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Deficient repair of 8-hydroxyguanine in the BxPC-3 pancreatic cancer cell line

Simon G. Nyaga, Althaf Lohani, and Michele K. Evans*

Laboratory of Cellular and Molecular Biology, National Institute on Aging, National Institutes of Health, Biomedical Research Center, 251 Bayview Boulevard, Baltimore, MD 21224, USA

Abstract

Elevated levels of oxidatively induced DNA lesions have been reported in malignant pancreatic tissues relative to normal pancreatic tissues. However, the ability of the pancreatic cancer cells to remove these lesions has not previously been addressed. This study analyzed the effectiveness of the pancreatic cancer cell line, BxPC-3 to repair 8-hydroxyguanine (8-OH-Gua) relative to a nonmalignant cell line. We show that BxPC-3 cells repair 8-OH-Gua less effectively than the nonmalignant cells. This repair deficiency correlated with significant downregulation of the hOGG1 protein and the corresponding mRNA (30-fold lower than *GAPDH*) in BxPC-3 cell line. The repair defect was complemented *in vivo* by transient transfection of the *hOGG1* gene and *in vivo* by recombinant hOGG1. These results are the first to show a deficiency of 8-OH-Gua repair in BxPC-3 cells, implicating this defect in the risk factor of pancreatic cancer.

Keywords

Pancreatic cancer; Oxidative stress; Oxidative DNA damage; DNA repair; Base excision repair; Reactive oxygen species; 8-Hydroxyguanine; hOGG1; Pancreatic cancer risk and antioxidant; enzymes

Pancreatic cancer is one of the leading causes of cancer mortality among men and women in the United States with more than 37,000 new cases estimated in 2008 [1]. Diagnosis is usually in the late stages of the disease, consequently, the mortality rate of pancreatic cancer is approximately equal to the incidence rate. Despite its rapid mortality, the causes of pancreatic cancer remain poorly understood. However, oxidative stress has been presumed to play a major role in the etiology of pancreatic carcinogenesis, reviewed in [2]. DNA base adducts associated with oxidative stress have been shown to be present in pancreatic tissues [3,4]. Furthermore, higher levels of oxidatively induced DNA adducts were detected in the pancreas of pancreatic cancer patients relative to the controls [5,6]. These reports implicate oxidatively induced DNA damage in the etiology of pancreatic cancer and suggest that the repair of these lesions may be a critical factor in pancreatic cancer.

The common oxidatively induced DNA lesion, 8-OH-Gua, is primarily produced through the oxidation of guanines by reactive oxygen species (ROS). 8-OH-Gua is a mispairing lesion that is potentially carcinogenic. In order to maintain proper genetic integrity and to minimize cancer risk, this lesion must be efficiently repaired. In human cells, 8-OH-Gua is repaired predominantly by hOGG1, a DNA glycosylase/AP lyase that is specific for 8-OH-Gua opposite cytosine [7–9]. The repair of 8-OH-Gua in mammalian cells is primarily accomplished via the base excision repair (BER) pathway [10]. The repair of 8-OH-Gua in pancreatic cancer cells

*Corresponding author. Fax: +1 410 558 8268. E-mail address: E-mail: me42v@nih.gov (M.K. Evans).

had not been studied prior to this study although this lesion has been shown to accumulate in pancreatic cancer tissues. This study analyzed the ability of a frequently studied pancreatic cancer cell line, BxPC-3, to repair 8-OH-Gua relative to a nonmalignant cell line.

Materials and methods

Cell lines and culture conditions

BxPC-3 is a pancreatic adenocarcinoma cell line that was purchased from American Type Culture Collection (ATCC, Manassas, VA) and was cultured in RPMI 1640 media supplemented with 10% FBS. AG11134 is a primary nonmalignant mammary epithelial cell line purchased from Coriell Cell Repository (Camden, NJ) and was cultured in serum-free mammary epithelial growth media following the supplier's instructions (Cambrex, Walkersville, MD). All the cell lines were cultured at 37 °C in the presence of 5% CO₂.

