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Regulation of p53-targeting microRNAs by polycyclic aromatic hydrocarbons: Implications in the etiology of multiple myeloma

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Abstract

Multiple myeloma (MM) is a common and deadly cancer of blood plasma cells. A unique feature of MM is the extremely low somatic mutation rate of the *p53* tumor suppressor gene, in sharp contrast with about half of all human cancers where this gene is frequently mutated. Eleven miRNAs have been reported to repress *p53* through direct interaction with the 3' untranslated region. The expression of nine of them is higher in MM plasma cells than in healthy donor counterparts, suggesting that miRNA overexpression is responsible for *p53* inactivation in MM. Here, we report that the environmental carcinogen benzo[a]pyrene (BaP) upregulated the expression of seven *p53*-targeting miRNAs (miR-25, miR-15a, miR-16, miR-92, miR-125b, miR-141, and miR-200a), while 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) upregulated two of them (miR-25 and miR-92) in MM cells. The miR-25 promoter was activated by both BaP and TCDD, and this response was mediated by the aryl hydrocarbon receptor (AhR). We screened 727 compounds that inhibit MM cell survival and down-regulate the expression of *p53*-targeting miRNAs. We found that (-)-epigallocatechin-3-gallate (EGCG), a constituent of green tea and a major component of the botanical drug Polyphenon® E, reduced the expression of four *p53*-targeting miRNAs, including miR-25, miR-92, miR-141, and miR-200a. Collectively, these data implicate polycyclic aromatic hydrocarbons and AhR in the regulation of *p53*-targeting miRNAs in MM and identify a potential therapeutic and preventive agent to combat this deadly disease.

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Keywords

toxicants; polycyclic aromatic hydrocarbons; microRNAs; p53; multiple myeloma

Introduction

Multiple myeloma (MM) is an incurable hematological neoplasm characterized by excessive infiltration of abnormal plasma cells in the bone marrow and overproduction of monoclonal immunoglobulins (Igs). About 45,000 people in the United States are living with MM. The American Cancer Society estimated that more than 21,000 new cases of MM will be diagnosed and that more than 10,000 patients will die of MM each year. MM patients have a 5-year survival rate of ~40% even with the use of successful drugs, such as the proteasome inhibitor bortezomib.

MM is regarded as an environmental disease, yet its etiology remains elusive. Individuals exposed to radiation are at increased risk for MM [1]. Researchers have also investigated the association of MM with smoking, with some suggesting a strong association [2,3] while others refuting this relationship [4-6]. Such inconsistencies likely reflect unidentified confounders that impact epidemiological relationships. For instance, individuals can be exposed to benzo[a]pyrene (BaP) and other polycyclic aromatic hydrocarbons (PAHs) from alternate environmental sources, such as vehicle exhaust, burned wood or foods, and industrial processes[7-9], and this may confound the interpretation of smoking studies. An expert panel reviewing ~40 epidemiologic studies on another PAH 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a contaminant in Agent Orange, and the risks of MM concluded that there is an elevated risk of MM associated with TCDD exposure in three fourths of all studies examined [10]. This can be interpreted as compelling evidence for an association between exposure and disease. Similar to cigarette smoking, caution must be exercised, however, because a quarter of the studies did not support such a relationship.

The *p53* tumor suppressor gene (also known as *TP53* in human) is regarded as a “guardian of the genome” because of its crucial role in coordinating cellular responses to genotoxic stress, as well as other stresses such as hypoxia, expression of certain oncogenes, and cytotoxic insult. As a transcription factor, p53 regulates expression of a battery of genes which promote apoptotic cell death, cell cycle arrest, cellular senescence, and other processes [11]. The fundamental role of p53 in tumor suppression is underscored by the fact that it is one of the most frequently mutated proteins in human cancer. p53 protein does not function properly in most human cancers, yet in only half of these tumors p53 is inactivated directly as a result of mutations. In many cases, p53 is found to be inactivated indirectly through amplification of the *MDM2* gene encoding the murine double minute 2 (Mdm2) protein or its homolog *MDMX*, allelic deletion of *CDKN2A* encoding the p14^{ARF} protein, viral proteins that inactivate p53 directly or indirectly, or mislocalization of p53 to the cytoplasm [12]. Various studies of *p53* mutations in MM patients displaying clinical heterogeneity in the cohort or small sample sizes (all with <100 patients) reported a prevalence of *p53* mutations ranging from 0 to 20% [13]. The latest comprehensive study with the largest number of newly diagnosed patients reported that *p53* mutations are rare as

only 9 of the 268 samples (3%) tested were positive for mutations [14]. Moreover, other known mechanisms of p53 inactivation also have relatively low incidence in MM [15]. Together, these data suggest that p53 inactivation in MM is unconventional.

