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Influence Of Quercetin-Rich Food Intake On Microrna Expression In Lung Cancer Tissues

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INFLUENCE OF QUERCETIN-RICH FOOD INTAKE ON MICRORNA EXPRESSION IN LUNG CANCER TISSUES

By

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Abstract

Background: Lung cancer is one of the most common cancers worldwide and has the highest cancer-related mortality rate. Epidemiologic studies, including the Environment and Genetics in Lung Cancer Etiology (EAGLE) study, have reported that frequent consumption of quercetin-rich foods is inversely associated with lung cancer incidence. Although experimental studies suggest that quercetin intake might modulate microRNA (miR) expression, this mechanism has not been fully examined. We investigated this hypothesis in lung carcinogenesis using EAGLE lung cancer tissues.

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. Individuals were categorized into sex-specific tertiles of quercetin-rich food intake. 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 grouped individual miRs into biologically functional families and conducted analyses using Functional Class Scoring (FCS). We accounted for multiple testing using 10,000 global permutations (significance at $p\text{-value}_{global} < 0.10$). All analyses were conducted separately by histology (adenocarcinoma and squamous cell carcinoma) and by smoking status (former and current smokers). **Results:** Family-based analyses showed that a quercetin-rich diet strongly differentiated miR expression profiles of the let-7 family among adenocarcinomas ($p\text{-value}_{FCS} < 0.001$). Other significantly differentiated miR families included miR-146, miR-26, and miR-17 ($p\text{-values}_{FCS} < 0.05$). In the 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 consumers (23 expected by chance; $p\text{-value}_{global} = 0.047$). Additionally, we identified 25 miRs to be differentially expressed among former smokers with adenocarcinoma (22 expected by chance; $p\text{-value}_{global}=0.076$). **Conclusions:** We observed differential expression of key biologically functional miRNAs between high-versus-low consumers of quercetin-rich foods in adenocarcinoma cases. Our findings, the first of this kind, warrant confirmation from larger studies.

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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. Quercetin and quercetin-rich foods may prevent carcinogenesis via several mechanisms [6], including free radical scavenging, pro-apoptotic and anti-proliferation pathway mediation, modification of anti-inflammatory responses, and activation of detoxifying Phase II enzymes [7-10].

New findings suggested that polyphenol compounds like quercetin are more likely to interact with cellular signaling cascades that regulate transcription factors.[11] More specifically, *in vitro*[12, 13] and *in vivo*[14] studies showed that they modulate a wide range of miR expressions and may consequently influence carcinogenesis. [13, 14] MiRs are short, non-coding, single-stranded RNAs involved in gene expression of multiple target mRNAs.[15] Misregulated miRs have been implicated in many cancers where they act to promote overexpression of oncogenes and underexpression of tumor suppressor genes.[16] For example, the let-7 class of miRs function as tumor suppressors by repressing cell proliferation and regulating both RAS and c-myc oncogenes.[17] In lung cancer, we previously showed that the let-7 family is differentially expressed by histology and is associated with survival in the Environment And Genetics in Lung Cancer Etiology (EAGLE) study.[15]

Polyphenolic compounds, including quercetin, have been shown 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 cancer cells.[23] Additionally, 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 biological 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,[24] we investigated the influence of quercetin-rich food consumption on miR expression signatures in lung tissues of lung cancer patients. Given the importance of let-7 in lung carcinogenesis and their association with polyphenols, 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.[25] Briefly, EAGLE is a population-based case-control study of lung cancer conducted in the Lombardy region of Italy between 2002 and 2005 (http://dceg.cancer.gov/eagle). Confirmed, incident, lung cancer cases (N=2100) were recruited from 13 hospitals that examined ~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.[15] Briefly, the miR expression data were derived from formalin-fixed paraffin-embedded (FFPE) tissue samples in 144 lung adenocarcinoma and 120 squamous cell carcinoma cases from EAGLE. The 264 individuals included in the current study were a subgroup with both dietary quercetin information and miR expression data**.** The miRs were analyzed using a custom-made, two-channel oligo-array that included a total of 713 human, mammalian, and viral mature antisense miRs standardized to one EBV cell line. Individual miRs with low overall signal intensity (<100) or low signal/noise ratio were excluded. Of the 440 human miRs, a total of 198 miRs were retained in the final analysis and are reported in Supplemental Table S1.

