Long-term Soy Consumption and Tumor Tissue microRNA and Gene Expression in Triple Negative Breast Cancer

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Abstract

Background—Soy food intake may have protective effects against breast cancer risk, including estrogen receptor negative breast cancer. However, the underlying molecular mechanisms remain unclear.

Methods—To evaluate the association of soy intake with the expression of microRNAs and genes in the tumor tissue of triple-negative (lacking expression of estrogen receptor, progesterone receptor and HER2) breast cancer (TNBC) patients, a total of 800 microRNAs (miRNAs) and 302 gene expressions were measured by the NanoString nCounter Assays in formalin-fixed paraffin-embedded (FFPE) tumor tissues from 272 TNBC patients. Soy intake during the one-year period prior to cancer diagnosis was assessed by a validated food-frequency questionnaire. The association of soy intake with the expression of miRNAs and genes was evaluated by performed linear regression analysis with adjustments for patient age and TNM.

Results—A total of 14 miRNAs and 24 genes were significantly associated with soy food intake (p<0.05): 13 of the 14 miRNAs (92.9%) and 9 of 24 genes (37.5%), including tumor suppressors miR-29a-3p and *IGF1R,* showed over-expression for those women with a high soy intake, while the remaining miRNAs and genes, including oncogenes *KRAS* and *FGFR4,* showed under-expression. Furthermore, cell growth-related genes showed a predominant under-expression

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pattern when comparing tumor samples from women with a high soy food intake to samples from those who had a lower soy food intake.

Conclusions—Our study suggests that long-term pre-diagnosis soy intake may lead to increased expression of tumor suppressors and decreased expression of oncogenes, especially cell growth-related genes, in breast tumor tissues.

Keywords

triple-negative breast cancer; soy consumption; miRNA expression; gene expression

Introduction

Soy foods contain abundant amounts of isoflavones, which have estrogen-like structures and may act as natural estrogen receptor (ER) modulators that interact with ERs (1, 2). Soy foods have also been shown to have many non-estrogen related anti-cancer properties, such as inhibiting cell proliferation and inducing apoptosis (3). Previous epidemiological studies, including our own work, have found that soy intake was inversely associated with breast cancer risk (4-6), and post-diagnosis soy consumption was associated with a reduced risk of breast cancer recurrence and mortality (7, 8), including that of ER-negative breast cancer. These observations suggest that soy food may be a potential natural and alternative approach to the chemoprevention of breast cancer (8, 9). However, evidence of the potential anti-cancer benefit of soy food consumption is not entirely consistent (9, 10). Furthermore, the underlying molecular mechanisms of soy food intake on breast cancer risk and prognosis are still not fully understood.

Previous *in vitro* studies have shown that soy isoflavones may exhibit an anti-cancer effect by acting on specific miRNAs (11, 12). However, the effects of long-term soy food intake on miRNA expression in the tumor tissues of humans are not well understood. In addition, several experimental studies have suggested that soy isoflavones may also regulate genes that control cell cycle, apoptosis, and survival (13-15). Recently, Shike and colleagues reported that short-term (7-30 days) soy supplementation immediately prior to cancer surgery was associated with increased expression of cell-cycle genes in breast cancer tissue, provoking concerns of a potential cancer-promoting effect (16). However, the study was limited by its abbreviated window of exposure and sudden high dose of exposure (17). In the present study, we investigated the effects of long-term pre-diagnosis soy food intake on the expression of 800 miRNAs and 302 pre-selected genes in tumor tissues from 272 TNBC cases in a population-based cohort study of breast cancer patients.