We hypothesized that p53 is inactivated in MM cells by the overexpression of p53-targeting microRNAs (miRNAs). miRNAs are short 20-25 nucleotide RNA molecules that negatively regulate gene expression by targeting the 3' untranslated region (UTR) of mRNAs [16]. In this work, we itemized all the miRNAs that directly suppress the human p53 gene and their expression levels in MM. We then determined whether miRNA expression is modulated by two environmental toxicants—BaP and TCDD. Finally, we performed a small-scale screening to identify FDA-approved drugs on MM cell survival and the expression of p53-targeting miRNAs. Collectively, this work implicates PAHs and AhR in miRNA deregulation and MM etiology and suggests a putative therapeutic option against this deadly disease.

Results

p53-targeting miRNAs are overexpressed in MM

Over the past few years, eleven miRNAs have been identified by our group and others to negatively regulate p53 expression by directly targeting the 3'UTR: miR-25, miR-30d [15], miR-125b [17], miR-504 [18], miR-380-5p [19], miR-92 [20], miR-141 [20], miR-1285 [21], miR-200a [22], miR-15a and miR-16 [23] (Table 1). Recent antagomir experiments in this laboratory demonstrated direct binding of miR-25, miR-30d, and miR-200a to the p53 3'UTR [15,22]. A number of miRNAs reported to directly target p53 in rodents [24] were excluded from this investigation due to low conservation between the p53 UTR sequences between species. Rigorous review of all published work on miRNA expression profiling of MM specimens strikingly showed that expression of nine of these p53-targeting miRNAs was higher in MM cells than plasma cells obtained from healthy donors in at least one report: miR-25 [15,25], miR-30d [15,25,26], miR-125b [25-27], miR-92 [25], miR-200a [26,28], miR-504 [26], miR-141 [26], miR-15a [25], and miR-16 [25] (Table 1). These findings support the hypothesis that p53 inactivation in MM cells is mediated by miRNA overexpression. As p53 is well-integrated in a complex molecular network and involved in a number of positive and negative feedback loops [29], the activation of p53 can induce the expression of p53-targeting microRNAs. To this end, we transfected pCMV-p53 (Clontech) carrying a functional p53 gene or pCMV-p53m153 with an inactive p53 into 293T, p53-null H1299, and MM1.s cells and determined the expression of 11 p53-targeting miRNAs by qPCR. None of the miRNAs were significantly up- or down-regulated indicating that p53-targeting miRNAs are not regulated by p53.

Upregulation of p53-targeting miRNAs by BaP and TCDD

We incubated MM1.s (an MM cell line that expresses WT p53 gene) with 3μM BaP for 24 hr. This concentration represents an environmentally relevant dose that affords optimal activation of aryl hydrocarbon receptor (AhR) [30]. Microarray analysis was performed to profile the expression of all miRNAs in treated cells compared to vehicle controls treated with an equal volume of DMSO. Nineteen miRNAs were upregulated and eight down-

