Abstract

Green tea has been shown to be a potent chemopreventive agent against lung tumorigenesis in animal models. Previously, we found that treatment of A/J mice with either green tea (0.6% in water) or a defined green tea catechin extract (polyphenon E; 2.0 g/kg in diet) inhibited lung tumorigenesis. Here, we described expression profiling of lung tissues derived from these studies to determine the gene expression signature that can predict the exposure and efficacy of green tea in mice. We first profiled global gene expressions in normal lungs versus lung tumors to determine genes which might be associated with the tumorigenic process (TUM genes). Gene expression in control tumors and green tea–treated tumors (either green tea or polyphenon E) were compared to determine those TUM genes whose expression levels in green tea–treated tumors returned to levels seen in normal lungs. We established a 17-gene expression profile specific for exposure to effective doses of either green tea or polyphenon E. This gene expression signature was altered both in normal lungs and lung adenomas when mice were exposed to green tea or polyphenon E. These experiments identified patterns of gene expressions that both offer clues for green tea’s potential mechanisms of action and provide a molecular signature specific for green tea exposure. (Cancer Res 2006; 66(4): 1956-63)

Introduction

Lung cancer is the leading cause of cancer death in the U.S. (1). Exposure to tobacco is involved in 90% of lung carcinomas. Smokers’ risk of lung cancer is 20 times that of persons who have never smoked (2). Former heavy smokers retain an elevated risk for lung cancer even decades after they stopped smoking. One potential strategy to prevent lung cancer in high-risk populations is to use chemopreventive agents to regress existing intraepithelial neoplastic lesions, prevent the progression of these lesions to cancer, or inhibit the development of new lesions (3). Green tea contains flavanols or catechins including epigallocatechin gallate (EGCG; refs. 4–6). These polyphenols have various biological activities including antioxidation, modulation of enzyme systems for metabolizing chemical carcinogens, inhibition of nitrosation reactions, scavenging of activated metabolites of chemical carcinogens, and inhibition of tumor promotion (4–6). Although epidemiologic studies on the cancer-preventive effects of tea produced inconsistent results, a subset of studies suggested that tea consumption might reduce the risk of human lung, skin, breast, and gastrointestinal cancers (4–6). A prospective cohort study over 10 years in Japan showed that the consumption of 10 or more cups of green tea per day delayed the onset of cancer in both smokers and never smokers (7).

Preclinical studies have shown the inhibitory action of green tea or green tea extracts against tumorigenesis on different organ sites such as skin, lung, oral cavity, esophagus, forestomach, stomach, small intestine, colon, liver, pancreas, and mammary gland (8–10). Green tea and one of its components, EGCG, has been shown to inhibit 1-(4-methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)–induced mouse lung tumorigenesis by 63% and 28%, respectively (11). We have recently shown the chemopreventive efficacy of green tea and polyphenon E in A/J mice (12, 13). In the green tea study, administration of green tea as the sole drinking source beginning 1 week after carcinogen administration significantly reduced tumor multiplicity in A/J mice (ref. 12; Fig. 1). Tumor multiplicity for the mice treated with a carcinogen was 5.1 and decreased to 2.4 in mice treated with green tea (P < 0.0001). In the polyphenon E study (13) treatment of mice with 2% of polyphenon E in diet caused a significant decrease in tumor multiplicity (10.8 tumors/mouse in controls to 5.9 tumors/mouse in those treated with polyphenon E; P < 0.05). The lung tissues derived from these studies were used to determine the potential mechanisms of action and gene expression signature that can predict both the efficacy and pharmacodynamics of green tea in mice.

Although there are many suggested mechanisms of action for green tea, including the induction of apoptosis and cell cycle arrest, the exact mechanisms for the action of green tea are not yet clear. Expression profiling is a powerful technique to uncover patterns or pathways indicative of potential mechanisms of action. Additionally, we will identify gene expression profiles that may be predictive of the chemopreventive efficacy of green tea for lung cancer, as well as uncover a specific expression signature for green tea exposure. The results from our study could be used for either clinical trials or perhaps in epidemiologic studies.