Materials and methods

Study population

This study utilized the resources generated from the Shanghai Breast Cancer Survival Study (SBCSS), which is a longitudinal, population-based cohort study of breast cancer survival in Shanghai, China (details described elsewhere (8)). Briefly, the SBCSS included a total of 5,042 women with incident breast cancer, aged 20 to 75 years, who were recruited to the study between March 2002 and April 2006, approximately 6 months after diagnosis

(response rate: 80%). Participants were followed up by in-person surveys at 18, 36, 60 and 120 months after cancer diagnosis, in combination with periodic record linkage with the Shanghai Vital Statistics Registry. Included in the current study are 272 TNBC cases who had both pre-diagnosis soy food intake information and sufficient tumor tissue RNA collected prior to any cancer therapy. Sample selection and preparation have been previously published (18) and a study flow chart can be found in the online supplementary materials. The study protocol was approved by the institutional review boards of Vanderbilt University and the Shanghai Municipal Center for Disease Control and Prevention and all participants provided written informed consent.

The measurement of Soy food intake

A validated food-frequency questionnaire (19) was used to assess usual soy and other dietary intake. Questions related to pre-diagnosis soy food intake were only administered to a sub-cohort of study participants, including 321 TNBC cases, per study design. Soy food intake during the year before cancer diagnosis was measured by soy protein intake, which was estimated by summing the product of individual soy food intake amount and its protein content based on the Chinese Food Composition Tables 2002 (8). Clinical information and tumor characteristics including patient age, TNM stage, tumor grade, chemotherapy usage, and ER/PR hormone receptor status were extracted from medical charts. HER2 status was measured in the Vanderbilt Molecular Epidemiology Core Laboratory (20).

miRNA and Gene Expression Analysis

Expression profiles of the 800 miRNAs and 302 pre-selected genes were measured by the NanoString nCounter Assays using total RNA isolated from formalin-fixed paraffin-embedded (FFPE) breast tumor tissues (details described elsewhere (18)). The Human v2 miRNA assay and custom-designed gene Expression Assay (NanoString) were used to measure the tumor tissue level of miRNA and gene expression following the NanoString standard protocol. The detailed normalization process in miRNA and gene expression and downstream statistical analysis are described in the Supplementary materials.

Results

Among the study participants, 272 had gene expression data, and 244 cases had miRNA expression data. The age at diagnosis for cases ranged from 26-75 years. The median soy protein intake assessed during one year period prior to diagnosis was 10.8 grams/day. We did not observe significant associations between soy food intake and demographics or clinicopathological characteristics, including patient age, tumor grade and TNM stage (Table 1).

miRNAs significantly associated with soy food intake

A total of 14 miRNAs showed a significant association pattern with soy food intake (p < 0.05; Figure 1A and Table 2). Of them, 13 miRNAs, (miR-223-3p, miR-219-5p, miR-3690, miR-4421, miR-188-3p, miR-3168, miR-29a-3p, miR-2110, miR-759, miR-590-3p, miR-891b, miR-142-3p and miR-150-5p) were positively correlated with soy intake; only one miRNA (miR-1253) showed an inverse association (Figure 1A, Table 2). Functional

analysis using IPA revealed that seven miRNAs, including the four well-known cancer-related miRNAs described above, and another three miRNAs (miR188-3p, miR-219-5p and miR-5903p), are involved in the regulation of a *TP53* cancer-related network (Figure 1B). This network includes several cancer-related genes, including *TP53*, *AGO2* and *DDX20*. In addition, miR-150-5p and miR-142-3p were found to target downstream gene AGO2 (Figure 1B), which had been reported to lead to decreased tumor suppressor *PTEN* in hepatocellular carcinoma (47).