Preparation of nuclear extracts

Approximately 2.0×10^8 cells were harvested and pelleted by centrifugation at 1600 rpm at 4 °C for 5 min using a tabletop centrifuge (Beckman Coulter, Allegra, 6R). The cells were washed and then lysed by homogenization using a glass–glass homogenizer (Thomas Scientific) in buffer A (10 mM HEPES, pH 7.4; 0.21 M Mannitol; 0.07 M sucrose; 0.15 M spermine; 0.75 mM spermidine; 1 mM EDTA; 0.5 mM DTT and protease inhibitor cocktail. The homogenate was spun at 500g for 7 min at 4 °C and the nuclear pellet sonicated (Ultrasonic Processor GE 130, Sonics & Materials, Inc., Newtown, CT) in half volume of buffer B (20 mM HEPES, pH 7.9, 25% glycerol, 0.2 mM EDTA; 0.5 mM DTT, and 0.2 mM PMSF) on ice. The sonicated samples were incubated on ice for 30 min before centrifugation for 30 min at 25,000g at 4 °C (ultracentrifuge, Beckman XL-90). The supernatant was aliquoted and stored in –80 °C. Total protein yield was assessed by Bradford assay (Bio-Rad, Hercules, CA).

Western blot analysis

The protein samples were heated at 95 °C for 5 min then separated by electrophoresis through 12% Tris-glycine gels (Invitrogen, Carlsbad, CA). The protein bands were transferred onto PVDF membranes (Invitrogen, Carlsbad, CA). The membranes were blocked for 1 h in 5% dry milk in PBS Tween (0.2%) and then probed with the following primary antibodies: hOGG1 and catalase (Calbiochem, La Jolla, CA). The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies and then developed using enhanced chemiluminescence plus Western detection kit (Amersham Biosciences). The membranes were stripped at 50 °C for 30 min in stripping buffer (62.5 mM Tris-HCl, pH 7.4, 100 mM β -mercaptoethanol, 2% SDS) and re-probed with antibody against β -actin (Santa Cruz Biotechnology), after washing with anti-goat horseradish peroxidase-conjugated secondary antibody (Vector Laboratories).

Quantitative real-time RT-PCR

Total RNA was isolated from AG11134 and BxPC-3 cells using TRIzol reagent following the instructions manual (Invitrogen, Carlsbad, CA). First strand synthesis was performed to generate cDNA from 2.0 μ g of the total RNA following the RETROscript protocol (Ambion, Austin, TX). The Power SYBR Green 1 assay and the 7300-system detection software (AB Applied Biosystems, Foster City, CA) were used to detect real-time PCR products of the *hOGG1* and *GAPDH* mRNAs. The primers used were *hOGG1*, forward, 5'-CCTACACCTCAGGAAAGCC-3' and reverse, 5'-GGGAAGATAAAACCATCCTTAG-3' and *GAPDH*, forward, 5'-GAAGGTGAAGGTCGGAGTC-3' and reverse, 5'-GAAGATGGTGATGGGATTTTC-3'. The PCR cycling parameters were performed as follows: 50 °C, 2 min for AmpErase UNG (uracil N glycosylase) incubation, 95 °C, 10 min

for Amplitaq Gold activation, and 40 cycles for the melting (95 °C, 15 s) and annealing/extension (60 °C, 1 min) steps. The PCRs were performed in 96-well plates and the comparative (C_T) method was used to determine the expression level of *hOGG1* in BxPC-3 relative to AG11134 using *GAPDH* as the normalization control.

End labeling of oligonucleotides

The oligonucleotides used had 8-OH-Gua (shown in bold font) opposite C and had the following sequences: 5'-ATACGCATATACCGCT**8-OH-Gua**TCGGCCGATCTCCGAT-3'. Approximately, 100 ng of the single-stranded oligonucleotides were 5' end-labeled with 30 μ Ci of [γ - 32 P] ATP using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). The labeled single-stranded oligonucleotides were converted to double-stranded form by annealing to a 4-fold excess of the complementary oligonucleotides and heating to 90 °C for 5–10 min and slowly cooled to room temperature.

