Overexpression of *hMTH1* mRNA: a molecular marker of oxidative stress in lung cancer cells

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Abstract Human *MutT* homologue (*hMTH1*) mRNA was overexpressed in SV-40-transformed non-tumorigenic human bronchial epithelial cells (BEAS-2B cells) and in 11 out of 12 human lung cancer cell lines relative to normal human bronchial epithelial cells. Expression levels of *hMTH1* mRNA were inversely proportional to cellular levels of 8-oxo-deoxyguanosine. Together, these results suggest that *hMTH1* gene expression may represent a molecular marker of oxidative stress that could ultimately be used to elucidate the temporal relationships between oxidative stress, genomic instability and the development of lung cancer.

Key words: Human *MutT* homologue; Gene expression; Lung cancer; Molecular marker; Oxidative stress; 8-Oxo-deoxyguanosine

1. Introduction

Reactive oxygen species (ROS) such as superoxide and hydrogen peroxide are continually formed in vivo by normal cellular metabolism. Under normal conditions, the cell is able to detoxify ROS via a number of pathways that utilize chemical antioxidants (e.g. ascorbate, glutathione, α-tocopherol and uric acid) and/or antioxidant enzymes (e.g. catalase, glutathione peroxidase and superoxide dismutase). Following exposure to exogenous toxicants such as air pollution, cigarette smoke or radiation, cellular redox homeostasis may be disrupted, resulting in oxidative stress, a condition characterized by an increased flux of ROS [1]. At elevated levels, ROS may act as endogenous carcinogens by the following mechanisms: (1) induction of oxidative DNA damage resulting in base-pair mutations or chromosome aberrations; (2) activation of cytoplasmic or nuclear signal transduction pathways; and (3) modulation of stress genes that control effector genes related to cellular proliferation, differentiation and apoptosis [2].

One specific modification of DNA linked to ROS is the formation of 8-oxo-deoxyguanosine adducts (8-oxo-dG, also known as 8-hydroxy-deoxyguanosine) resulting from attack of either a hydroxyl radical or singlet oxygen on deoxyguanosine [3]. Significantly elevated levels of 8-oxo-dG have been detected in carcinomas of the breast [4], lung [5,6] and kidney [7]. These data provide support for the hypothesis that DNA of certain cancers is persistently exposed to a higher level of oxidative stress than adjacent normal tissue [8]. However, the use of 8-oxo-dG as a biomarker of oxidative stress has been challenged because of the lack of consistency in its measurement; levels detected in normal human cells have ranged from < 0.1 to > 200 8-oxo-dG per 10^6 base pairs [9]. Therefore, a more reliable molecular marker of oxidative stress in cancer is needed.

Several enzymes have been identified in *E. coli* that act to either remove 8-oxo-dG from DNA (MutM, MutY) [10] or prevent incorporation of the oxidized free nucleotide (MutT) [11]. Incorporation of 8-oxo-dG into DNA results in A:T to C:G transversion mutations following DNA replication; the bacterial *mutT* gene encodes an enzyme with 8-oxo-GTPase activity that hydrolyzes 8-oxo-dGTP to 8-oxo-dGMP [11]. The human homologue of *mutT*, termed *hMTH1*, has been cloned and localized to chromosome 7p [12]. To date, there has only been one report in the literature relating *hMTH1* expression and cancer; Okamato et al. [13] detected elevated levels of *hMTH1* mRNA in human renal cell carcinomas relative to adjacent non-tumorous kidney tissue. Further, they found that advanced-stage tumors exhibit significantly higher levels of *hMTH1* mRNA relative to early-stage tumors, suggesting the level of oxidative stress in renal cell carcinomas increases with the stage of the disease. The purpose of the current investigation was to extend the studies of Okamoto et al. [13] to determine the relationship between *hMTH1* mRNA expression and 8-oxo-dG levels in lung cancer.