Genes significantly associated with soy food intake

Correlation analysis shows the expressions of 24 genes to be significantly associated with soy food intake (p < 0.05; Figure 1C and Table 3). Of these, 15 genes exhibited under-expression for women with a high soy intake, while 9 genes showed over-expression (Table 3). Of these identified genes, soy food intake was associated with decreasing expression of oncogenes *KRAS* and *FGFR4* but with increasing expression of *IGF1R*, consistent with findings from previous *in vitro* studies (21-24). Functional enrichment analysis using IPA for the down-regulated genes revealed that the two most significantly-enriched pathways are hereditary breast cancer signaling ($p < 1.08 \times 10^{-4}$) and IL-2 signaling ($p < 6.84 \times 10^{-4}$), both of which were driven by oncogenes *KRAS* (25), *IL2RB* (26) and *GADD45B* (27) (Figure 1D). For up-regulated genes, the most significantly-enriched pathway was nucleotide excision repair ($p < 1.54 \times 10^{-2}$), which was driven by the tumor suppressor gene *ERCC1* (28) (Figure 1D). It should be noted that we only included selected genes in the study which may have resulted in missing some significant soy-food intake associated genes and thus a reduced statistical power for the IPA enrichment analysis.

Comparing differentially-expressed genes reported in the Shike *et al* study with our study

To further examine whether soy intake leads to over-expression of cell-cycle genes as recently reported by Shike *et al* (16), we carried out further analyses focusing on comparisons of the genes that differed significantly between soy supplement and control groups in their study. Notably, the medians of the lowest and highest quartiles of protein intake in our study were 4.9 and 21.2 (grams/day), respectively. The median protein intake in the soy protein supplement used in the Shike *et al* study was 25.8 grams/day. Thus, we applied the comparisons of the highest versus lowest quartile groups because intake level of the former is more comparable to the amount of soy supplement administered in their study. Of the 99 genes that differed significantly between soy supplement and control groups in the Shike *et al* study, 10 with > 2 fold change (FC), including one with under-expression and nine with over-expression, were included in our study (Figure 2A). We found that the gene *MAPT,* which was under-expressed in the Shike *et al* study, also had a lower expression in the tumor tissue of women in the highest soy protein intake group in our study, although the difference was not significant (p = 0.8, FC = 1.12, Figure 2A). Of the remaining nine over-expression genes, an opposite direction-of-expression difference pattern, i.e., lower gene expression in the higher soy protein intake group, was observed for all except one gene (*TOP2A*) in our study samples (Binomial test, p = 0.04), although none of the individual gene expression differences reached the significance threshold (Figure 2A). For example, cell-cycle genes *MYBL2* and *CDC20* exhibited under-expression with FCs of 1.33 and 1.27

respectively in our study, but showed over-expression with 2 and 3.07 FC in the soy supplementation group relative to the placebo group in Shike *et al*.

Under-expression of cell growth-related genes associated with soy food consumption

We also found that eight cell growth-related genes were significantly and differentially expressed in the tumor tissue (Wilcoxon rank-sum test, $p < 0.05$; Table 4) in the group comparisons. Among them, growth factor *TYMP* and five cell growth-related genes, including *KLF6*, *IL2RB*, *NR1H3*, *ABCB1* and *EAF2,* had a lower expression in tumor tissue from women with high soy food intake, whereas transporter *SNX13* and cell-growth gene *DDAH1* had a higher expression in the high soy food intake group (Figure 2B, Table IV). Most genes that were significant in the group differential analyses were also significantly correlated with soy intake in the regression analysis (Table 4). In addition, breast cancer tissue from women in the highest quartile of soy protein intake had an overall slightly higher proportion of cell growth-related genes that were under-expressed (57.8 % of total 166 cell-growth genes, Figure 2C) as compared to women in the lowest quartile of intake (Fold change - FC $4^{th}/1^{st} > 1$). The proportion of under-expressed genes in the high soy protein intake group increased when a higher FC cutoff was applied (e.g., 60.6% or 78.9% cell growth-related genes were under-expressed when the FC cutoff was 1.2 or 1.5, respectively). We further performed differential gene expression analyses for the second and third quartiles versus the lowest quartile of protein intake for cell-growth genes. A similarly predominant down-regulation pattern for these genes was observed based on a hierarchical cluster analysis (Figure 2C). In general, the cell growth-related genes are necessary in the control of cell growth and division, while down-regulation of those genes can lead to reduced cancer cell proliferation.