BER repair assay

The 5' end-labeled duplex oligonucleotides containing a single 8-OH-Gua were incubated at 37 °C with 50 μ g of nuclear extracts. The positive control reactions were performed by incubating the damage-containing oligonucleotides with excess hOGG1 (Trevigen, Gaithersburg, MD) for 1 h. The negative control reactions were performed by incubating undamaged duplex oligonucleotides with 50 μ g of extracts for 4 h. The repair assays were performed in buffer containing 20 mM HEPES, pH 7.4, 5% glycerol, 0.1 mM NaCl, 5 mM EDTA, 5 mM DTT, 100 μ g/ml BSA, and 25 μ g/ml poly(dI).poly(dC). Aliquots were removed at 0 h (before addition of extracts), 2 h and 4 h and the reactions stopped with 0.25% SDS followed by phenol/chloroform/isoamylalcohol purification as described previously [11]. The reaction products were separated by electrophoresis through 20% polyacrylamide gels containing 7 M urea at 15 W for approximately 80 min. The band images were scanned and analyzed by PhosphorImager.

Transient transfection of BxPC-3 with pFLAG-CMV-4-OGG1

BxPC-3 cells (2.0×10^6) were cultured at 37 °C in 100 mm $\times$ 20 mm tissue culture dishes. After 24 h the cells were transfected with pFLAG-CMV-4 plasmid carrying the *hOGG1* gene or the vector alone using DMRIE-C reagent following the method described earlier [12] and the supplier's instructions (Invitrogen, Carlsband, CA). The expression of hOGG1 was determined by Western blot analysis and the repair activity was analyzed following the BER assay.

Stimulation of 8-OH-Gua specific activity by hOGG1

BxPC-3 nuclear extracts (0 μ g, 30 μ g, 50 μ g, and 75 μ g) were incubated at 37 °C for 1 h with 20 ng of a duplex 5' end-labeled oligonucleotide containing a single 8-OH-Gua in the presence or absence of 30 ng of hOGG1 (Trevigen Inc., Gaithersburg, MD). The reactions were stopped with 0.25% SDS and heating at 90 °C for 10 min. An equal volume of oligonucleotide loading buffer (95% formamide, 5% glycerol and 0.02% bromophenol blue, and 0.02% xylene cyanol) was added to the reactions and the reaction products separated by electrophoresis through 20% polyacrylamide gels containing 7 M urea. The band images were captured on phosphorImager cassettes and analyzed by Imagequant (Amersham Biosciences).

Sequencing of the hOGG1

Genomic DNA was isolated from approximately 1.2×10^7 of BxPC-3 cells using Midi (tip 100/G) purchased from Qiagen, Valencia, CA). PCR amplification and subsequent sequence analyses were performed for all the exons including the intron regions in the proximity of the splice sites and the promoter region of the *hOGG1* (Seqwright, Houston, TX).

Statistical analyses

Statistical analyses were performed using Statistica 7.1 (Statsoft Inc., Tulsa, OK). All the tests and corresponding *p*-values were two-sided and the differences between groups were tested for significance using two-way ANOVA. The error bars represent $\pm$ standard error of means (SEM) of at least three independent experiments. A $p < 0.05$ was considered statistically significant.

Results

BER of 8-OH-Gua

We assessed the ability of the pancreatic adenocarcinoma cell line, BxPC-3, to repair an oligonucleotide-containing the common oxidatively induced DNA lesion, 8-OH-Gua relative to the nonmalignant cell lines, AG11134. BxPC-3 nuclear extracts repaired 8-OH-Gua at a significantly reduced rate compared to the AG11134 cell line both qualitatively and quantitatively (Fig. 1A–C). This deficient repair of 8-OH-Gua by BxPC-3 is specific since this cell line repaired an AP site-containing oligonucleotide at a rate comparable to that of the nonmalignant cell line (data not shown).

