;28:630–8.
41. Lu J, He ML, Wang L, Chen Y, Liu X, Dong Q, et al. MiR-26a inhibits cell growth and tumorigenesis of nasopharyngeal carcinoma through repression of EZH2. Cancer Res 2011;71:225–33.
42. Liu XX, Li XJ, Zhang B, Liang YJ, Zhou CX, Cao DX, et al. MicroRNA-26b is underexpressed in human breast cancer and induces cell apoptosis by targeting SLC7A11. FEBS Lett 2011;585:1363–7.
43. Herbst RS, Heymach JV, Lippman SM. Lung cancer. N Engl J Med 2008;359:1367–80.
44. Lam TK, Cross AJ, Consonni D, Randi G, Bagnardi V, Bertazzi PA, et al. Intakes of red meat, processed meat, and meat mutagens increase lung cancer risk. Cancer Res 2009;69:932–9.

Published OnlineFirst October 3, 2012; DOI: 10.1158/1055-9965.EPI-12-0745

Cancer Epidemiology, Biomarkers & Prevention

AACR American Association for Cancer Research

Influence of Quercetin-Rich Food Intake on microRNA Expression in Lung Cancer Tissues

Tram K. Lam, Stephanie Shao, Yingdong Zhao, et al.

Cancer Epidemiol Biomarkers Prev 2012;21:2176-2184. Published OnlineFirst October 3, 2012.