Discussion

In this cohort of 272 TNBC patients, we found that soy food intake during the one-year period prior to cancer diagnosis was associated with increasing expression of tumor-suppressor miRNAs and genes, but with decreasing expression of oncogenes, especially cell growth-related genes, in TNBC tumor tissue. These results reveal possible molecular mechanisms by which soy food intake influences the risk and prognosis of breast cancer.

Although soy isoflavones are known as estrogen receptor modulators, there are also other non-estrogen mediated anti-cancer mechanisms which may explain the associations between soy food intake and breast cancer risk and prognosis. Our findings are consistent with previous studies suggesting that soy consumption may have anti-cancer effects beyond its estrogen-related effect (29). One of the potential mechanisms is via epigenetic modification (32, 33). Previous studies have shown that isoflavones are capable of demethylating CpG sites in the promoter region of tumor-suppressor miRNAs and genes (14, 34). Altered miRNA expression may in turn influence their downstream-regulated genes and affect cancer risk and prognosis. Genisteins, major isoflavones in soy food, have been found to increase tumor-suppressors miR-34a and miR-574-3p in pancreatic cancer cells, resulting in inhibition of cell growth and induction of apoptosis (11, 13). In the prostate cancer cell line, isoflavones were shown to increase miR-29a and miR-1256, leading to the inhibition of cell

growth and invasion (30). Genisteins were shown to inhibit the oncogenes miR-1260b and miR-151, leading to the suppression of cancer cell growth (15, 31). In addition, soy foods are rich in folate, anti-oxidants and other phytochemicals that have known anti-cancer properties (35). The associations observed in our study could also be attributed to these soy constituents instead of soy isoflavones.

The majority of our identified miRNAs associated with soy intake may play regulatory roles in the inhibition of carcinogenesis. For example, miR-29a-3p has been previously shown in an *in vitro* study to inhibit prostate cancer cell growth and invasiveness (14). No *in vitro* study has been conducted for evaluation of the association between any other miRNA and soy intake. MiR-29a-3p, miR-223-3p, miR-142-3p and miR-150-5p are well-known for their involvement in the process of cancerogenesis. Previous studies have shown that miR-223-3p may inhibit cell proliferation by targeting *IGF-1R* in endometrial carcinoma (36), and is possibly associated with both recurrence and survival in TNBC (37). Both miR-142-3p and miR-150-5p have been suggested to play a dual role in the promotion and inhibition of carcinogenesis (38-41). As soy intake was associated with over-expression of specific tumor-suppressor miRNAs, one might expect soy food consumption to be related to their downstream target genes. In fact, we did find in our study that soy food intake was also associated with a decreasing expression of these miRNA targets, including cell-growth genes *CYLD* (miR-590-3p), *KLF6* (miR-590-3p), *GADD45B* (miR-219-5p), and *IL2RB* (miR-150-5p). These results lend strong support to the hypothesis that down-regulations of cell growth-related genes mediated by their upstream miRNAs may be one of the mechanisms by which soy food influences breast cancer etiology and prognosis.

Of the total of 24 identified genes significantly associated with soy food intake, we examined whether those genes function as either tumor suppressor or oncogenes based on a literature review and the Cancer Gene Census catalogue (http://cancer.sanger.ac.uk/census/). Several genes, including *KRAS, FGFR4, IL2RB* and *GADD45B*, function as oncogenes, while ERCC1 is reported to function as a tumor suppressor. Correlations for those genes observed in our study are in the expected direction of the hypothesized mechanisms. The functional roles for the remaining genes are either not well known or bidirectional in carcinogenesis. For example, *KLF6* plays an important role in the regulation of cell proliferation and apoptosis, and as such has been considered a tumor suppressor. However, a recent report suggests that an oncogenic splice variant (KLF6-SV1) of *KLF6* could lead to increased progression and metastasis of breast cancer (42). In our study, soy protein intake was inversely associated with the expression of *KLF6*.

