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Increased expression of MAD2 occurs in association with DNMT1 protein stabilization and aberrant CpG island promoter hypermethylation during carcinogenesis in immortalized bronchial epithelial cells

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Increased expression of MAD2 occurs in association with DNMT1 protein stabilization and aberrant CpG island promoter hypermethylation during carcinogenesis in immortalized bronchial epithelial cells.

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Introduction:

Physicians and scientists must join together and research the specific effects of environmental carcinogens in relation to cancer. A great model to study environmental carcinogenesis is carcinogen-induced lung cancer. We know that 30% of cancer deaths are accredited to lung cancer and that cigarette smoking is the number one cause of lung cancer, other forms of cancer, and cancer deaths worldwide (1). Second hand smoke is also a very serious health concern. Second hand smoke has been chosen as a cancer-causing agent by the U.S. Environmental Protection Agency, the National Toxicology Program and the International Agency for Research on Cancer (IARC) because this extremely deadly agent is known to contain hundreds of chemicals known to be toxic or carcinogenic (1).

There is a common misconception that smokers are the only people that get cancers associated with tobacco smoke. A serious tragedy associated with second hand smoke is its effects on America's youth. An astounding 60 percent of children in the United States ages 3-11 years are exposed to secondhand smoke (1). Because there are so many children in the U.S. being innocently exposed to tobacco smoke, this would lead one to conclude that there may possibly be a surge in lung cancer incidences in the future (1). Occupational workers are also at risk because 30 percent of indoor workers in the United States are not covered by smoke-free workplace policies (1). The research regarding tobacco carcinogens and the specific effects on lung cancer must be more greatly emphasized, however, because lung cancer is a disease where everyone is fair game, smoker or not. Therefore, lung cancer is a serious public health concern that must be addressed by both physicians and scientists. Novel treatments and early diagnosis could affect lung cancer mortality. Understanding how premalignancy progresses to lung cancer could aid efforts in both early detection and treatment.

In order to fully understand the complete deadliness of tobacoo smoke, one must start with examining its most dangerous components. Cigarette smoke has more than 50 agents known to cause cancer (2). Two of these cancer causing agents that are well

studied are polyaromatic hydrocarbons (PAHs) and tobacco-specific nitrosamines (TSNAs) (2). Both PAHs and TSNAs are known to create truly dangerous mutations in the cell. Some of these mutations included are the oncogene Kras and others are in the p53 gene (3-8). Of course, mutagenesis and chromosomal instability is important in the etiology of cancer, but perhaps it is not the only factor that should seriously be considered.

Epigenetic-mediated silencing of several genes implicated in cancer progression has become a major carcinogenic mechanism (9). Epigenetics are changes in gene expression that occur without altering the genomic sequence. Some of genes that have been found to be epigenetically silenced in lung cancer include classic tumor suppressor genes such as p16, cell-cell adhesion genes such as E-cadherin, etc. DNA methylation and histone modification are the best-studied epigenetic alterations studied in cancer. Furthermore, DNA methylation is the best-studied and most widely accepted epigenetic alteration that occurs in cancer. CpG island methylation occurs by removing a methyl group from S-adenosyl methionine (SAM) to produce 5-methylcytosine. This reaction is catalyzed by the cytosine DNA methyltransferases (DNMTs) to generate 5-methylcytosine in regions known as CpG islands. A CpG island is a small portion of DNA where the frequency of CG sequence is higher than in other parts of the genome. In addition, the “p” indicates a phosphodiester bond separating C and G. CpG island methylation is key in cancer because these islands are found around the promoters of “housekeeping genes” essential for general cell functions. Even though CpG islands are more often than not unmethylated in the promoter regions of these genes, the cytosine in CpG islands can become abnormally methylated in cancer by the DNMTs. The DNMTs that are best characterized in cancer are DNMT1, DNMT3A and DNMT3B. DNMT3A and DNMT3B are the de novo methyltransferases because they can methylate unmethylated DNA and DNMT1 functions as a maintenance methyltransferase because of its higher affinity for hemimethylated DNA, though there is evidence that DNMT1 can act as a de novo methyltransferase (10). It is known that the de novo DNMTs, DNMT3A and DNMT3B, are essential for embryonic development because disruption of these genes in mice is fatal (11). DNMTs contribute to epigenetic gene silencing by means other than CpG island promoter hypermethylation. The DNMTs also recruit chromatin remodeling (CR) complexes and histone deacetylase (HDAC) to the promoter region of

a gene. The entire scenario is as follows: when a gene is going through transcription, chromatin must be acetylated to allow entry for transcription factors. When abnormal promoter methylation of CpG islands in gene promoters occurs, transcriptional repressors known as methylcytosine-binding proteins (MBPs) binds the methylated DNA (12-14). The MBPs and the DNMTs make possible the recruitment of CR and HDAC, which altogether leads to histone deacetylation, chromatin compaction, and cessation of transcription because transcription factors cannot bind to the promoter region (12-14). Extensive studies support that genes are epigenetically silenced in lung cancer by CpG island promoter hypermethylation. The genes p16, p14, MGMT, DAPK, BRCA1, GSTP1 and TIMP-3 were all found to be silenced by CpG island promoter hypermethylation from lung primary tumor samples (15). Methylation of the p16, RASSF1A, and HCAD genes in sputum collected from 121 cancer-free women with an average smoking history of 40 pack years and a 25% incidence of COPD (16). In another study, several genes were found to be methylated in NSCLC cell lines, including MSX1, BNC1, CTSZ, ALDH1A3, CCNA1, LOX and SOX15 (17). These studies demonstrate that epigenetic alterations do occur in cancer.