regulated in BaP-treated cells (Table 2). Among 10 miRNAs found to be upregulated 2-fold or more, there were seven p53-targeting miRNAs: miR-25, miR-15a, miR-16, miR-92, miR-125b, miR-141 and miR-200a. We noted that miR-25 and miR-92 belong to a family of highly conserved miRNAs located in three gene clusters (Fig. 1A) [25,31]. miR-25 is encoded in the miR-106~25 cluster only, while miR-92 is encoded in both the miR-17~92 cluster and the miR-106a~363 cluster. Given the similarity in sequences (Fig. 1B), it is presumed that the miR-106b~25 gene cluster and its two paralogs exert similar, if not identical, biological functions. miR-106b of the miR-106b~25 and miR-17 of the miR-17~92 were also induced by BaP treatment (Table 1). We next validated the modulation of miRNAs using quantitative real-time PCR (qPCR) in MM1.s cells treated with 3µM BaP or 10nM TCDD (another AhR ligand implicated in MM etiology). We confirmed that all seven p53-targeting miRNAs were upregulated by BaP, yet only miR-25 and miR-92 were upregulated by TCDD (Fig. 1C). Such differences between BaP and TCDD suggest that complex mechanisms are operational in the regulation of miRNA expression by PAHs. We also examined the expression of four other miRNAs (miR-106b, miR-17, miR-106a, and miR-18b) from the three clusters, respectively, as all three clusters are overexpressed in MM plasma cells [25]. The expression of miR-106b and miR-17, but not that of miR-106a and miR-18b, was upregulated by BaP and TCDD (Fig. 1C). These results indicate that the expression of two miRNA gene clusters (miR-106b~25 and miR-17~92) but not that of the miR-106a~363 cluster is upregulated by BaP and TCDD.

The promoter to miR-25 contains an AhRE binding site

Among the three miRNA gene clusters, the miR-106b~25 cluster is the best characterized in terms of gene structure and promoter function [32]. Therefore, subsequent studies were completed focusing on this cluster. The three miRNAs are located in an intron of the *mini-chromosome maintenance protein 7 (MCM7)* gene and a core aryl hydrocarbon-responsive element (AhRE) sequence “GCGTG” was identified in the promoter. AhRE is the binding site for a heterodimer of AhR and AhR nuclear translocator (ARNT). PAHs such as BaP and TCDD are ligands for the cytosolic AhR and regulate gene expression through activation of AhR/ARNT heterodimer binding to DNA [33]. Upon binding of ligand, the activated AhR releases the Hsp90 dimer and translocates to the nucleus where it heterodimerizes with ARNT. The AhR/ARNT complex then binds to AhREs in the 5' flanking region of target genes and acts as a transcriptional regulator. To test whether the promoter of miR-25 is responsive to AhR ligands, we obtained this promoter, located upstream of a *luc* gene, from Dr. Kiyono (Fig. 2A) [32]. We then co-transfected the promoter construct with pRL-TK containing a constitutive expressed Renilla luciferase gene (*Rluc*) into HeLa cells and treated them with 3µM BaP or 10nM TCDD for 24 hr, respectively. Dual-luciferase measurements followed to determine whether PAH treatment increased reporter expression. As shown in Fig. 2B, both BaP and TCDD increased gene expression of the reporter under control of the miR-25 promoter. We next performed mutagenesis to alter the core AhRE site (Fig. 2A) or deletion of the 234-bp DNA fragment containing the site [32]. Ensuing reporter assays showed that reporter upregulation by BaP and TCDD was abolished when the AhRE site was deleted or mutated (Fig. 2B). Lastly, we down-regulated AhR (~40%) and ARNT (~30%) by transfection of MM1.s cells with siRNAs directed against these proteins. We found that upregulation of the endogenous miR-25 by BaP (3µM) or TCDD (10nM) was

overturned in MM1.s cells following AhR or ARNT knockdown (Fig. 2C). These results support the hypothesis that miR-25 gene expression is regulated by BaP and TCDD through interactions of the ligand activated AhR-ARNT heterodimer with regulatory sites in the miR-25 promoter.