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.[25] 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, apricots, 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).[26] We created a summary measure of quercetin-rich foods by adding the frequency of intake reported over one year for the individual food item.

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[15] and miR expression was found to be associated with smoking status;[27] therefore, all our analyses were performed for smoking-specific (former and current smokers) and lung cancer subtype (adenocarcinoma and squamous cell carcinoma). Although we examined the influence of quercetin-rich food intake on miR expression in never smokers, the results were unstable and not reported due to too few individuals (n=29).

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), smoking intensity (pack-years, continuous), consumption of non-quercetin-rich fruits and vegetables (continuous), red/processed meats (continuous), and lifetime alcohol consumption (continuous). Selection of 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 Supplemental Table S2.

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 10,000X where the number of significant miRs from ANOVA testing was recorded (n^P). The global p-value is then defined as one plus the number of times in which n^p was at least as large as the number of original significant miRs divided by10,000.

Family-miR-based analyses: We grouped each miR into known biological functional families using MiRBase release 17.0 (http://microrna.sanger.ac.uk/). We first assembled miRs into families based on unique 'seed sequence' (nucleotides 2-7 at the 5'end) and identified 21 miR families (Supplemental Table S3). We then restricted our analyses to 9 miR families with at least one miR identified at a *p*-value < 0.05 in the individual-miR-based analyses (Table 3). 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).[28] This raw score was then compared to 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 $p\text{-value}_{FCS} < 0.05$. We further evaluated the statistical significance using the more conservative Bonferroni *p*-value (0.05/9= 0.006).

Permutations were performed in the statistical package R and we considered a permuted $p_{global} < 0.10$ to be statistically significant. All other analyses were performed 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 adenocarcinoma and squamous cell carcinoma. 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*-values < 0.05) between high-versus-low consumers of quercetin-rich foods (Table 2). Likewise, 19 miRs were differentially expressed for former (12 miRs) and current (7 miRs) smokers (see Supplemental Table S4).

Table 3 presents analyses examining the association of quercetin-rich diet with miR expression within histologic subtypes for former and current smokers separately. We identified 48 unique miRs that were differentially expressed between highest-vs-lowest quercetin-rich consumers for all groups (*p*-value < 0.05, Table 3). 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).

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; $p\text{-value}_{global} = 0.047$, Table 3). For SQ cases, we identified 15 miRs ($p\text{-value}_{global} > 0.10$).

Quercetin-associated miR expression profiles appeared to be more prevalent among former smokers with AD. In this group, we identified 25 miRs with a p-value < 0.05 (22 expected by chance; $p\text{-value}_{global} = 0.076$). The largest fold-change was observed for miR-26b, a proapoptotic miR (fold-change = 2.00; p-value = 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 at a p-value ≤ 0.05. Moreover, the majority of let-7 family members were up-regulated with increasing frequency of quercetin intake (Figure 1).

In comparison to AD cases, far fewer miRs were identified at a p-value < 0.05 among SQ cases. None of these groups were statistically significant when we calculated the global p-value ($\text{p-values}_{global} > 0.10$).

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-value$_{FCS}$ < 0.001, Table 4) followed by miR-146 (p-value$_{FCS}$ = 0.002), miR-26 (p-value$_{FCS}$ = 0.010), and miR-17 (p-value$_{FCS}$ = 0.031). Both the let 7 and the miR-146 families remained significant after Bonferroni correction for multiple comparisons (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 miR17 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 up-regulated. In contrast, the expression of miR-146a was downregulated.