2. Materials and methods

2.1. Cell culture

Normal human bronchial epithelial (NHBE) cells (strains 2129 and 4501) and bronchial epithelial cell growth medium (BEGM) were purchased from Clonetics (San Diego, CA). Non-tumorigenic human bronchial epithelial cells immortalized by transfection with SV40 virus (BEAS-2B cells) were obtained from Dr. Curtis C. Harris at the National Cancer Institute. The following lung cancer cell lines were purchased from ATCC (Rockville, MD): A427 (adenocarcinoma, AC); A549 (AC); Calu-3 (AC); SKLU-1 (AC); Calu-6 (anaplastic carcinoma, APC); Calu-1 (epidermoid carcinoma, EC); NCI-H82 (small cell lung cancer, SC); NCI-H128 (SC); NCI-H209 (SC); NCI-H520 (squamous cell carcinoma, SCC); and SW900 (SCC). NHBE and BEAS-2B cells were grown in BEGM. All lung cancer cell lines were grown in RPMI 1640 (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), 100 U/ml penicillin (Sigma, St. Louis, MO), and 0.1 mg/ml streptomycin (Sigma).

2.2. Total RNA isolation

Cells grown in monolayer were expanded to approximately 90% confluence. Growth medium was removed and the cells were lysed directly in the culture flask by treatment with TRI REAGENT (Molecular Research Center, Cincinnati, OH). Cells grown in suspension

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were sedimented prior to lysis. The resulting cell lysates were snap frozen in liquid nitrogen and stored at −80°C. Total RNA was isolated from the lysates by a standard method [14].

2.3. Preparation of an hMTH1 cDNA probe

Total RNA from NHBE cells was reverse-transcribed using the SuperScript Preamplification System for First Strand cDNA Synthesis (Gibco BRL). Polymerase chain reaction (PCR) primers corresponding to bases 97–116 (5′-AGCCTCAGCGAGTTCTCCTG-3′) and 248–266 (5′-GATCTGGCCCACCTTGTGC-3′) of the published *hMTH1* cDNA sequence [15] were purchased from Southwest Scientific Resources (Albuquerque, NM). A GeneAmp PCR reagent kit and AmpliTaq Gold were purchased from Perkin Elmer (Foster City, CA). A 170 bp amplimer was prepared from 3 μg first strand cDNA. The amplimer was isolated by electrophoresis, ligated into the pCR2.1 vector and cloned using the TA cloning kit (Invitrogen, Carlsbad, CA). Dideoxy sequencing confirmed that the DNA sequence of the insert was homologous to the published sequence of *hMTH1* [15]. For Northern analysis, 100 ng of *hMTH1* cDNA was labeled with α-^{32}P-dCTP (Amersham, Arlington Heights, IL) using the Random Primed DNA labeling kit (Boehringer Mannheim, Indianapolis, IN). Unincorporated α-^{32}P-dCTP was removed by filtration through a Bio-Spin 30 chromatography column (Bio-Rad, Hercules, CA).

2.4. Northern analysis of hMTH1 mRNA expression

Northern blots were prepared by a standard protocol [16] using Zeta-Probe nylon membranes (Bio-Rad). The blots were pre-hybridized in a constant temperature shaking water bath at 65°C for 4 h in 100 ml of 250 mM sodium phosphate buffer (pH 7.2) containing 7% (w/v) sodium dodecyl sulfate (SDS). The blots were then hybridized overnight with the ^{32}P-labeled *hMTH1* cDNA probe, and washed at room temperature for 1 h in 1000 ml of 25 mM sodium phosphate buffer (pH 7.2) containing 1% (w/v) SDS. Autoradiographs were prepared by placing the blots on Kodak BioMax film at −80°C. *GAPDH* cDNA probe was generously provided by Dr. William A. Palmisano. Probe labeling and hybridization were as described except that a second wash was performed at 65°C for 1 h. Autoradiographs were scanned into a PC using a flatbed scanner and analyzed using QuantiScan software (Biosoft, Cambridge, UK).