A small-scale screening assay to identify therapeutic agents for MM therapy

In addition to MM, miR-25 is also overexpressed in other cancers [34]. Croce and colleagues identified miR-25 as the second most differentially expressed miRNA in 31 types of solid cancers [35]. Therefore, we have initiated a screening assay to identify small molecules that target miR-25 in MM cells. To date, we have screened 727 FDA-approved compounds from an NIH Clinical Collection compound library [36]. The collection was assembled by NIH via the Molecular Libraries Program, an NIH Roadmap Initiative, to enable the use of compound screens in biomedical research. These clinically tested compounds are highly drug-like, with known safety profiles, and are excellent starting points for drug repurposing. All of the compounds have been approved for at least Phase I trials and a handful of them are not approved for prescription or over-the-counter use. In our screen, we placed 90,000 MM1.s in 96-well plates and incubated them with each compound at a final concentration of 1.0 μ M (dissolved in DMSO). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were performed to determine cell viability after 48 hr. Of 727 compounds, 52 reduced the viability of MM1.s cells by 75% (Fig. 3A). We then screened the same 52 compounds for their ability to modulate miR-25 expression and identified (-)-epigallocatechin-3-gallate (EGCG) and eight other compounds (acarbose, methylandrostenediol, flumazenil, rifabutin, diazepam, tramadol, ampiroxicam, and glimepiride) which reduced miR-25 expression significantly in MM1.s cells (Fig. 3B). In addition, four compounds (methylandrostenediol, flumazenil, ampiroxicam, and glimepiride) were found to increase p53 mRNA levels. Lastly, we measured the p53 protein levels upon treatment of MM cells with these compounds and found that all nine compounds elevated p53 protein levels more than 50% in MM1.s cells (Fig. 3C).

EGCG inhibits miR-25 expression

Among the nine compounds identified in our screening, six are approved by the FDA for indications other than cancer, two are approved for medical use in other countries, and one is used as a food supplement (Table 2). We searched PubMed for the p53 relevance of these compounds and found that only EGCG has been linked to p53 based on its ability to upregulate p53 in multiple human cancer cell lines [37-40]. In addition, EGCG has been shown to reduce the mutagenic effect of BaP in mice [41]. We first examined the expression of seven BaP-induced p53-targeting miRNAs in MM1.s cells following treatment with EGCG and found that EGCG inhibited the expression of miR-25, miR-92, miR-141, and miR-200a, as well as miR-106b and miR-17, miRNAs within the sample clusters of miR-25 and miR-92 (Fig. 4A). We next examine whether the modulation of miR-25 by EGCG involves regulation of its promoter. Using the same reporter assay described above, we found that EGCG reduced the promoter activity of the reporter (Fig. 4B). Next, we co-treated MM1.s cells with BaP/TCDD and EGCG and found that EGCG treatment inhibited miR-25 induction by BaP or TCDD (Fig. 4C). This suggests that EGCG functions as an

antagonist to PAHs capable of inducing the expression of miR-25 in in hydrocarbon-treated cells.

Discussion

MM is one of the most common hematological neoplasms. It constitutes about 1% of human cancers and 2% of all cancer deaths. MM is preceded by monoclonal gammopathy of undetermined significance (MGUS), which is one of the most common pre-cancerous conditions afflicting 3% of the United States population over 50 years old. MGUS progresses to incurable MM at a relentless rate of ~1% per year [42], and yet little information is available about the molecular events that mediate MM progression. Comparing to most other cancer types, the p53 mutation rate is extremely low in MM. Yet p53 is most likely inactivated in MM due to several reasons. Although p53 mutation is rare in MM, MM patients with p53 mutation or lower p53 mRNA levels show extremely poor prognoses [14,43]. Activation of p53 in MM by an Mdm2 antagonist induced p53 target gene expression and apoptotic cell death [44]. Another recent publication revealed that p53 mRNA levels and p53 gene deletion are correlated with MM prognosis; while introduction of ectopic p53 expression decreased MM cell proliferation and cell viability [43]. These data indicate that should p53 be fully functional, MM cells would in fact undergo apoptosis. We hypothesized that overexpression of miRNAs inactivates p53 in MM and in this study we summarized all reported p53-targeting miRNAs (Table 1). A total of eleven p53-targeting miRNAs were identified, nine of which were found to be upregulated in MM. We reported earlier that the expression levels of two p53-targeting miRNAs (miR-25 and miR-30d) were inversely correlated with p53 mRNA levels in MM plasma cells [15]. Moreover, inhibiting miR-25 or miR-30d expression increased p53 protein levels and elevated cellular apoptosis in MM cells with a wild-type p53 [15]. Interestingly, an improved progression-free survival is identified in MM patients with low expression of *DICER*, a gene essential for maturation of all miRNAs [26], underscoring that miRNA overexpression may be important to MM prognosis.