Materials and Methods

Reagents

Benzo[a]pyrene [B(a)P, 99% pure] and tricaprylin were purchased from Sigma Chemical Co. (St. Louis, MO). NNK (99% pure) was from Chemsys Science Laboratories (Lenexa, KS). Bulk green tea extract powder was obtained from the National Cancer Institute. Polyphenon E was obtained from Tokyo Food Techno Co., Ltd. (Tokyo, Japan). Chemical carcinogens were prepared immediately before use in bioassays: NNK was dissolved in warmed PBS, and B(a)P was prepared in tricaprylin.
Microarrays

RNA samples were further purified, labeled, and processed according to standard manufacturer's recommendations. Singleton cRNA preparations were produced from 30 μg of total RNA from each specimen and 10 μg equivalent aliquots were hybridized to each Affymetrix oligonucleotide array (Santa Clara, CA). The labeled cRNA from NNK-induced mice was hybridized onto the Murine Genome U74Av2 Array (MG-U74Av2), which consists of >12,000 genes and expressed sequence tags on one array. The labeled cRNA from B(a)P-induced mice was hybridized onto the Mouse Genome 430A 2.0 Array (ME430Av2), which contains 22,960 genes and expressed sequence tags on one array. Arrays were then scanned and digitized. Sixteen slides from the NNK-induced model and 15 slides from the B(a)P-induced model were obtained for data analysis. In the B(a)P-induced model, one slide from a tumor tissue treated with polyphenon E had poor data quality and thus was excluded from data analysis.

The raw fluorescence intensity data within CEL files were processed with Robust Multichip Average algorithm (14), as implemented with R packages from Bioconductor. This algorithm analyzes the microarray data in three steps: a background adjustment, a quantile normalization, and finally, a summation of the probe intensities for each probe set using a log scale linear additive model for the log transform of (background corrected, normalized) PM intensities.

RT-PCR

To evaluate the reliability of the array results, 12 genes were randomly selected from the genes differentially expressed between normal and tumor tissues in the microarray assay for further confirmation by real-time PCR. Two micrograms of total RNA per sample, collected as described above, were converted to cDNA using the SuperScript First-Strand Synthesis system for RT-PCR (Invitrogen). RT-PCR assay was done using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). One microliter of cDNA was added to a 25 μL total volume reaction mixture containing water, SYBR Green PCR Master Mix, and primers. Each real-time assay was done in duplicate on Stratagene Mx3000. Data was collected and analyzed on the Stratagene Mx3000 software. GAPDH was used as an internal standard.

The GAPDH value, a reflection of the number of cycles needed to reach a threshold of fluorescence was subtracted from the cycle value for the individual gene whose expression was being assessed.

DNA Analyses

Matching mouse probes from two microarray systems. Because two microarray platforms were used, the probe sets should be corresponded. The batch query tool provided by Affymetrix was used for the correspondence between probe sets (15). There are a total of 8,904 pairs of probe sets corresponding to each other (representing the same gene) on the two microarray systems MG-U74Av2 and ME430Av2. All of the following comparisons between the two mouse models are based on these probe set pairs.

Identifying differentially expressed genes. The following ANOVA model was used to test if a gene has significantly different transcription levels between different tissues and/or tea treatment in the two mouse models. Let \( y_{ijk} \) be the gene expression level of gene \( n \) from tissue \( i \), treatment \( j \) and sample \( k \). The gene expression level \( y_{ijk} \) can be expressed as,

\[
y_{ijk} = \mu_n + \tau_i(n) + \gamma_j(n) + \delta_{ijk} \]

where \( i = 1 \) or 2, represents cancer or normal tissue; \( j = 1 \) or 2, represents tea treatment or control; \( k = 1 \) to 4, represents replicates in each group. The gene effect \( \mu_n \) captures the overall mean expression level of gene \( n \) across the tissues and tea treatments. The term \( \tau_i(n) \) accounts for gene-specific tissue effects representing overall differences between two tissues. The term \( \gamma_j(n) \) accounts for tea effects that capture overall differences between tea
treatment and control in the samples. The term $CT_{ij}$ accounts for the interaction effect between tissue and tea treatment. The term $\epsilon_{ijkn}$ represents random errors. For each gene, we did 10,000 permutation tests and obtained empirical $P$ values for each variance component. The ANOVA and permutation tests were implemented using the R statistical package (16).

Pathway analysis. The visualization tool GenMAPP 6 was used to illustrate pathways containing differentially expressed genes. The differential gene expression was based on tea treatment versus nontreatment expression change by the ANOVA analysis ($P < 0.05$).