Our study does not support the finding of Shike *et al* that soy food intake is associated with over-expression of cell growth-related genes in tumor tissue. However, it should be noted that our study differs from Shike *et al* in several aspects. First, our study population is Chinese women, almost all of whom have had some soy food exposure during their lifetime, while women in the Shike *et al* study had only consumed soy for one to four weeks immediately prior to surgery. Sudden exposure to a high dose of soy at a vulnerable window may lead to a quite different consequence than that produced from long-term exposure (17). Second, because almost none of the women in our study were non-soy food eaters, we had to rely on comparisons between highest and lowest quartile. Thus, a stronger association would

be expected had the comparisons been made between high soy food consumers and non-consumers. Third, our study included only TNBC cases, while most women in the Shike *et al* study were diagnosed with ER-positive breast cancer. Thus, our findings may not directly apply to ER or PR positive breast cancers. However, previous studies have suggested that soy intake is associated with breast cancer risk and prognosis irrespective of hormone receptor status (43-45).

It should also be noted that our findings are based on an observational study. Soy food consumption was not randomly assigned. Thus, potential confounding from factors related to self-selection of high/low soy food consumption cannot be ruled out. In addition, the sample size of our study is relatively small, and assessment of dietary intake is inherently prone to misclassification–both of these factors can compromise statistical power. Furthermore, because a large number of genes and miRNAs were evaluated in the study, some of the findings could be due to chance. Only the gene *E2F2* reaches the threshold of significance if a false discovery rate $P < 0.05$ is applied. Another limitation of our study is that we included only 302 genes, selected either to define the intrinsic subtype of breast cancer or for their potential to predict TNBC outcomes. Although a large proportion of these genes are related to cell-cycle regulation, they represent only a small proportion of genes in the relevant pathways. Thus, more studies with increased sample sizes and comprehensive evaluations of gene expression are warranted.