2.5. DNA isolation, enzymatic digestion and analysis of 8-oxo-dG

Cells grown in monolayer were expanded to approximately 90% confluence. Medium was removed and the cells were washed twice with HBS. NHBE cells were dissociated by adding 0.5 M urea, washing with HBS, and then adding E-PET (BRFF, Ijamsville, MD) [17]. BEAS-2B cells and lung cancer cells were dissociated by treatment with trypsin (Sigma). Dissociated cells and cells grown in suspension were sedimented by centrifugation, washed once with HBS, snap frozen in liquid nitrogen and stored at −80°C. DNA was isolated from the cell pellets using a Genomic DNA preparation kit (Qiagen, Valencia, CA) and precipitated with two volumes of 100% ethanol. The precipitated DNA was washed twice with 70% ethanol, dried under vacuum, solubilized in deionized water, and quantified by UV spectroscopy. The DNA samples were digested with 20 μg nuclease P1 (Sigma) in 20 mM sodium acetate buffer (pH 5.2) at 37°C for 30 min and then treated with 1.3 units of *Escherichia coli* alkaline phosphatase (Sigma) in 100 mM Tris-HCl (pH 7.5) at 37°C for 60 min [18]. The resulting nucleotide mixture was filtered through a Nalgene syringe filter (4 mm) and analyzed by HPLC-ECD using a standard analytical method [19]. Instrument specifications and conditions: HPLC, Hewlett Packard 1090; UV detector, Hewlett Packard 1040 diode array detector; UV wavelength, 254 nm; EC detector, ESA Coulochem model 5100A; ESA 5020 guard cell; EC cell, ESA 5010 analytical cell; column, Spherisorb ODS2, 5 μm, 25 cm×4.6 mm; solvent, 92% (v/v) 50 mM potassium phosphate (pH 5.5)/8% (v/v) methanol; flow rate, 1 ml/min. The amount of deoxyguanosine (dG) was measured by UV absorbance at 245 nm while the amount of 8-oxo-dG was simultaneously measured by the ECD. The level of 8-oxo-dG and dG in each DNA sample was determined by peak height comparison following injection of known quantities of 8-oxo-dG (Cayman Chemicals) and dG (Sigma) standards.

Table 1
Amount of 8-oxo-deoxyguanosine in human lung cells

Cell line	Pathology[a]	8-oxo-dG/10^5 dG[b]
NHBE, strain 2129	N	6.35
NHBE, strain 4501	N	4.84
BEAS-2B	INT	2.68
A427	AC	2.21
A549	AC	4.00
Calu-3	AC	3.22
SKLU-1	AC	2.72
Calu-6	APC	3.49
Calu-1	EC	3.47
NCI-H82	SC	1.04
NCI-H128	SC	3.46
NCI-H520	SCC	3.00
SW900	SCC	3.56

[a]Abbreviations for tumor pathology: N, normal; INT, immortalized non-tumorigenic; AC, adenocarcinoma; APC, anaplastic carcinoma; EC, epidermoid carcinoma; SC, small cell lung cancer; SCC, squamous cell carcinoma.
[b]The number of 8-oxo-deoxyguanosine residues per 10^5 normal deoxyguanosine residues was determined by high pressure liquid chromatography with electrochemical detection (HPLC-ECD).

3. Results

Expression levels of *hMTH1* mRNA and *GAPDH* mRNA were measured by Northern analysis in NHBE cells, BEAS-2B cells and 12 lung cancer cell lines (Fig. 1). The numbers shown in Fig. 1 indicate the ratio of *hMTH1* mRNA to *GAPDH* mRNA relative to the ratio calculated for NHBE cells. Elevated levels of *hMTH1* mRNA were detected in BEAS-2B