While it is unknown what fundamentally switches a cell to a transformed phenotype, there is a lot of evidence that epigenetic deregulation in the cell is an early event in lung cancer (18). Hypermethylation of the p16 gene promoter was observed in 94% of NNK-induced rat adenocarcinomas (19). The methylation of p16 was detected in adenomas and hyperplastic lesions, which could mean that methylation of this gene is an early event in lung cancer (19). In another study, exposing mice to NNK and then bisulfite sequencing the DAPK promoter from both premalignant and malignant lesions revealed dense methylation in the DAPK promoter (20). Furthermore, this demonstrated that DAPK is inactivated at the earliest histological stages of adenocarcinoma development by epigenetic changes (20). In a recent study, the prevalence of methylation of the p16, DAPK and RASSF1A genes in lung adenocarcinoma from smokers, former uranium miners and never smokers was examined (21). Results from this study showed that there is a significantly higher prevalence for p16 methylation seen in central vs. peripheral lung tumors (21). At least 1 of the 3 genes was methylated in 35% of stage 1 tumors (21). Interestingly, 2 genes were methylated in 40% of tumors and 3 genes in 16% of tumors (21). A case-control study of lung cancer cases from a high-risk

population for evaluating promoter methylation of 14 genes in sputum was done and found 6 of 14 genes were associated with a 50% increased lung cancer risk (22). The 6 genes most prevalently methylated in people at risk with lung cancer were p16, MGMT, DAPK, GATA5, PAX 5β and RASSF1A – all truly important genes for normal cell function (22). Considering the evidence cited above, understanding the mechanisms between CpG island promoter hypermethylation and carcinogenesis is an important area in the medical and scientific field.

Although we know that, in lung cancer, genes appear methylated, exactly how mutagenic exposure leads to epigenetic alterations is truly a mystery. A key to finding this "missing link" can be seen in the DNA methyltransferases. DNMT1 is the best-studied and most abundant DNA methyltransferase. The key reason as to why DNMT1 could be a key player in mutagen-mediated epigenetic gene silencing is due to its affinity for damaged DNA. A recent study concluded that inflammation-induced 5-halogenated cytosine damage products could induce inappropriate CpG island methylation via DNMT1 (23). In another study, the investigators looked at the silencing of Cdc25C and Cdc2 in wild-type HCT116, HCT116 with DNMT1 knockout, and HCT116 with DNMT1 and DNMT3B knockout combined (24). The scientists showed, after mutagen exposure that resulted in p53 stabilization, both Cdc25C and Cdc2 expression was reduced in wild-type HCT116 cells but not in p53-null, DNMT1 knockout or DNMT1and DNMT3B knockout cells (24). Furthermore, trapping nuclear DNMT1 and DNMT3B relieved p53-mediated repression of Cdc25C and Cdc2 (24). These results concluded that de novo methylation was mediated epigenetically via p53. In another study, irradiated cells expressing GFP-tagged DNMT1, DNMT3A, and DNMT3B, exclusivey, with red fluorescent protein-tagged proliferating cell nuclear antigen (PCNA) revealed that DNMT1 and PCNA accumulate at DNA damage sites as early as 1 min after irradiation while DNMT3A and DNMT3B were not observed at these damage sites (25). Therefore, understanding the role of DNMT1 in carcinogenesis is a crucial area of investigation and previous studies warrant further examination on its role in mutagen-induced transformation.

The mechanism behind DNMT1 protein increases is unclear. Several studies have looked at the DNMT1 mRNA level but with inconclusive results. A recent study used

the MCF7 breast cancer cell line to show that the DNMT1 protein levels were increased, but not in relation to the DNMT1 mRNA levels (26). Furthermore, the increase in protein is a result of a deletion of a destruction domain in the N-terminal portion of the protein that is usually tagged for ubiquitination, but when it is deleted the protein is not degraded in its normal manner (26). Even though this paper did show that DNMT1 is increased through protein stabilization, and not mRNA overexpression, the mechanism as to why this is so is in itself unclear.

MAD2 has been implicated as a mediator for DNMT1 protein stabilization (27). MAD2 is a mitotic checkpoint mediator and inhibitor of CDC20. MAD2 can bind to CDC20 and inhibit its ability to recruit substrates to the anaphase-promoting complex (APC), thus preventing proteins that are targets of the APC from being properly degraded. Using Western Blot and immunohistochemistry, this study was able to show that MAD2 caused stabilization of DNMT1 in the MCF7 breast cancer cell line and that MAD2 is increased in several breast cancer tissues (27). Overexpression of MAD2 in transgenic mice leads to a wide variety of neoplasias, including appearance of broken chromosomes, anaphase bridges, and whole-chromosome gains and losses, as well as acceleration of myc-induced lymphomagenesis (28). Therefore, studying the relation between MAD2 overexpression and DNMT1 overexpression during transformation is quite an interesting idea and an important topic of this study investigation.

In this study, the mRNA levels of MAD2 are going to be examined. Although looking at mRNA levels is not always in correlation with protein expression, a protein will generally be expressed in correlation with the mRNA. Microarrays use mRNA and are a very accepted tool when determining gene expression. In addition, working with RNA does not require a lot of cells, unlike protein harvests for Western Blots, and does not depend on having a suitable antibody. Therefore, since the previous study did not observe MAD2 mRNA levels, it would be interesting to see if this transcript increases in cancer progression. Furthermore, it would be interesting to see if this transcript would increase early during cancer progression since methylation has been called an early event in cancer. Therefore, the relation between increases in DNMT1, MAD2 and transformation will be examined.