We next assessed the impact of two environmental toxicants, BaP and TCDD, on the expression of p53-targeting miRNAs as these two pollutants have been implicated in the etiology of MM by epidemiological studies [2,3,10]. We found that BaP upregulated the expression of seven p53-targeting miRNAs in MM cells, while TCDD only elevated two of them, albeit miR-25 was increased by both hydrocarbons. miR-25 is also overexpressed in plasma cells from MGUS patients [25]. This suggests that BaP and TCDD play a significant role in deregulation of p53-targeting miRNAs and by extension in the etiology of MM. As both BaP and TCDD regulate gene expression through AhR and ARNT, and only the promoter to miR-106b~25 is well-defined [32], we determine whether miR-25 expression is modulated through AhR signaling. Our reporter and AhR/ARNT knockdown assays suggest that modulation of miR-25 expression by BaP and TCDD is likely mediated through the classical AhR signaling pathway. We do not know how the other miRNAs are regulated by PAHs as their promoters are not well-defined. Other components of AhR signaling may participate in miRNA regulation. For example, the AhR repressor (AHRR) and AhR form a negative regulatory loop that is evolutionarily conserved in vertebrates [45]: AhR regulates the expression of AHRR, while AHRR acts as a transcriptional repressor of AhR function

[45,46]. The biological and toxicological functions of AHRR in plasma cell maturation and myeloma pathogenesis are not well understood. Next-generation sequencing of MM did not reveal significant mutations in genes involved in AhR signaling [47]. However, we cannot exclude the possibility of AHRR involvement in the regulation of *miR-25* and other miRNA genes as *AHRR* is known to be silenced by hypermethylation in other types of human cancers [48]. The non-mutagenic consequences of PAH exposure likely contribute to miRNA regulation. *First*, PAHs modulate the expression of several miRNA genes through epigenetic mechanisms involving DNA methylation and histone modification [49-51]. *Second*, reactive intermediates of BaP induce reactive oxygen species (ROS) to disrupt the balance of cellular oxidants and anti-oxidants, potentially causing miRNA gene expression changes by redox signaling [52,53]. TCDD may also modulate miRNA expression through ROS production [54] and other mechanisms [55]. Thus, the differential miRNA expression by BaP and TCDD may be mediated by differences in epigenetic modulation and redox signaling. This will be a point of study in future investigations.

Most translational researchers studying miRNAs use modified DNA and/or RNA oligonucleotides as the main weapons to restrain the action of oncogenic miRNAs or mimic tumor-suppressive miRNAs [56,57]. The mechanism underlying this methodology is known: base pairing between antisense and oncogenic miRNAs or between tumor suppressing miRNA mimics and target mRNAs. Another strategy is the use of an adeno-associated virus (AAV) to deliver an miRNA or its antisense [58]; however, many AAV-based therapies work well in mice but fail in humans and no AAV-based therapies are commercially available. We have studied targeting of miRNAs with small molecules including repurposed FDA-approved drugs as an anti-neoplasia strategy. The rationale behind this is to identify compounds that down-regulate the expression of p53-targeting miRNAs, opposite of those induced by BaP. We have successfully performed a small-scale screening and demonstrated that lovastatin, a well-tolerated medication that lowers cholesterol levels, upregulates the expression of a potent tumor suppressor miR-33b, suppressing the expression and function of oncogenic c-Myc in medulloblastoma cells [36]. In the present study, we performed a similar screening to target miR-25 as it is the second most overexpressed miRNA in 31 types of solid tumors [35] and in MM [15,25]. We identified nine compounds that inhibited the expression of miR-25 and elevated p53 protein levels in MM cells. Among them, we singled out EGCG, a component from green tea, which has been reported to elevate p53 expression in human cancer cell lines and to reduce cancer progression in mouse models [37-40,59]. EGCG is the major active component (~55%) of Polyphenon® E, a botanical drug indicated for the treatment of external genital and perianal warts (*Condylomata acuminata*) in immunocompetent patients aged ≥ 18 years. We found that EGCG downregulated the expression of miR-92, miR-141, and miR-200a, in addition to miR-25. Using reporter assays, we discovered that the promoter activity of the miR-25 cluster is reduced by EGCG treatment. In addition, EGCG treatment directly attenuated BaP/TCDD-mediated miR-25 upregulation in MM cells. These experiments provide valuable information about the potential use of EGCG in MM prevention and therapy. EGCG can be safely administered to human subjects at a single dose as high as 1,600 mg, with plasma levels reaching 2,500 ng/ml and remaining above 1,000 ng/ml (~2µM) for hours [60]. Tea drinkers consume 2-10 cups per day [61], and one cup of green tea contains approximately