Discriminant analysis. $k$-nearest neighbors ($k$-NN) algorithm was used to select classifier. In the $k$-NN algorithm, a series of competitive models were built with a wide range of features (1-200 genes). Then, the predicting error rate of each of these models was estimated by using "leave-one-out" cross-validation approach. Finally, the best models were chosen based on their predicting error rates. Fisher’s test was used to evaluate the significance of these model predictors. The obtained classifier was then used for predicting tea status of samples, which were implemented in Gene Expression Pattern Analysis Suite V1.1.7

Results

Gene expression changes associated with tumorigenesis which were reversed by green tea. Gene expression analyses using Affymetrix microarrays showed a significant difference between mouse lung tumors and normal lungs. Specifically, 2,738 genes were differentially expressed when comparing normal lung tissue and $B(a)P$-induced lung tumors with $1,329$ up-regulated and $1,409$ down-regulated genes ($P < 0.005$). Similarly, 3,712 genes were differentially expressed when comparing normal

Figure 2. Gene expressions altered by tea treatments ($P < 0.005$). A, genes underexpressed in tumors and restored at the normal expression levels after tea treatments.

6 http://www.genmapp.org/

lungs and NNK-induced tumors (1,721 up-regulated and 1,991 down-regulated genes; $P < 0.005$). Comparing the tissue effects of these genes in both chemically induced mouse lung tumor models, we found that the expression levels of 2,036 significant genes were consistently altered in both models, including 946 up-regulated and 1,090 down-regulated genes. This result indicates that the gene expression changes, and presumably, the mechanism of lung carcinogenesis for both B(a)P and NNK are largely similar. Most of the common altered genes in both models are related to several important pathways of lung carcinogenesis (Supplementary Table S1). Interestingly, a subset of these differentially expressed genes in tumors was reversed towards levels found in normal lungs when animals were treated with either green tea or polyphenon E. Figure 2A and B show 88 such genes in the NNK model, 25 of them are most likely associated with the tumorigenic process from their functions, including 19 underexpressed, and 6 overexpressed genes in tumors. Detailed information for these genes is listed in Supplementary Table S2. Dual specificity phosphatase 1 (Dusp1), DNA topoisomerase I (Top1), Fyn proto-oncogene (Fyn), regulator of G protein signaling 2 (Rgs2), G1 to S phase transition 1 (Gpt1), heat shock protein 8 (Hspa8), E26 avian leukemia oncogene 2, 3' domain (Ets2), cyclin-dependent kinase inhibitor 2C (Cdkn2c), and v-abl Abelson murine leukemia oncogene 1 (Ab1) are related to cell cycle. In B(a)P mouse model, we found that seven genes met the criteria, including three overexpressed genes (Rbbp6, Tnfrsf9, and Kif20a) and four underexpressed genes (Ccb12, Drg1, Sf3a2, and Ryk) in tumors. Because these alterations in expression seemed to reverse the levels observed during tumorigenesis, they are candidates to be involved in the mechanism by which green tea or polyphenon E blocks tumorigenesis.

GenMAPP is a novel tool for visualizing expression data in the context of biological pathways (18). We imported our data set into the program and illustrated the pathways modulated by treatment with green tea. We found that genes in cell cycle pathways were significantly altered by tea treatment in both mouse models. Figure 3A represents the cell cycle pathway following treatment with green tea. Following treatment with green tea, genes in S phase and G2 phase were down-regulated with tea treatment; genes in G1 phase were up-regulated. In addition, green tea seems to affect the inflammatory response pathway (Fig. 3B).

Identification of a green tea gene expression signature. Using k-NN algorithm ($k = 3$; Fisher’s exact test, $P = 1.5 \times 10^{-5}$),
we successfully selected 14 and 91 gene expression sets that are most closely associated with green tea and polyphenon E exposure, respectively, to readily discriminate lung tissues (normal lungs and lung tumors) exposed to polyphenon E or green tea from those of vehicle control mice. When we did hierarchical cluster analyses using the 14-gene and 91-gene classifiers, the tissues with green tea exposure clustered together and there are no classification errors in either treatment. The results were displayed by TreeView (Fig. 4A and B). Furthermore, we identified a classifier composed of 17 genes which are most closely associated with tea status from all of the 31 samples (Fisher’s exact test, \( P = 3.3 \times 10^{-8} \)) using the integrated expression data. This 17-gene classifier could correctly discriminate from all samples (normal lung or lung adenomas) of mice exposed to both forms of green tea (green tea and polyphenon E).