The current models studying transformation are limited, primarily due to the finite life span of bronchial epithelial cells (BECs). SV40 immortalized BECs (BEAS2Bs) have been well studied but are inadequate for a progressive transformation model due to their genomic instability. Recently, human bronchial epithelial cell lines (HBECs) were immortalized by insertion of the hTERT catalytic subunit and cyclin dependent kinase 4 (cdk4) (received from Dr. Jerry Shay, Southwestern Medical Center, Dallas, TX). HBECs can go through numerous passages, have an intact p53 checkpoint and do not suffer from the same chromosomal instability as BEAS2Bs (29). These cell lines, therefore, are really great for measuring progression to transformation.

The purpose of this study is to identify the effect that exposing human immortalized bronchial epithelial cell lines to polyaromatic hydrocarbons and nitrosamines, two different and prevalent types of DNA damage, has on the expression of DNMT1 protein and MAD2 mRNA, as well as gene-specific promoter hypermethylation of 31 candidate genes and cellular transformation. This study will focus on the relationship between appearance of CpG island promoter hypermethylation, appearance of transformation by growth in soft agar, alterations in the levels of DNMT1 and changes in MAD2 mRNA levels. These studies will demonstrate that carcinogen-induced transformation of immortalized bronchial epithelial cells occurs in concert with increase of DNMT1 protein, MAD2 mRNA and the number of aberrantly methylated genes implicated in cell cycle, growth control and adhesion.

Materials and Methods

Cell culture and carcinogen exposures

Human immortalized bronchial epithelial cells (HBECs) (received from Dr. Jerry Shay, Southwestern Medical Center, Dalla, TX) from two different people (HBEC1: smoker without lung cancer, HBEC2: smoker with cancer) were cultured in Keratinocyte Serum Free Media (KSFM) (Invitrogen, Carlsbad, CA) supplemented with EGF, bovine pituitary

extract and 1% penicillin/streptomycin (Invitrogen) and plates were coated with FNC (Athena Enzyme Systems, Baltimore, MD) to promote adhesion. During the 12-week carcinogen exposure study, HBECs were supplemented with an additional 15% fetal bovine serum to account for any possible nutritional changes the cells will acquire during carcinogenesis (Invitrogen). HBECs were passaged by washing cells twice with 1X phosphate buffered saline (PBS), harvested with 1X trypsin-EDTA (Cambrex Bio Science, Walkersville, MD), neutralized with trypsin neutralizing solution (TNS) (Cambrex Bio Science), and split 1:5 when cells reached 90% confluency. Benzo(a)pyrenediolepoxide (BPDE) (Midwest Research Institute's (MRI) NCI Chemical Respository) was stored in DMSO (Invitrogen) at 4°C in the dark. Methylnitrosourea (MNU) (AppliChem, Darmstadt, Germany) was stored in a dry powder form and resuspended in DMSO (Invitrogen) prior to treatment.

Cytotoxicity was assessed using an 3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay (Sigma, St. Louis, MO) according to the manufacturer's directions. HBECs were grown in triplicate in 6-well plates at a concentration of 5.0×10^5 cells/well 24 h prior to exposure. HBECs were exposed to BPDE at concentration of 0.05, 1, 0.25, and 0.5μM as well as DMSO control (1part/2000) in a total of 2 ml of media, in triplicate. HBECs were exposed to MNU at a concentration of 0.5, 1, 2.5 and 5mM MNU as well as DMSO (1part/2000) as a control in a total of 2 ml of media, in triplicate. For the combination carcinogen treatments, HBECs were exposed to 0.05μM BPDE/1mM MNU, 0.1μM BPDE/1mM MNU, 0.1μM BPDE/0.5mM MNU and 0.25μM BPDE/2.5mM MNU as well as DMSO control (1part/2000) in a total of 2ml of media, in triplicate. Cells were exposed to the carcinogens for 1 h at room temperature. After 1 h exposure, the media was removed, cells were washed twice using 1× PBS, and then fresh media was added. Cell viability using the MTT assay (Sigma) was used 24 h post carcinogen exposure. The color change was read on a colorimeter using a VersaMax plate reader (Molecular Devices) at 570nm. Percentage of viable cells was calculated as follows: ([Absorbance of treated sample]/[Absorbance of control]) × 100.

Growth in soft agar tumorigenicity assay

HBECs in culture exposed to carcinogens for 12 weeks were collected and counted using a hemocytometer. HBECs exposed to carcinogens for 12 weeks were suspended in 0.3% agar (Sigma) containing 15% FBS and KSFM media at 6.0×10^3 cells per well on a 6-well plate. The HBECs in 0.3% agar were placed onto an already hardened agar layer (0.7% agar with 15% FBS-containing KSFM media). All experiments were run in triplicate. Cells were growth at 5% CO_2 at 37°C for 21 days. Colonies were counted under a microscope. All colonies were averaged between the three wells run per exposure with standard deviations calculated using Microsoft Excel. Colonies were photographed using a digital camera-equipped microscope.

Semi-quantitative RT-PCR

Total RNA from cells was extracted using Trizol reagent (Sigma) as instructed by manufacturer. The cDNA was synthesized using 1μg of total RNA using Superscript First-Strand synthesis for RT-PCR (Invitrogen). Semi-quantitative RT-PCR was conducted as previously described (30). Primer sequences are available upon request.