73.5 mg of catechins with the majority represented by EGCG. Therefore, the dosages used in this study (1 and 5 μ M) are physiologically relevant and well within the range of human consumption. We present a model of environmental toxicant-induced miRNAs overexpression in MM pathogenesis (Fig. 5): the wild-type *p53* tumor suppressor is inactivated by a group of miRNAs that are induced by PAHs to promote MM initiation and progression. As EGCG can reverse the elevated expression of four *p53*-targeting miRNAs, we propose the use of EGCG, pharmacological grade green tea extract Polyphenon E®, or green tea beverages as a therapeutic agent against MM and a preventive option for susceptible individuals with MGUS.

MATERIALS AND METHODS

miRNA expression profiling

Total RNAs were isolated and sent to LC Sciences (LCS) for a “color reversal” experiment using triplicates. Each RNA sample was labeled with either Cy3 or Cy5 and samples hybridized to three dual-color miRNA microarray chips: the Human miRNA Detection Array (MRA-1001, LCS). The array contains probes to detect mature miRNA sequences as well as pre-miRNAs in the Sanger miRNA registry 9.0 (<http://microrna.sanger.ac.uk/sequences/>). Microarray analysis was performed by LCS including background subtraction and data normalization to the statistical median of all detectable transcripts. Based on the *p*-value 0.01 (univariate *t* test), a list of differentially expressed transcripts was produced in each chip. The differentially expressed miRNAs having consistent calls on all three arrays were grouped together in the merged list (Table 1).

Cell culture and assays

HeLa cells were cultured in DMEM with 10% fetal bovine serum (FBS). MM1.s (courtesy of Dr. Nancy L. Krett, Northwestern University, Chicago, IL) were cultured in RPMI1640 with 10% FBS. All medium contained 1x antibiotic-antimycotic (Invitrogen, Carlsbad, CA). Transfection of MM1.s was performed with Lipofectamine LTX (Invitrogen) using the manufacture's protocol. Efficiency of transfection was estimated to be 50-60% with a miRNA-size oligo conjugated with Cy5. siRNAs against AhR and ARNT (Dharmacon SMARTpool, ONTARGETplus) were purchased from Thermo Scientific (Pittsburgh, PA). Reporter and MTT assays were performed according to a previous report [36] and Western blotting of *p53* was performed as described [15]. Briefly, total protein was isolated from cells from 6-well plates using M-PER mammalian protein extraction reagent (PIERCE, Rockford, IL). Protein concentration was measured by the Bicinchoninic Acid method (BCA) (PIERCE, Rockford, IL). (30-50) μ g of protein were separated on 12% to 15% Bis-Tris polyacrylamide gels (Bio-Rad, Hercules, CA) and then transferred to PVDF membranes (Bio-Rad). Protein membranes were incubated in blocking buffer [1x Tris-buffered saline, pH 7.5, 5% nonfat dried milk, 0.05% Tween 20] for 2 hour at room temperature, followed with anti-*p53* (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti- β -actin antibody (Sigma-Aldrich) for overnight at 4 °C. The membranes were washed with 1x Tris-buffered saline containing 0.05% Tween 20, incubated with horseradish peroxidase-linked goat anti-mouse Ig (Santa Cruz) or goat anti-Rabbit Ig (Cell Signaling) for 1 h at room temperature, washed, and visualized with the SuperSignal West Dura/ Femto Chemiluminescent

Substrate kit (PIERCE). The quantitation of protein expression levels was determined by ImageJ (<http://rsb.info.nih.gov/ij>) with arbitrary units (AU) reflecting the signal density from the blot.

Quantitative real-time PCR

We used TaqMan miRNA assays (Applied Biosystems Inc., Forest City, CA) to detect the expression level of mature miRNAs [62]. The quantification of mRNA was carried out using predesigned TaqMan Gene Expression Assay (Applied Biosystems Inc.). U6 snoRNA was used for normalization of miRNA levels and β -actin mRNA for *p53* mRNA levels.