Conclusions

We found that high pre-diagnosis soy food consumption may prevent breast carcinogenesis through the increased expression of tumor-suppressor miRNAs and genes, and decreased expression of oncogenes, especially cell-growth genes, in TNBC tumor tissue.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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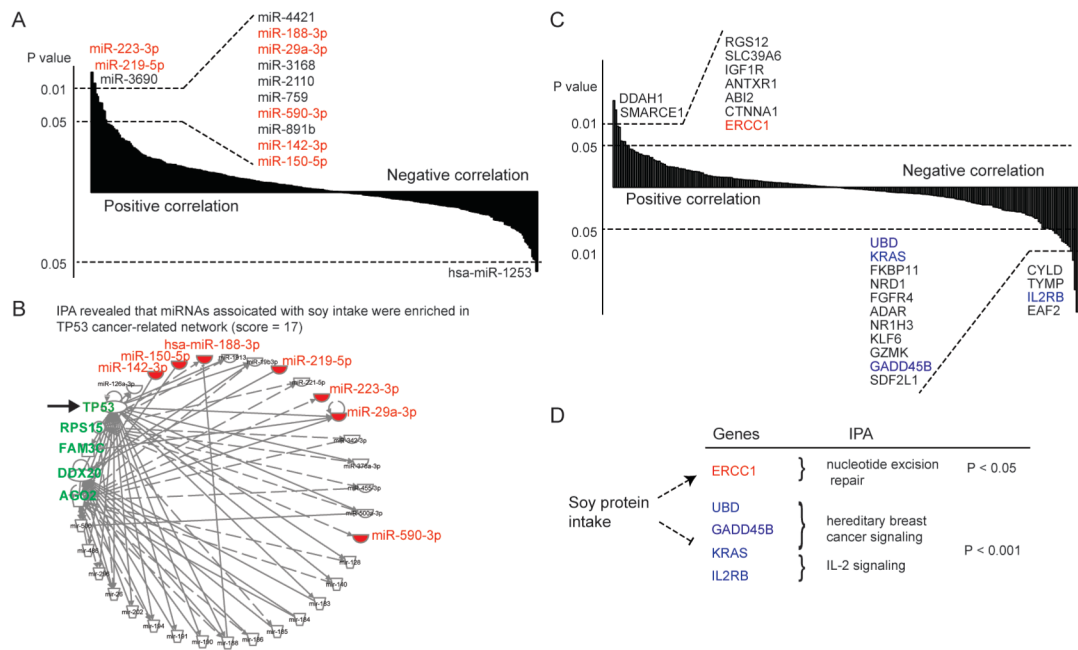
Figure 1.
miRNAs and genes significantly associated with soy food intake. (A) A total of 14 miRNAs showed significant association with soy food intake. Of these, 13 miRNAs showed a significantly positive correlation pattern between their expression levels and soy intake, while one showed an inverse association. B) *TP53* cancer-related network was shown for those significant miRNAs, including miR-233-3p, miR-219-5p, miR188-3p, miR-29a-3p, miR-5903p, miR-142-3p and miR-150-5p, which are highlighted in red. C) A total of 24 genes showed a significant association pattern with soy food intake. Of these, 15 genes showed an inverse correlation pattern between their expression levels and soy food intake, while 9 genes showed positive associations. D) Soy food intake and its association with the regulation of genes and pathways. The up-regulations and down-regulations of genes related to these pathways are highlighted in red and blue, respectively.
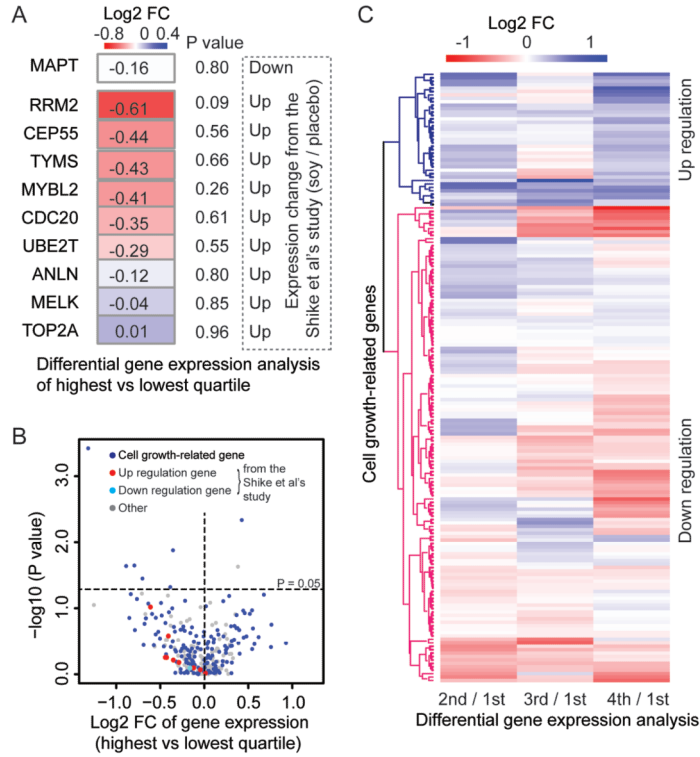
Figure 2.
Differentially-expressed genes in tumor tissue from women with different levels (quartiles) of soy food consumption. (A) Differential gene expression analysis comparing the highest and lowest quartiles of soy intake for 10 significant genes reported in the Shike *et al* study. P value denotes the significance of gene exposure differences across soy food intake. The dashed box lists the direction of gene expression change between soy protein intake and placebo reported in the Shike *et al* study. B) Heatmap of log2 FC of gene expression based on comparisons of the 4th, 3rd and 2nd vs the 1st (lowest) quartile of soy intake for cell growth genes. C) Distribution of log2 FC and gene expression significance, comparing the highest vs lowest quartiles of soy food intake.