Western blot analysis

Cells were harvested and protein extracts were prepared using the Nuclear and Cytoplasmic Extraction Reagents (NE-PER) (Pierce). Approximately 20 μg of nuclear protein extract was electrophoresed and transferred to PVDF membranes overnight at 4°C. The membranes were hybridized with antibodies against human DNMT1 (1:1000 dilution; New England Biolabs, Beverly, MA), beta-actin (1:1000 dilution, Sigma) and proliferating cell nuclear antigen (PCNA) (1:1000 dilution, Upstate Cell Signaling Solutions, Lake Placid, NY) in 1×Tris-buffered saline with Tween 20 (1×TBS-T) buffer (0.1 M Tris, 1.5 M NaCl, and 1% Tween 20) with 5% nonfat dry milk 2 h at room temperature. The membranes were washed three times for 5 min with 1×TBS-T buffer at

room temperature and incubated with secondary antibodies as follows: anti-mouse-IgG-HRP (1:10,000 dilution for PCNA and beta-actin; Calbiochem), anti-rabbit-IgG-HRP (1:10,000 dilution for DNMT1; Calbiochem). All secondary antibodies were incubated with the membrane for 30 min at room temperature. Afterwards, the membranes were washed three times for 10 min with 1×TBS-T at room temperature. Proteins were detected with the Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer) and by exposure to Blue basic autoradiography film, double emulsion (ISC Bioexpress).

Methylation-Specific PCR (MSP)

Nested MSP was used to detect methylated alleles in DNA. We used our nested MSP assay described previously (31) because of its increased sensitivity for the detection of promoter hypermethylation in biological fluids. In order to conserve DNA and effort, stage 1 multiplex PCR reactions amplifying four genes at once were done. DNA (~100ng) was used for stage 1 PCR following modification with bisulfite. A total of 31 genes were selected for methylation detection. Conditions for all stage 1 multiplexes were optimized through primer design and PCR conditions to achieve equal product intensity. These optimal conditions ensured a similar sensitivity for the detection of methylated alleles across genes in the stage 2 MSP assay. All stage 2 PCR reactions were conducted at annealing temperatures (68-70°C) that exceed the melting temperature of the primers to ensure the highest specificity for amplification of only methylated alleles present in the DNA sample. Cell lines positive and negative for methylation of these 14 genes and water blanks (bisulfite-modified and unmodified water) were used as controls for the MSP assays.

Immuno-slot-blot for N7-meG

DNA was isolated from cultured cells using the PureGene DNA extraction kit (Gentra Systems Inc., Minneapolis, MN, USA). Briefly, cell pellets were thawed and lysed in lysis buffer supplemented with 20 mM 2,2,6,6-tetramethylpiperidinoxyl (TEMPO; Aldrich). Protein was precipitated and removed by centrifugation, and the DNA/RNA in the supernatant was precipitated with isopropyl alcohol. The DNA/RNA pellet was

resuspended in lysis buffer with 20 mM TEMPO and incubated with RNase A (100 mg/ml) at 37°C for 30 min, followed by protein and DNA precipitation. The pellet was resuspended in sterilized distilled water with 2 mM TEMPO, and DNA was stored at –80°C. Experimental samples (1 µg) or a reference standard containing a known amount of N7-meG were denatured in 200 µl TE buffer plus 20 µl 2N NaOH at 37°C for 15 min. This treatment generates 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine (imidazole ring-opened 7-meG) from 7-meG. After addition of an equal volume of 2 M ammonium acetate, samples were applied to NC filters using a Minifold II (Schleicher & Schuell). The filters were soaked in 5x SSC (0.75 M NaCl, 75 mM sodium citrate, pH 7.0) for 15 min, dried and baked in a vacuum oven for 2 h at 80°C. Filters were incubated for 2 h at 37°C with blocking buffer [20 mM Tris–HCl (pH 7.5), 0.1 M NaCl, 1 mM EDTA, 0.5% casein and 0.1% deoxycholic acid], followed by a 2 h incubation at 37°C in blocking buffer containing antibody (1:10,000) to imidazole ring-opened 7-methylguanosine (Elder et al., 1998). The filters were washed extensively in washing buffer [20 mM Tris–HCl (pH 7.5), 0.26 M NaCl, 1 mM EDTA, 0.1% Tween-20] and treated with a labeled polymer (peroxidase-labeled polymer conjugated to goat anti-rabbit and goat anti-mouse immunoglobulins) (Daco) diluted 1:1000 in hybrid buffer. After rinsing the NC membrane, the enzymatic activity on the membrane was visualized by enhanced chemiluminescence reagents (Amersham). The NC filter was exposed to X-ray film, and the developed film was analyzed using a Kodak image analysis system.

Results

Effect of carcinogen exposure on viability of immortalized bronchial epithelial cell lines

In order to study the effect of carcinogen exposure on transformation of HBECs, it was necessary to identify a carcinogen exposure protocol that would be non-toxic. The carcinogens chosen, methylnitrosouea (MNU) and benzo(a)pyrenediolepoxide (BPDE), are direct-acting carcinogens derived from or generate reactive intermediates that mirror tobacco carcinogens (benzo(a)pyrene and NNK, respectfully). HBEC1 is from a smoker without lung cancer and HBEC2 is from a smoker with lung cancer. HBEC1 and HBEC2 were exposed to different amounts of MNU and BPDE (together and exclusively). The

cell viability was measured using an 3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay (Sigma, St. Louis, MO). All experiments were done in triplicate according to the manufacturer's instructions. The highest dose of carcinogen where viability was not statistically different from non-treated cells was the doses used for the subchronic exposure study. BPDE (0.1 μM) and MNU (1mM), did not effect cell viability whereas 0.25μM BPDE and 2.5mM MNU, exclusively, exhibited a reduced viability of approximately 30% (data not shown). When both BPDE and MNU were used to transform cells, the doses were reduced by half (0.05 μM of BPDE, 0.5mM MNU) (data not shown).

Once the dose of carcinogen was established, the time interval between exposures was defined. Cells were treated with MNU and the formation and removal of N7 methylguanosine adducts were determined using an immuno slot blot assay. N7-methylguanosine adducts were measured with a t1/2 for repair of adducts at approximately 48h (data not shown). Therefore, to avoid causing toxicity, cells were exposed to carcinogen once a week.