Discussion

We previously observed higher consumption of quercetin-rich foods to be associated with lower risk of lung cancer in a large population-based case-control study in Italy. 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-associated miR expression signatures for 48 unique miRs among AD and SQ cases who were former or current smokers. The identified miRs have been shown to decrease cellular proliferation, reduce tumor metastasis, and increase apoptosis in smoking-specific analyses (see Supplemental, Table S5 for specific miRs). In family-miR-based analyses, we found that the large majority of members of the let 7 family were upregulated among former smokers with AD who consumed a higher intake of quercetin compared with low consumers (p-value$_{FCS}$ < 0.001). We also

observed similar family-based results for miR-146, miR-26, and miR-17 families (p-value$_{FCS}$ < 0.05) in this group.

Due to its association with lung cancer, 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-value < 0.006. Members of the let-7 family are known to function as tumor suppressors in lung carcinoma by repressing NSCLC cell proliferation[17, 29] and by negatively regulating the RAS oncogene.[30] Among the let-7 miRs in the present study, let-7a, a known suppressor of k-RAS and c-Myc oncogenes,[31] 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-associated 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.[32] Investigators have shown that the miR-17-92 cluster is frequently overexpressed in lung cancer.[33] Expression of miR-17 is associated with poorer prognosis and cellular proliferation [34] while miR-106b targets p21 and subsequently promotes cell cycle progression.[35] Additionally, suppression of miR-20a induces apoptosis in lung cancer.[36] 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 was observed to negatively regulate pro-inflammatory

chemokines.[37] Additionally, miR-146a is one of two 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, a pro-inflammatory miR, 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.[40] However, it has also been suggested to function as a tumor suppressor by repressing cell proliferation.[41] In the present study, 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 G1 phase arrest and repression of c-Myc [38] as well as induce apoptosis in breast cancer cells.[39] 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 adenocarcinoma from squamous cell carcinoma with prognostic implications in EAGLE.[15] 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 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 which is less associated with smoking than SQ.[42] Furthermore, AD cases included in the present study on

average smoked less intensely than SQ cases. The anti-carcinogenic 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 of the association of dietary quercetin with 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.

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 prior to the study, we cannot exclude the possibility that lung cancer diagnosis had influenced the patients' responses. However, this potential recall bias should not have differentially affected the adenocarcinoma or squamous cell carcinoma cases and could not explain the different association with quercetin by histologic type. Moreover, we derived a quercetin-rich diet from food items available in the EAGLE's FFQ and not measured quercetin content directly; thus, we cannot rule out the contribution of other flavonoids or nutrients that are found in those foods. Confirmation in a larger prospective study with both dietary quercetin information and miR expression data is warranted.

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 potential insights

into the observed inverse association between quercetin-rich food consumption and lung cancer risk.

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

Subject characteristics	Adenocarcinoma				Squamous Cell Carcinoma			
	T1 (n=57)	T2 (n=47)	T3 (n=40)	*p* -value	T1 (n=48)	T2 (n=42)	T3 (n=30)	*p* -value
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)	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]
Intake								
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% due to rounding; **Bolded p-values** indicated statistical significance; T1-T3 = 1st tertile through 3rd 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, servings/day; alcohol, grams/day)

[c] ANOVA test.

[d] Chi-square test.

[e] Non-parametric 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.

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	T3 mean	Fold Change	p-value
Adenocarcinoma (n=97)				
hsa-miR-502	1.089	1.224	1.124	0.0169
hsa-mir-564	1.76	1.566	0.89	0.0297
hsa-miR-124a	1.261	1.074	0.852	0.0440
hsa-miR-125a	1.869	2.813	1.505	0.0450
Squamous Cell Carcinoma (n=78)				
hsa-miR-510	1.327	1.158	0.872	0.0026
hsa-mir-605	8.312	3.591	0.432	0.0040
hsa-miR-155	0.006	0.008	1.399	0.0122
hsa-miR-373	0.995	0.913	0.917	0.0142
hsa-miR-453	1.817	1.635	0.899	0.0166
hsa-miR-502	1.374	1.1	0.801	0.0172
hsa-miR-18b	0.073	0.108	1.483	0.0197
hsa-miR-183	3.189	2.179	0.683	0.0215
hsa-mir-573	1.306	1.134	0.869	0.0243
hsa-miR-524	1.077	0.921	0.855	0.0358
hsa-mir-612	0.843	0.901	1.069	0.0420
hsa-miR-363	0.927	1.132	1.222	0.0464