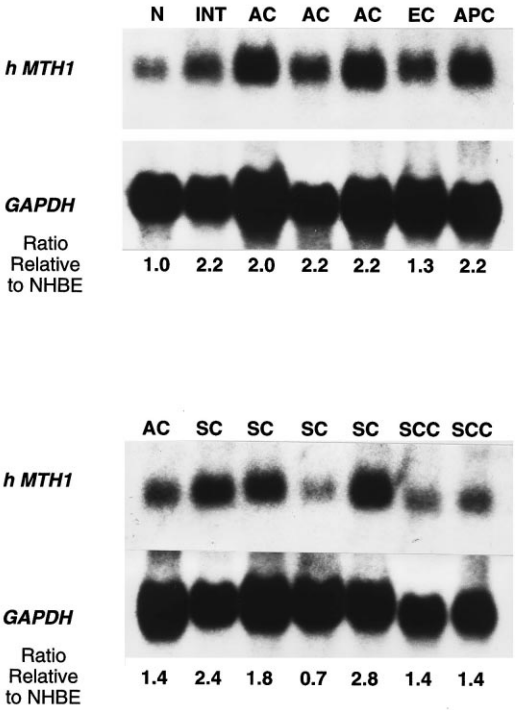

Fig. 1. Northern analysis of *hMTH1* mRNA expression in NHBE, BEAS-2B, and human lung cancer cells. Relative Ratio=ratio of *hMTH1* mRNA to *GAPDH* mRNA relative to this ratio determined for NHBE cells. Abbreviations for tumor pathology: N, normal; INT, immortalized non-tumorigenic; AC, adenocarcinoma; EC, epidermoid carcinoma; APC, anaplastic carcinoma; SC, small cell lung cancer; SCC, squamous cell carcinoma. Identity of cells: top panel (left to right) NHBE, BEAS-2B, SKLU-1, Calu-3, A427, Calu-1, and Calu-6; bottom panel (left to right) A549, NCI-H82, NCI-H128, NCI-H146, NCI-H209, SW900, and NCI-H520.

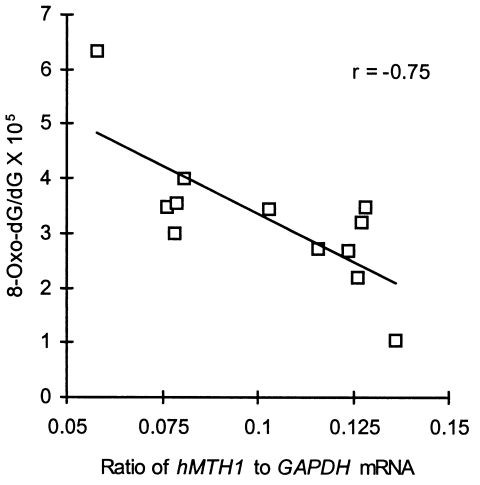

Fig. 2. Relationship between 8-oxo-deoxyguanosine levels and *hMTH1* expression for human lung cancer cells.

cells and 11 out of 12 lung cancer cell lines. One small cell lung cancer line (NCI-H146) exhibited a lower *hMTH1* to *GAPDH* mRNA ratio than NHBE cells. Consistent elevated transcription of the *hMTH1* gene was demonstrated by probing a second set of Northerns that were blotted with RNA from eight of the same cell lines and four different cell lines (data not shown); *hMTH1* mRNA was overexpressed in 12 out of 12 lung cancer cell lines (four adenocarcinomas, one anaplastic carcinoma, one epidermoid carcinoma, and six squamous cell carcinomas). Overall, the gene was overexpressed in 94% (15 out of 16) of lung cancer cell lines evaluated.

Levels of 8-oxo-dG were measured by HPLC-ECD in nucleoside mixtures prepared from DNA of two strains of NHBE cells (2129, 4501), BEAS-2B cells and 10 lung cancer cell lines. The results (Table 1) are expressed as the number of 8-oxo-dG residues per 10^5 normal deoxyguanosine (dG) residues. The NHBE cells exhibited higher levels of 8-oxo-dG than BEAS-2B cells and all 10 lung cancer cell lines. The average value for NHBE cells was 5.60 ± 0.75 (mean $\pm$ absolute deviation, $n = 2$). The average value for the lung cancer cell lines was 3.02 ± 0.85 (mean $\pm$ standard deviation, $n = 10$). However, the use of only two different strains of NHBE cells precludes the ability to ascribe statistical significance to these data. Values of 8-oxo-dG/10^5 dG (Table 1) were plotted against *hMTH1* to *GAPDH* mRNA ratios (Fig. 1) to determine whether there is a relationship between total 8-oxo-dG levels and *hMTH1* mRNA expression. Fig. 2 shows that an inverse linear relationship ($r = -0.75$) exists between these parameters.

4. Discussion

The hypothesis of persistent oxidative stress in cancer states that DNA of certain cancers is persistently exposed to a higher level of oxidative stress than that of adjacent normal tissues [8]. Okamoto et al. [13] have provided support for this hypothesis by showing that *hMTH1* mRNA is overexpressed in renal cell carcinomas and that transcription of this gene increases with clinical stage of the disease. The present study extends their work to lung cancer. We have measured expression levels of *hMTH1* mRNA relative to *GAPDH* mRNA in NHBE cells, BEAS-2B cells, and 12 lung cancer cell lines (Fig. 1). Since elevated levels of *GAPDH* mRNA have been detected in human lung cancer tissues [20], the use of *GAPDH* as a marker when comparing normal and cancer cells results in a conservative estimate of *hMTH1* mRNA overexpression. Our results show that *hMTH1* mRNA levels are elevated in human lung cancer cell lines (Fig. 1), suggesting that the level of oxidative stress is indeed elevated in lung cancer cells relative to NHBE cells. Further, the fact that *hMTH1* is overexpressed in BEAS-2B cells, an SV40-immortalized non-tumorigenic human bronchial epithelial cell line, suggests the exciting possibility that oxidative stress may be a characteristic of pre-malignant cells.