Transformation of immortalized bronchial epithelial cell lines

HBEC1 and HBEC2 were treated once a week for 12 weeks. After 12 weeks of exposure, cells were plated in soft agar. During carcinogen treatment, the cells began to assume a morphology resembling transformed cells (Fig. 1). Colony formation, indicative of cell transformation, was apparent in carcinogen treated HBEC1 and HBEC2, but significant differences in transformation efficiency were seen (Fig. 2). Four to 16-fold fewer colonies developed from carcinogen exposed HBEC1 than HBEC2 cells and no transformation was evident in HBEC1 cells treated with BPDE. HBEC1 produced fewer colonies overall than HBEC2. In addition, HBEC1 could not be transformed by BPDE while HBEC2 was transformed by this carcinogen. Colony formation in soft agar occurs from single, abnormal cells. Therefore, each colony represents one cell in the population of cells exposed to carcinogen that has been transformed. In the soft agar assay, 6.0×10^3 cells per well were plated in each well of a 6-well plate. All samples were run in triplicate. For the case of BPDE exposed to HBEC2 cells, 158 colonies

developed from the 1.8 ×10^4 cells that were seeded in the soft agar. This demonstrates that, when cells are exposed to non-toxic doses of carcinogens for an extended period of time, only a small population of cells obtains the transformed phenotype, mimicking the field cancerization effect seen in the lungs of smokers.

Detection of alterations in DNMT1 protein and MAD2 mRNA levels

We were next interested in looking at DNMT1 levels in correlation with mRNA expression for MAD2. Our results show that DNMT1 protein levels, a key contributor to epigenetic integrity in the genome, were increased in all the transformants. When it was determined that this protein was increased in transformants, relative to the non-transformed passage controls, the next question was to determine when the increase in DNMT1 protein occurred. There is approximately a 5-fold increase in DNMT1 between week 6 and week 9 in HBEC1 exposed to MNU and BPDE and then increased to 9-fold in the transformants (cells pulled from soft agar) (Figs. 3a and 3b). Although β-actin tends to overestimate the levels of DNMT1 increase at weeks 9 and 12, there is still an obvious increase in DNMT1, when normalized to PCNA.

Next, we looked at the mRNA levels for MAD2 during carcinogen exposure. The MAD2 mRNA levels increased in correlation with the increases in DNMT1 protein. Like DNMT1, MAD2 mRNA is increased in all transformants. In HBEC1 exposed to both MNU and BPDE, there is approximately a 30% increase in mRNA between week 6 and week 9, a 2-fold increase at week 12 and then a 5-fold in the transformants (Figs. 3c and 3d). Since MAD2 is an effecter of CDC20, we looked to see if there were decreases of CDC20 mRNA upon increases of MAD2 transcript. There were no changes in mRNA levels for CDC20 upon carcinogen exposure (data not shown).

Key genes are methylated in transformants

After observing the increases in DNMT1 protein, the next step is to examine genes potentially methylated in promoter as a result of carcinogen-induced transformation.

Table 1 lists the 31 candidate genes being studied. Table 2 shows genes that were methylated in HBEC1 and HBEC2, exclusively, prior to exposure. Table 3 shows the genes that became methylated in the transformed cells (directly pulled from soft agar and expanded in cell culture) as a result of carcinogen exposure. The data show genes involved in cell adhesion are being targeted for methylation. In each transformed cell line, at least 2 out of the 7 cell adhesion genes being analyzed are methylated. The p53 regulator, Reprimo, and transcription factor FOXA2, are found to be methylated in 2 out of the 5 transformed cells.

Association between methylation, increased DNMT1 protein, increased MAD2 mRNA levels and colonies in soft agar

The question as to the association between promoter hypermethylation of tumor suppressor genes, appearance of transformants in a population of cells exposed to carcinogen, DNMT1 protein and MAD2 mRNA levels was examined during carcinogen treatment. In order to determine an association between DNMT1 protein increases, appearance of aberrant methylation of genes (31 candidate genes studied) and transformation, we graphed all these parameters (Fig. 4). These data demonstrate a clear association between DNMT1 protein increase, appearance of aberrant gene methylation, MAD2 mRNA increases, and appearance of abnormal cells in a population. For HBEC1 exposed to MNU and BPDE, MAD2 mRNA levels began to increase as early as week 6. There was an increase in DNMT1 protein at week 6 following an increase to approximately 5-fold at week 9. Sparse colony growth also begins to appear at week 6 when the p16 exon 2 deletion was detected. Aberrant gene methylation was apparent at week 12 of exposure, in association with a significant increase of colony growth. These data demonstrate a clear association between DNMT1 protein levels, MAD2 mRNA increase, and aberrant gene promoter hypermethylation and colony formation in soft agar.

Conclusion

Our results demonstrate that epigenetic gene silencing of genes involved in cell signaling, growth and adhesion occurs during the transformation process and DNMT1 is most likely a critical player in this process. In conjunction with appearance of colonies in soft agar, one sees both an increase of appearance of methylated genes and increases in DNMT1, an indication of epigenetic changes in a cell. In correlation with this increases are increases in MAD2 mRNA levels, implicated in the stabilization of DNMT1.