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Figure 1.
miRNA expression quantification using qPCR in MM1.s cells treated with BaP or TCDD. (A) Schematic representation of gene structure of miR-25 cluster and its orthologs. (B) miRNA sequences of the three miRNA clusters. (C) miRNA expression in MM1.s treated with BaP or TCDD. miRNAs that have the same seed sequence (bold) are grouped together and color-coded.

Figure 2.

The promoter of miR-25 contains an AhRE site. (A) Mutagenesis analysis of miR-25 promoter. (B) A reporter assay in HeLa cells demonstrated that BaP and TCDD increased the promoter activities. (C) Inhibition of miR-25 induction in MM1.s cells by TCDD or BaP treatment when AhR or ARNT was knocked down. MM1.s cells were transfected with siAhR or siARNT and 24 hrs later, cells were treated with TCDD (10nM) or BaP (3μM) and miR-25/AhR/ARNT expression was determined after another 24 hrs. Y axis indicates the relative levels of miR-25 or AhR/ARNT mRNA levels with values normalized to that from

cells transfected with a siRNA negative control (siControl) and treated with TCDD/BaP.
“V” denotes a vehicle control (transfected with siControl and treated with DMSO).

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Figure 3.

Screening small molecules that inhibit miR-25 expression in MM cells. (A) Cell proliferation of MM1.s treated with 721 compounds at 1.0 μ M final concentration. (B) qPCR to measure the levels of miR-25 and p53 mRNA in treated MM1.s cells. (C) Western blotting to measure the p53 protein levels in treated MM1.s cells.

Figure 4.

EGCG inhibits the expression of miR-25. (A) qPCR to measure the expression of miRNAs in MM1.s treated with 1.0 μM EGCG (B) Reporter assay of the miR-25 promoter in HeLa cells treated with EGCG (C) miR-25 expression in MM1.s cells treated with BaP (3.0 μM) / TCDD (10 nM) and/or EGCG (5.0 μM). Y axis indicates the relative levels of miR-25 in MM cells with treatments normalized to that with mock treatment.

Figure 5.
Schematic representation of the potential role of BaP and miRNA overexpression in MM pathogenesis and that of EGCG in MM prevention and intervention.

Table 1

Overexpression of p53-targeting miRNAs in MM

<i>p</i> 53 3'UTR and miRNA ¹	Consequential pairing ¹	Seed Match ²	Overexpressed in MM	
			Yes or No	Reference
Pos. 93-98	5' CUUUGAACCCUUGCU-- UGCAAUA ::	7mer-1A		
hsa-miR-25	3' AGUCUGGCUCUGUUC ACGUUAC		Yes	[15,25]
<u>hsa-miR-92a</u>	3' UGUCCGGCCCUUGUUC ACGUUAU		Yes	
Pos. 287-292	5' UGCAGUUAAGGGUUAUUUACA 	7mer-1A		[15,25,26]
<u>hsa-miR-30d</u>	3' GAAGGUCAGCCCCUA--CAAAUGU		Yes	
Pos.734-739	5' AAGACUUGUUUUAUGC UCAGGGU 	7mer-m8		
<u>hsa-miR-125b</u>	3' AGUGUUCAAUCC CAGAGUCCCU		Yes	[25-27]
Pos. 736-741	5' GACUUGUUUUAUGCUCAGGGUCA 	8mer		
<u>hsa-miR-504</u>	3' CUAUCUCACGUCUGGUCC CAGA			
Pos. 1065-1070	5' CCACGUCCAGCUGGAAGGGUCA 		Yes	[26]
<u>hsa-miR-504</u>	3' CUAUCUCACGUCUGGUCC CAGA			
Pos. 804-809	5' UGGGUCUCGCUUUGUUGCCCAGG : :	7mer-m8		
hsa-miR-1285	3' UCCAGAGUGAAACAACGGGUCU			
Pos. 967-972	5' GGGGUCUCACAGUGUUGCCCAGG : :		No	Not reported
hsa-miR-1285	3' UCCAGAGUGAAACAACGGGUCU			
Pos. 1084-1089	5' GUCAACAUCUUUACA UUCUGCAA 	Low-complementary seed match	Yes	
<u>hsa-miR-15a</u>	3' GUGUUUGGUAAUAC ACGACGAU		Yes	[25]
<u>hsa-miR-16</u>	3' GCGGUUAUAAAUGC ACGACGAU			
Pos. 1161-1167	5' AGAGAUGGGGUCUCACAGUGUUG... 	7mer-m8	Yes	
<u>hsa-miR-200a</u>	3' UGUAGCAAUGGUCUG UCACAAU		Yes	[26,28]
<u>hsa-miR-141</u>	3' GGUAGAAAUGGUCUG UCACAAU			
3'UTR as reported	5' GTCAAGTCTCTGCTGGCCCAGCCAAAC			
hsa-miR-380-5p	3' CGCGUACAAGAUACCAGUUGGU			
3'UTR as reported	5' TGACAACCTCTTGGTGAACCTTAGTACCTAAA		No	Not reported
hsa-miR-380-5p	3' CGCGUACAAGAUACCAGUUGGU			