To further evaluate the performance of the 17-gene classifier obtained by the \( k \)-NN algorithm, we comparatively applied four other clustering methods to integrated expression data: (a) \( k \)-mean with Pearson correlation coefficient, (b) a self-organizing map with Gaussian function neighborhood, (c) support vector machines with linear kernel, and (d) hierarchical clustering. All four methods made no misclassification errors. Figure 4C presents the results of hierarchical clustering.

**Confirmation of gene expression data using semiquantitative RT-PCR.** To confirm the significance of the genes identified to be differentially expressed using microarrays, we did RT-PCR analysis. Of the selected genes, nine of them (Lamr1, Sftpb, Clu, P4ha1, U46068, Hba-a1, Car4, Aldh2, and Pon1) were identified as
having significant tissue effects in both mouse models by ANOVA analysis, whereas Ngef, Cyr61, and Scgb1a1 were identified in either mouse model ($P < 0.005$). All of them were confirmed by RT-PCR. Figure 5 is the comparison of the fold changes produced by DNA microarrays with the relative expression ratio obtained from RT-PCR.

**Discussion**

In this report, we did gene expression analyses on both normal lungs and tumors treated with either green tea or polyphenon E to examine mechanistic questions regarding the efficacy of green tea or polyphenon E. We showed that a subset of the differentially expressed genes in tumors treated with green tea were reversed towards levels found in normal lungs, suggesting that they might be involved in mediating the chemopreventive effect of green tea or polyphenon E. We also identified a green tea gene expression signature that can readily discriminate green tea–treated lung tissues from those without exposure to green tea in mouse models.

As can be seen in Fig. 1, treatment with either polyphenon E (2.0% in diet) or green tea (0.6% in water) administered beginning 1 week following the last dose of carcinogen, decreased lung tumor multiplicity by 45% and 55%, respectively. Presuming consumption of 5 mL of water and a similar amount of feed, a 25 g animal would consume roughly 1.4 g/kg of green tea and roughly 5.0 g/kg of tea polyphenols. Given the fact that polyphenols represent roughly 15% to 20% of the dry weight of

**Figure 4.** Hierarchical clustering of mice treated with either green tea or polyphenon E. **A,** 15-gene classifier from animals treated with green tea. **B,** 91-gene classifier from mice treated with polyphenon E. **C,** 17-gene classifier from the integrated gene expression data for all 31 samples in animals treated with either green tea or polyphenon E.
tumor tissues (normal lungs and lung adenomas) that we were not observing effects on carcinogen metabolism. Employing the delayed administration of these agents was to insure that we would differentiate tea exposed (green tea or polyphenon E) and they might prove applicable to epidemiologic studies assessing the effects of green tea or polyphenon E.

We examined genes whose expressions were altered in tumors (TUM genes) and determined whether green tea or tea polyphenols were able to reverse the gene expression changes associated with the tumorigenic process. The obvious rationale to such an approach is that these genes are mechanistically likely to be involved in the mechanism by which green tea or polyphenon E blocks tumorigenesis. As can be seen in Fig. 2, there are a substantial number of genes which met this criteria in the NNK model. However, the genes which were modulated by green tea versus polyphenon E had limited overlap. When we took the results into GenMAPP, we found that although both agents affected cell cycle related genes, they seemed to preferentially affect different specific genes. In addition to affecting cell cycle related genes, green tea but not polyphenon E seemed to affect genes related to the inflammatory pathway. At this time, the identified genes and pathways must be considered candidate genes requiring further specific examination. EGCG, the major polyphenol from green tea, can inhibit DNA methyltransferase (DNMT) activity and reactivate methylation-silenced genes in cancer cells (20). The average level of mRNA for DNMT2 was significantly lower in hepatocellular carcinomas, colorectal cancers, and stomach cancers than in non–cancerous tissue (21, 22). In our results, DNMT2 was also found underexpressed in lung tumors and green tea was able to reverse the gene expression towards levels in normal lungs.