The reason for DNMT1 overexpression could potentially be due altered levels in MAD2, though the increase in MAD2 is not likely implicated through the Rb pathway. This idea stems from the fact that MAD2 has a binding site for the E2F transcription factor. If there are increases in the E2F transcription factor, this will lead to increased transcription for MAD2. A problem for this scenario in the HBECs is that Rb is already disabled due to overexpression of CDK4, increased inhibition of Rb, and thus increases in free E2F. However, if E2F is the key regulator for MAD2 overexpression and Rb is already disabled, this would lead one to conclude that MAD2 should already be overexpressed and, potentially, we should see increases in DNMT1 in the HBECs and perhaps even transformation. Neither are the cases. We see increases in MAD2 mRNA levels upon carcinogen exposure, in conjunction with increases in DNMT1 protein appearances of colonies in soft agar. Regulation of MAD2 is most likely a complex process where deregulation of several key pathways is important in its aberrant expression. In a recent study, increased MAD2 expression was correlated with cellular sensitivity to cisplatin, which was associated with activation of the MEK pathway (32). Treatment of cells expressing high levels of MAD2 with an MEK inhibitor, U0126, led to cellular protection against cisplatin-induced apoptosis (32). Therefore, not only is MAD2 potentially sensitive to different methods of deregulation but it is also sensitive to carcinogen exposure. In another study, suppression of MAD2 increased sensitivity to DNA-damaging agents, indicating that MAD2 may be a key factor in regulating cellular response to DNA damage in cancer cells (33). Since the HBECs were exposed to DNA damaging agents, and DNMT1 is implicated in DNA damage, it would be logical to conclude that MAD2 would also increase in response to DNA damage while the

regulation of MAD2 gene expression during carcinogen exposure is most likely a complex topic worthy of further investigation.

These results are integral to the understanding of the etiology of cancer for several reasons. The HBECs resemble stem cells, making them great models for looking at cancer progression. In addition, we believe our transformation model accurately portrays field cancerization in vitro. For example, in HBEC1 exposed to MNU and BPDE, there were approximately 35 colonies counted out of a total of 18,000 cells (6000 cells per well, done in triplicate). Since one colony represents a single cell, only about 0.2% of the cells exposed to carcinogen became transformed. At week 6 of this treatment regiment, we saw the appearance of one methylated gene in conjunction with the p16/14 deletion. When a 5-fold increase of protein was seen at week 9 in conjunction with increases in colonies and number of methylated genes with a continuous trend into week 12 and the transformants, one becomes curious as to the reason for the changes over time.

The reason for this increase of DNMT1, MAD2 and methylation during carcinogen treatments could most plausibly be clonal expansion. Cells were plated sparsely so they could have a greater probablility of being damaged several times with carcinogen (cells were dosed once a week for 12 weeks). Since cells were so sparsely plated, cells (treated and controls) did not need to be passed too much. In addition, cells had a difficult time growing in weeks 2-5 of the treatments. Around week 6 is when most cells started to grow better. In the cell culture flask, one could observe fast-growing cells with fibroblastic-like morphologies emerging in a now heterogeneous-appearing cell population. As the fibroblastic-appearing cells started to overtake the normal-appearing cell population, as observed during treatments, the increases in number of methylated genes, DNMT1 protein levels and number of colonies in soft agar increased. During the end of treatment, it would appear as if all the cells appeared fibroblastic although, interestingly, only a small percentage would actually grow in soft agar. Of note, all the cells appeared fibroblastic after treatment except the HBEC1s, which maintained their normal morphology. However, when the colonies from HBEC1 exposed to MNU were pulled, the cells appeared fibroblastic while the HBEC1 exposed to both MNU and BPDE retained its normal morphology. Therefore, the description above supports the notion

that the increases in DNMT1 and methylation are attributed to emergence of the single, or few, transformed cells in a population and the increase occur over weeks because the transformed cells overtook the normal cell population.

When trying to inject cells that grow in soft agar into nude and SCID mice, exclusively, no tumors formed. These results lead us to the conclusion that further steps must be taken in order for a cell to exhibit a full transformed phenotype. However, it appears that increases in DNMT1 and CpG island promoter hypermethylation –epigenetic alteration – occur early in transformation. There were no mutations found in Kras and no changes in p53 protein expression (data not shown).

These studies combined demonstrate compelling evidence for the importance of epigenetic alterations in cancer progression. Furthermore, more research on therapeutics should targets mechanisms that aberrantly alter epigenetic pathways in cancer because this is clearly a system that drives carcinogenesis.

Limitations/Alterative Strategies

1. No protein data for MAD2. This Western was not done in order to support the notion that mRNA data was sufficient for analyzing MAD2 expression in cancer progression. However, an alternative strategy would have been to do the Western blot in order to match the mRNA data. The experiment was attempted, but the antibody did not work properly and did not produce any bands on the membrane. Although this same antibody was cited in other publications, the lot number was new.

2. Real time RT-PCR. Semi-quantitative RT-PCR was done instead of real-time RT PCR for the following reasons. Taqman real-time RT-PCR primers are very expensive. In addition, it is the experience of the Belinsky laboratory that Sybr-green does not provide much more accurate quantitation than semi-quatitative RT- PCR and band

quantification. An alternative strategy, however, would have been to examine the expression of MAD2 using a real-time RT-PCR technique.

3. Beta-actin normalizer for MAD2 expression. In this study, we used only beta-actin as the control and not PCNA. We decided to only use beta-actin because, when looking at DNMT1 protein, using either PCNA or beta-actin was sufficient to show increases in expression. An alternative strategy would have been to use PCNA as a normalizer to control for cell proliferation.

Future Directions

1. RNA interference on MAD2 to look at its effect of DNMT1 expression and transformation in lung cancer cell lines. Upon decreases in MAD2, one would expect to see DNMT1 protein stabilization to be reduced. In addition, transformation may be reduced in transformed cells in conjunction with loss of aberrant gene promoter hypermethylation and re-expression.

2. Overexpression of MAD2 to look at its effect of DNMT1 expression and transformation in lung cancer cell lines. Upon elevations of MAD2, one would expect to see DNMT1 protein to increase in stability. In addition, transformation may occur in non-transformed cells in conjunction with aberrant gene promoter hypermethylation and silencing.