Adjusted for age, sex, BMI, smoking status, non-quercetin-rich fruits and vegetables, red/processed meat, alcohol, and cigarette packyears

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

Adenocarcinoma

Former smokers (n=44)			Current smokers (n=42)		
miR name	Fold Change	*P*-value*	miR name	Fold Change	*P*-value*
hsa-mir-641	0.8280	0.0029	hsa-mir-580	0.7670	0.0028
hsa-miR-29b	1.0920	0.0033	hsa-miR-215	0.7660	0.0039
hsa-miR-146a	0.8900	0.0058	hsa-miR-194	0.6480	0.0107
hsa-miR-500	0.8390	0.0077	hsa-mir-598	0.5740	0.0157
hsa-let-7e	1.2700	0.0175	hsa-miR-518a-2	0.9290	0.0197
hsa-miR-134	0.9520	0.0196	hsa-miR-503	0.6770	0.0369
hsa-miR-26b	2.0030	0.0214	hsa-miR-146b	1.4970	0.0430
hsa-miR-302c	1.1470	0.0229	hsa-miR-381	0.8430	0.0471
hsa-miR-98	1.0060	0.0238			
hsa-let-7c	1.0920	0.0241			
hsa-miR-27a	1.2900	0.0254			
hsa-let-7a	1.4640	0.0259			
hsa-let-7g	0.8930	0.0262			
hsa-let-7i	1.4090	0.0279			
hsa-let-7f	1.3770	0.0303			
hsa-miR-195	0.9660	0.0311			
hsa-miR-16	1.4290	0.0316			
hsa-miR-146b	1.0390	0.0337			
hsa-miR-26a	1.7830	0.0343			
has-miR-19b	0.7640	0.0361			
hsa-mir-564	0.9410	0.0366			
hsa-miR-20a	1.0610	0.0411			
hsa-miR-106a	1.0470	0.0439			
hsa-miR-34a	1.1240	0.0455			
hsa-miR-92	1.1210	0.0481			

Squamous cell carcinoma

Former smokers (n=38)			Current smokers (n=40)		
miR name	Fold Change	*P*-value*	miR name	Fold Change	*P*-value*
hsa-miR-492	1.129	0.0115	hsa-miR-502	0.782	0.0103
hsa-miR-510	0.785	0.0212	hsa-mir-605	0.335	0.0127
hsa-miR-491	1.165	0.0227	hsa-miR-506	0.644	0.0166
hsa-mir-612	1.168	0.0252	hsa-miR-183	0.397	0.0282
hsa-miR-500	0.908	0.0284	hsa-miR-524	0.768	0.0294
hsa-mir-663	1.054	0.0341			
hsa-miR-503	0.923	0.0344			
hsa-miR-453	0.903	0.0348			
hsa-mir-654	1.137	0.0411			
hsa-mir-658	0.916	0.0467			

Note: miRs are ordered by P-value within strata

*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 packyears.

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

Family *Function*	**miRNA members**	**Adenocarcinoma**		**Squamous Cell Carcinomas**	
		Former	Current	Former	Current
		P-value*	*P*-value*	*P*-value*	*P*-value*
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 one miR that were significant at p-value <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, smoking status, non-quercetin-rich fruits and vegetables, red/processed meat, alcohol, and cigarette packyears.