As mentioned above, the preclinical studies showing the efficacy of tea and tea extracts as well as the more limited epidemiologic data have encouraged the use of clinical trials employing tea or tea extracts (9). Therefore, another major objective of this study was to identify potential pharmacodynamic markers which might be useful for clinical trials with this class of agents. Although there are genes that were modulated only in tumors or in histologically normal tissue, we queried the array data to define genes which were modulated in both tumors and histologically normal tissues. We initially defined potential pharmacodynamic markers for individual treatment agents, e.g., green tea or polyphenon E. Therefore, in Fig. 4A and B, we have defined genes for either green tea or polyphenon E that can differentiate tissues exposed to effective levels of an agent versus control. As can be seen, we can readily differentiate histologically normal or tumor tissue treated with green tea from control tumors or control normal lungs. Similarly, we found a group of genes that differentiate histologically normal or tumor tissue treated with polyphenon E from control tumors or control normal lungs. These genes might be useful in examining a clinical trial employing either of these specific agents. The fact that these gene candidates can be used in either histologically normal lung tissues or lesions would seem to be a plus because it allows one to use samples from either set of tissues. It will be of some importance to determine whether these samples are relevant for testing bronchial washings as well because they may be the most readily accessible tissue from a phase II study in lung (23, 24). Finally we mined the data to determine whether they might be applicable to a wide variety of tea compounds and they might prove applicable to epidemiologic studies assessing tea consumption where the specific makeup of the tea, caffeinated tea, there is ~1/10 of the amount of the polyphenols in the green tea preparation that was achieved in the polyphenon E treatment. The delayed administration of these agents was to insure that we were not observing effects on carcinogen metabolism. Employing the lung tissues (normal lungs and lung adenomas) that we generated from these studies, we isolated RNAs and proceeded to determine gene expression in histologically normal or tumor tissue from control mice or mice treated with green tea or polyphenon E to address a variety of questions.

We first profiled global gene expressions in normal lungs versus lung tumors to determine genes which might be associated with the tumorigenic process (TUM genes; ref. 19). There were a great number of genes (>2,000) which were differentially expressed between histologically normal lungs and lung tumors. Although some of the gene expression changes which we have observed are presumably due to the tumorigenic process itself, most of these differences may be due to comparing a variety of cell types in the normal lung with a much more limited number of cell types in the adenomas. Nevertheless, we observed a substantial number of changes in the major pathways related to transcription, cell proliferation, and cell signaling, many of which are likely to contribute to the tumorigenic process. We have designated the gene changes between the lesions and the corresponding histologically normal lungs as TUM genes. We have designated them as such both because we feel that they may be biologically relevant and because we used this subset of genes for examining the effects of green tea or polyphenon E.

Figure 5. Comparison of fold changes produced by DNA microarray with relative expression ratio obtained from RT-PCR for selected genes with differential expression between normal and tumor tissues. A, for B(a)P-lung tumor model; B, for NNK-lung tumor model. Positive fold change represents up-regulated and negative fold change represents down-regulation in tumor tissues.
or decaffeinated, may not be known. Thus, such a generalized set of markers might have some general applicability.

One should be aware of certain aspects of these pharmacodynamic markers. Firstly, because the gene changes are not necessarily involved in the mechanism of action of these agents, they are not necessarily efficacy end points. Thus, they only directly associated with the pharmacodynamic/physiologic end point (gene modulation) and that at doses which achieved these changes in an animal that you achieved efficacy. Therefore, if you fail to observe these changes in a clinical trial, you may feel that it is unlikely that the dose employed will be effective. Secondly, multiple genes should be examined simultaneously because some genes which are modulated in animal models by a given agent may not be similarly modulated in a human. However, with various genes, this is less likely to be a problem. Finally, these genes become potential pharmacodynamic markers for the agent in tissues other than the tissue examined. Thus, genes modulated in the lung by tea might be similarly modulated by tea in other tissues, which remains to be determined in these other tissues.

In summary, we found multiple gene changes associated with lung tumorigenesis. Interestingly, both green tea and tea polyphenols could reverse certain of these gene changes associated with tumorigenesis. However, the specific genes altered are significantly different for green tea and polyphenon E, implying potentially different mechanisms of action. Finally, we defined potential pharmacodynamics for both green tea alone and polyphenon E alone and additionally defined genes which were modulated by both treatments. We feel that these genes are potentially useful for ongoing and proposed clinical trials using tea or tea extracts in humans.

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References

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