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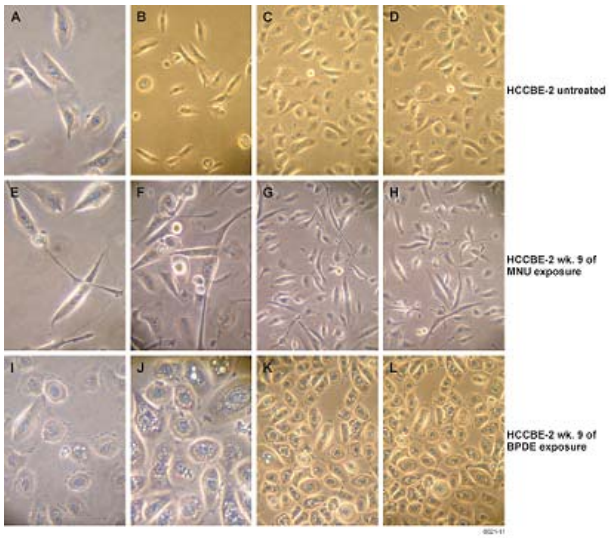

Fig. 1: Cell morphology data in HCCBE-2. A. Untreated cell, 20X, 50% confluence B. Untreated cell, 10X, 50% confluence C. Untreated cell, 10X, 80% confluence D. Untreated cell, 10X, 80% confluence E. Cell exposed to 1 mM MNU once a week for 9 week, 20X, 50% confluence F. Cell exposed to 1 mM MNU once a week for 9 week, 20X, 80-90% confluence G. Cell exposed to 1 mM MNU once a week for 9 week, 10X, 80-90% confluence H. Cell exposed to 1 mM MNU once a week for 9 week, 10X, 80-90% confluence I. Cell exposed to 0.1 µM BPDE once a week for 9 week, 20X, 50% confluence J. Cell exposed to 0.1 µM BPDE once a week for 9 week, 20X, 90-100% confluence K. Cell exposed to 0.1 µM BPDE once a week for 9 week, 20X, 90-100% confluence L. Cell exposed to 0.1 µM BPDE once a week for 9 week, 20X, 90-100% confluence

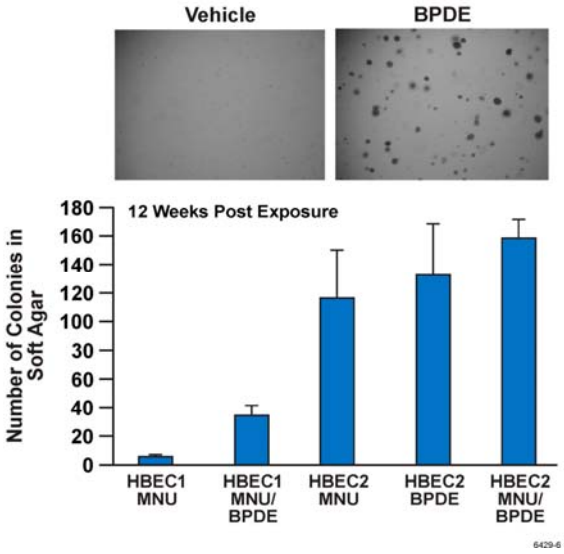

Fig. 2. Transformation of HBECs following subchronic carcinogen exposure. Top panel demonstrate HBEC2 colony formation of cells exposed to vehicle for carcinogen (DMSO) once a week for 12 weeks and to BPDE once a week for 12 weeks. The bottom panel demonstrates number of colonies in soft agar (N = 3) in both HBEC1 and HBEC2.

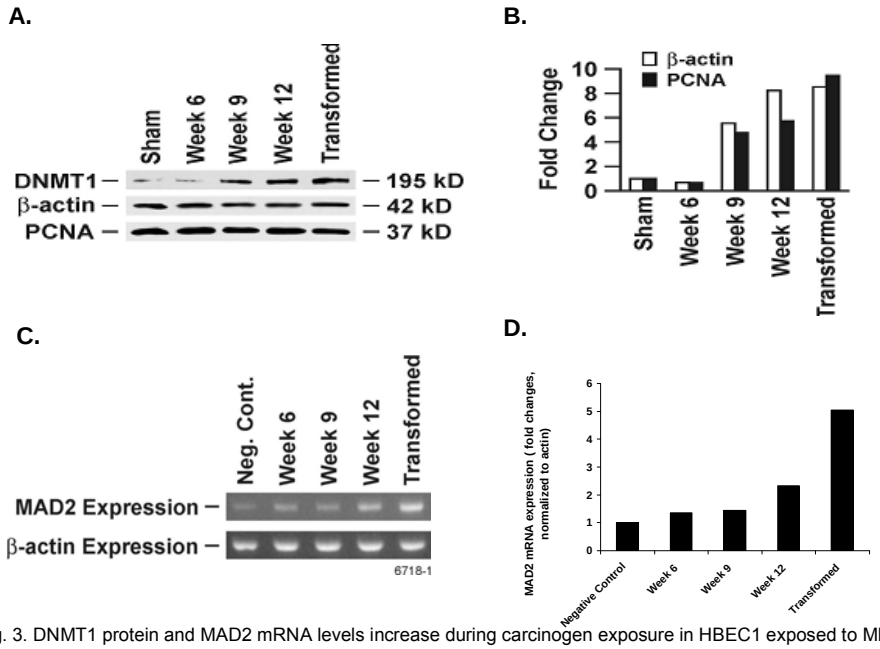

Fig. 3. DNMT1 protein and MAD2 mRNA levels increase during carcinogen exposure in HBEC1 exposed to MNU and BPDE. A. DNMT1 Western blot, B. Protein fold changes normalized to both actin and PCNA, C. MAD2 mRNA RT-PCR and D. MAD2 mRNA quantitation, normalized to actin.