Levels of 8-oxo-dG were higher in two strains of NHBE cells than in the BEAS-2B cells and in all 10 of the lung cancer cell lines analyzed (Table 1). This is in contrast to previous studies where elevated levels of 8-oxo-dG were detected in primary lung tumor samples [5,6]. The disparity between these results may be due to overestimation of 8-oxo-dG levels in the primary tumors due to the GC/MS method used in these two studies, but it is impossible to confirm this possibility since the HPLC-ECD method has not been used to measure 8-oxo-dG levels in primary human lung tumors. However, the HPLC-ECD method has been used by Barciszewski et al. [21] to measure 8-oxo-dG levels in normal and SV40-immortalized human fibroblasts in culture; the transformed cells were reported to have 2.1 residues of 8-oxo-dG per 10^5 dG residues while the normal cells had five-fold higher levels of 8-oxo-dG. Our data with BEAS-2B cells and NHBE cells show similar results (Table 1); the level of 8-oxo-dG in BEAS-2B cells is lower than in either of two strains of NHBE cells. In contrast, levels of 8-oxo-dG determined by the GC/MS method in HPV-immortalized human bronchial epithelial cells [22,23] are more than an order of magnitude higher than either our values or those of Barciszewski et al. [21]. These differences suggest that either the GC/MS method overestimates 8-oxo-dG levels in cultured cells or that the HPLC-ECD method underestimates these levels [24].

The fact that *hMTH1* mRNA expression is 2.2-fold higher in BEAS-2B cells compared to NHBE cells (Fig. 1) suggests that the differences in 8-oxo-dG levels between these cells (Table 1) can be ascribed to higher DNA repair efficiency of the immortalized cells. This was the conclusion drawn by Barciszewski et al. [21] after observing: (1) lower levels of 8-oxo-dG in DNA from immortalized fibroblasts compared to normal fibroblasts; and (2) no difference in the amount of 8-oxo-dG in culture media collected from normal and transformed fibroblasts. Instead of decreased misincorporation of 8-oxo-dGTP into DNA, their results seem to suggest that the efficiency of removal of 8-oxo-dG from DNA is enhanced, perhaps via up-regulation of human homologues of *mutM* and *mutY* genes [10]. However, measurement of 8-oxo-dG in culture media does not differentiate between 8-oxo-dGTP and 8-oxo-dGMP, the substrate and product of the hMTH1 enzyme. Therefore, 8-oxo-dG can only be used as a marker of oxidative DNA damage and not as an indicator of the efficiency of DNA repair.

A plot of 8-oxo-dG values against *hMTH1* expression (Fig. 2) shows the relationship between these two parameters in human lung cancer cell lines. The plot shows an inverse linear correlation between these parameters, suggesting that up-regulation of *hMTH1* gene expression protects the integrity of

cancer cell DNA by preventing misincorporation of 8-oxo-dGTP into DNA, thereby reducing the detectable level of 8-oxo-dG. These results are in agreement with Okamoto et al. [13] who found that *hMTH1* mRNA expression increased with clinical stage of renal cell carcinoma while 8-oxo-dG levels remained relatively constant [7]. Thus, the level of oxidative stress in renal cell carcinomas increases with the clinical stage of the disease; however, the cells apparently up-regulate expression of *hMTH1* in response to increased oxidative stress and thereby keep 8-oxo-dG levels relatively constant throughout the course of the disease. Our results suggest that the same type of protective mechanism is active in BEAS-2B cells and lung cancer cell lines.

Future work in our laboratory will examine the association between *hMTH1* mRNA levels, hMTH1 protein levels, 8-oxo-dGTPase activity and 8-oxo-dG levels in NHBE cells and lung cancer cell lines. Ultimately, *hMTH1* expression will be used to investigate the temporal relationship between oxidative stress, genomic instability and the development of lung cancer.

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