¹ Nucleotides in the *p*53 3'UTR are numbered starting from the one after the stop codon (NM_000546). Nucleotides complementary to the seed sequence (positions 2-7) of miRNAs are indicated. Position (pos.) numbers correspond to nucleotides that are complementary to the miRNA seed sequence (in bold). One miR-504 site overlaps with the miR-125b site.

²The 7mer-m8 site comprises the seed match supplemented by a Watson Crick match to miRNA nucleotide 8. The 7mer-A1 site comprises the seed match supplemented by an A across from miRNA nucleotide 1. The 8mer site comprises the seed match supplemented by both m8 and A1. “NO” “indicates that the seed match was abolished.”?, unconventional interaction. We could not find significant complementarity between miR-380-5p (also called miR-380*) and *p53* 3'UTR. miRanda and TargetScan algorithms miR-380-3p (also called miR-380) binding sites in *p53* 3'UTR but not at the sites reported by Swarbrick *et al* [19].

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Table 2

miRNAs regulated by BaP in MM1.s cells

Probe_ID	log2 (BaP/DMSO) *
hsa-miR-542-5p	3.7
hsa-miR-200a	2.4
hsa-miR-141	1.9
hsa-miR-125b	1.5
<i>hsa-miR-106b</i>	<i>1.2</i>
hsa-miR-25	1.1
hsa-miR-92	1.1
hsa-miR-15a	1.1
<i>hsa-miR-17</i>	<i>1.1</i>
hsa-miR-16	1.0
hsa-miR-768-3p	1.0
hsa-miR-10b	1.0
hsa-miR-26b	1.0
hsa-miR-768-5p	0.9
hsa-miR-594	0.9
hsa-let-7f	0.8
hsa-miR-183	0.7
hsa-miR-132	0.7
hsa-let-7a	0.6
hsa-miR-23b	-0.8
hsa-miR-222	-0.9
hsa-miR-29a	-1.3
hsa-miR-484	-1.4
hsa-miR-19b	-1.4
hsa-miR-19a	-2.6
hsa-miR-145	-2.7
hsa-miR-143	-3.1

* The log2 values of signal densities of miRNAs in MM1.s cells treated with BaP normalized to the vehicle control. miRNAs in bold are those target p53 and miRNAs in italic are those in the miR-106b~25 cluster or its orthologs but do not target p53.

Table 3

Compounds that inhibited miR-25 expression and upregulated p53 protein levels

Compounds	FDA indication	p53 relevance
Epigallocatechin gallate (EGCG)	Tropical use; Polyphenon E (>50% components are EGCG) is used to treated external genital warts	Activating p53 in multiple cell lines
Acarabose	None (Treatment of Type-2 diabetes in Europe & China)	None
Methylandrostenediol Flumazenil	None (Testosterone-like, body building) Treats drowsiness caused by sedative medicines (GABA antagonist)	None
Rifabutin	Antibiotic (tuberculosis and other bacteria)	None
Diazepam	Treats anxiety, panic attacks, insomnia, seizures	None
Tramadol	Treats pain in adults	None
Ampiroxicam	None (Approved in Japan; A prodrug to piroxicam, which is approved by FDA to treat osteoarthritis and rheumatoid arthritis)	None
Glimepiride	Treats type-2 diabetes	None