Table 1. Gene Evaluated for Promoter Hypermethylation in Transformed HBECs

- GATA4
- GATA5
- FOXA2
- BETA3
- REPRIMO
- IGFBP3
- PGR
- AP2 α
- ECAD
- HCAD
- PCDH10
- TSLC1
- DAL1
- LAMC2
- TUBB4
- DAPK
- RASSF1A
- RASSF2A
- DCR1
- DCR2
- SFRP1
- APC
- DAB2
- AK5
- 3-OST-2
- MGMT
- PAX5 α
- PAX5 β
- p16
- Novel 2
- XT3

Table 2. Genes Methylated Prior to Carcinogen Exposure in Immortalized Cell Lines

HBEC 1	HBEC2
GATA4	GATA4
DCR1	DCR1
	RASSF2A
	PGR

Table 3. Gene Methylation In Transformed Cells

HBEC1	HBEC 2	HBEC2	HBEC1	HBEC2
MNU		BPDE	MNU/BPDE	
HCAD	HCAD	HCAD	HCAD	
ECAD	ECAD	ECAD		ECAD
PAX5 α	PAX5 α	PAX5 α		PAX5 α
XT3	XT3	XT3		XT3
PCDH10		PCDH10	PCDH10	
PAX5ß		PAX5ß		
RASSF2A			RASSF2A	
IGFBP3				
	REPRIMO	REPRIMO		
	BETA3	BETA3		BETA3
	DAL1	DAL1		DAL1
		FOXA2	FOXA2	
			DCR2	

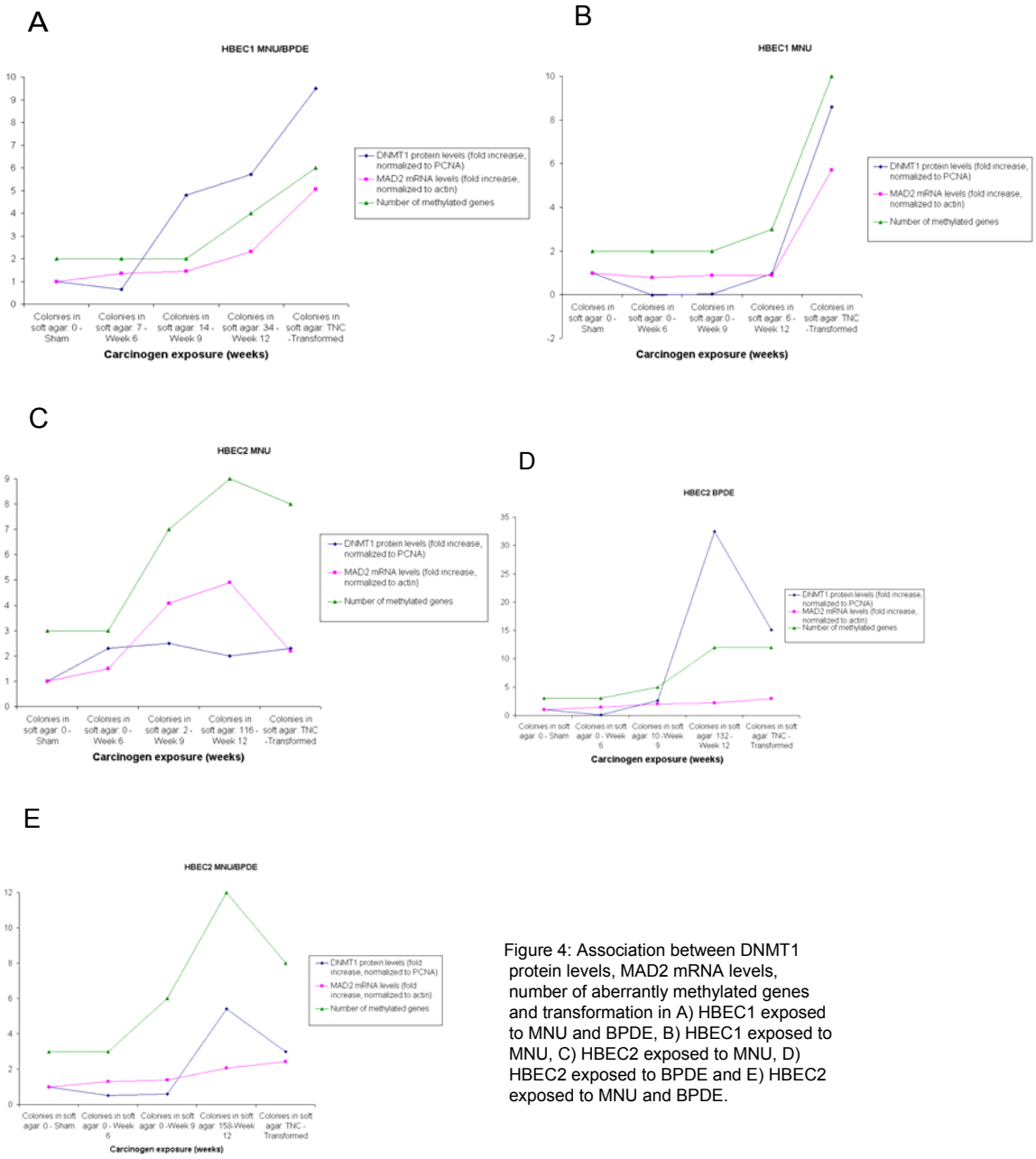

Figure 4: Association between DNMT1 protein levels, MAD2 mRNA levels, number of aberrantly methylated genes and transformation in A) HBEC1 exposed to MNU and BPDE, B) HBEC1 exposed to MNU, C) HBEC2 exposed to MNU, D) HBEC2 exposed to BPDE and E) HBEC2 exposed to MNU and BPDE.