Table 1

Soy Food Consumption by Demographic and Clinical Characteristics

Characteristic	N	soy food consumption mean (sd)	p value[*]
Age at diagnosis, yrs			
< 46	68	12.12 (8.10)	
46-51	68	12.59 (7.25)	
52-60	68	12.57(7.30)	0.33
>60	68	12.12(6.80)	
TNM stage			
0	6	14.91(10.56)	
I	88	13.74(7.35)	
IIA	92	11.11(6.9)	
IIB	52	12.80(7.57)	0.09
III, IV	25	10.81(7.02)	
Unknown	9	11.41(7.73)	
Grade			
I	34	13.98(9.24)	
II	86	12.11(7.08)	0.36
III	152	12.13(7.01)	

[*] Spearman's rank correlation test was used for characteristic variables with soy food intake.

Table 2
miRNAs significantly associated with soy food intake

miRNA	Regression analysis[*]		Correlation analysis[#]	
	Beta	p value	ρ	p value
miR-223-3p	0.025	0.005	0.172	0.007
miR-219-5p	0.023	0.008	0.142	0.027
miR-3690	0.023	0.008	0.163	0.011
miR-4421	0.022	0.013	0.158	0.013
miR-188-3p	0.021	0.015	0.149	0.020
miR-3168	0.021	0.019	0.124	0.053
miR-29a-3p	0.021	0.019	0.154	0.016
miR-2110	0.021	0.020	0.124	0.053
miR-759	0.020	0.027	0.118	0.066
miR-590-3p	0.019	0.028	0.121	0.060
miR-1253	-0.019	0.030	-0.108	0.092
miR-891b	0.018	0.042	0.116	0.072
miR-142-3p	0.017	0.049	0.087	0.177
miR-150-5p	0.017	0.050	0.107	0.096

[*]
Adjusted for patient age and TNM stage

[#]
Spearman's rank correlation analysis was performed between miRNA expression and soy food intake.

Table 3

Genes significantly associated with soy food intake

Genes	Regression analysis[*]	
	Beta	p value
DDAH1	0.025	0.002
SMARCE1	0.024	0.004
RGS12	0.021	0.012
SLC39A6	0.02	0.013
IGF1R	0.017	0.035
ANTXR1	0.017	0.036
ABI2	0.017	0.037
ERCC1	0.016	0.048
CTNNA1	0.016	0.048
EAF2	-0.031	1.1×10^{-4}
UBD	-0.016	0.048
KRAS	-0.017	0.044
FKBP11	-0.017	0.036
NRD1	-0.017	0.035
FGFR4	-0.018	0.031
ADAR	-0.018	0.026
NR1H3	-0.019	0.020
KLF6	-0.019	0.019
GZMK	-0.02	0.017
GADD45B	-0.02	0.015
SDF2L1	-0.02	0.014
CYLD	-0.021	0.010
TYMP	-0.023	0.005
IL2RB	-0.028	0.001

[*] Adjusted for patient age and TNM stage.

Table 4

Genes differentially expressed in tumor tissue based on comparison of the lowest vs highest quartile of soy food intake

Gene	Quartile analysis				Regression analysis[*]		Cell growth gene
	2nd/1st[&]	3rd/1st[&]	4th/1st[&]	p value (4th/1st)	Beta	p value	
EAF2	-0.291	-0.561	-1.318	3.7×10^{-4}	-0.031	1.1×10^{-4}	yes
DDAH1	0.402	-0.107	0.426	0.005	0.025	0.003	yes
KLF6	-0.329	-0.353	-0.355	0.013	-0.019	0.019	yes
SNX13	0.295	0.144	0.383	0.023	0.013	0.103	
NR1H3	-0.329	-0.267	-0.794	0.023	-0.019	0.020	yes
ABCB1	-0.115	-0.600	-0.887	0.023	-0.013	0.106	yes
IL2RB	-0.110	-0.649	-0.702	0.036	-0.028	0.001	yes
TYMP	-0.161	-0.222	-0.386	0.048	-0.023	0.005	

[*] Per Log2 fold change.

[&] Adjusted for patient age and TNM stage.