[a]Refer to Supplemental Table S5 for more detailed functions

*p-value based on Functional Class Score as described in the Methods section

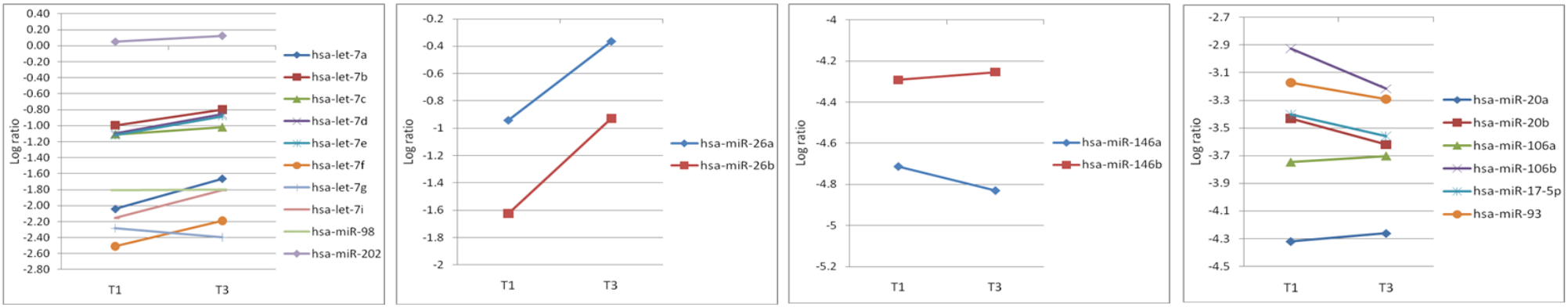

Figure 1. Mean expression levels for significant miR groups comparing the highest versus lowest tertile of quercetin-rich food intake in former smokers with adenocarcinoma

References

1. Murakami A, Ashida H, Terao J. Multitargeted cancer prevention by quercetin. Cancer Lett. 2008; **269**(2): 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**(4): 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**(10): 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**(9): 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**(3): 560-8.

6. Kandaswami C, Lee LT, Lee PP, Hwang JJ, Ke FC, Huang YT, et al. The antitumor activities of flavonoids. In Vivo. 2005; **19**(5): 895-909.

7. Murakami A, Ashida H, Terao J. Multitargeted cancer prevention by quercetin. Cancer Lett. 2008; **269**(2): 315-25.

8. 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**(2): 454-63.

9. 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**(10): 992-8.

10. 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**(4): 313-8.

11. 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**(2): 244-60.

12. 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**(3): 213-30.

13. Chen J, Xu X. Diet, epigenetic, and cancer prevention. Adv Genet. 2012; **71**: 237-55.

14. 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**(1): e29837.

15. 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**(2): 430-41.

16. 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**(9): 2999-3004.

17. 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**(10): 3903-8.

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**(3): 293-9.

19. Arola-Arnal A, Blade C. Proanthocyanidins modulate microRNA expression in human HepG2 cells. PLoS ONE. 2011; **6**(10): e25982.

20. Li Y, VandenBoom TG, 2nd, 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**(16): 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**(4): 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**(6): 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. 2012; **21**(2): 140-6.

24. 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**(4): 634-42.

25. 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.

26. Laboratory ND. Database for the Flavonoid Content of Selected Foods. In: United States Department of Agriculture ARS, editor. Beltsville Human Nutrition Research Center, Nutrient Data Laboratory, Beltsville, MD.; 2003.

27. 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**(3): 806-12.

28. Pavlidis P, Lewis DP, Noble WS. Exploring gene expression data with class scores. Pac Symp Biocomput. 2002: 474-85.

29. Roush S, Slack FJ. The let-7 family of microRNAs. Trends Cell Biol. 2008; **18**(10): 505-16.

30. 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**(5): 635-47.

31. 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**(7): 1023-8.

32. Mendell JT. miRiad roles for the miR-17-92 cluster in development and disease. Cell. 2008; **133**(2): 217-22.

33. Osada H, Takahashi T. let-7 and miR-17-92: small-sized major players in lung cancer development. Cancer Sci. 2011; **102**(1): 9-17.

34. 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**(8): 748-57.

35. 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**(7): 2167-74.

36. 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**(41): 6099-105.

37. 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**(8): 5689-98.

38. 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**(1): 225-33.

39. 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**(9): 1363-7.

40. 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**(14): 5776-83.

41. 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**(5): 630-8.

42. Herbst RS, Heymach JV, Lippman SM. Lung cancer. N Engl J Med. 2008; **359**(13): 1367-80.