Expression of hOGG1 protein and mRNA

To determine if the defective repair of 8-OH-Gua in BxPC-3 cells is associated with the level of hOGG1, we analyzed the expression of this enzyme in BxPC-3 cells relative to the nonmalignant cell line. Interestingly, BxPC-3 nuclear extracts expressed undetectable levels of hOGG1 compared to the nonmalignant cell line (Fig. 2A). These results are consistent with the defective repair of 8-OH-Gua in BxPC-3 cells.

To determine if the under-expression of hOGG1 could be associated with transcriptional defects, we analyzed the *hOGG1* mRNA levels by quantitative real-time RT-PCR. The *hOGG1* mRNA was significantly downregulated (>30-fold lower) in BxPC-3 cells relative to the control gene, *GAPDH* (Fig. 2B). Conversely, BxPC-3 cells and AG11134 cells expressed comparable levels of *hOGG1* mRNA (Fig. 2B) relative to *GAPDH*.

Complementation of 8-OH-Gua repair

To determine if the reduced repair of 8-OH-Gua in BxPC-3 cells could be associated with the reduced expression level of hOGG1, we transiently transfected BxPC-3 cells with a plasmid carrying the *hOGG1* gene. BxPC-3 cells transfected with *hOGG1* expressed a higher level of hOGG1, compared to BxPC-3 cells transfected with the vector alone (Fig. 3A). Nuclear extracts from BxPC-3 cells expressing a higher level of hOGG1 exhibited significantly higher 8-OH-Gua-specific excision repair activity (Fig. 3C and D) than the nuclear extracts from cells transfected with the empty vector (compare Fig. 3C with Fig. 3B and D). Furthermore, whole cell extracts from BxPC-3 cells transfected with *hOGG1* repaired 8-OH-Gua more effectively than whole cell extracts from BxPC-3 cells transfected with the empty vector (data not shown), consistent with the results obtained with nuclear extracts.

Stimulation of 8-OH-Gua excision by recombinant hOGG1

We speculated that recombinant hOGG1 would stimulate the repair of 8-OH-Gua on the basis of deficient 8-OH-Gua repair and the low protein level in BxPC-3 cells. Indeed, hOGG1 significantly stimulated the repair of 8-OH-Gua by BxPC-3 nuclear extracts (Fig. 4A and B). Conversely, the excision of 8-OH-Gua by BxPC-3 nuclear extracts in the absence of recombinant hOGG1 was significantly lower (Fig. 4A and B). These results further prove that the reduced repair of 8-OH-Gua in BxPC-3 cells is indeed associated with the low level of hOGG1 in these cells.

Genetic analysis of the hOGG1

To determine if there was a correlation between the defective repair of 8-OH-Gua and the genetic status of the *hOGG1*, we sequenced the coding and promoter regions of the *hOGG1* in BxPC-3 cells. There were no mutations in the promoter region of the *hOGG1*. However, we found three single nucleotide polymorphisms (SNPs): A/G, G/A and a deletion/A in exons 4, 7, and 8, respectively (data not shown). These SNPs did not correspond to amino acid changes suggesting that the reduced repair of 8-OH-Gua was not due to a genetic change in the hOGG1.

Discussion

We report here that the pancreatic adenocarcinoma cell line BxPC-3 is deficient in the repair of 8-OH-Gua relative to the nonmalignant cell line AG11134. The deficient repair of 8-OH-Gua was shown to be associated with defects in expression levels and activity of hOGG1 since BxPC-3 cells exhibited undetectable level of hOGG1 and had severely downregulated *hOGG1* mRNA. Furthermore, these defects were complemented *in vivo* by transfection of *hOGG1* and *in vitro* by addition of recombinant hOGG1.

The inefficient repair could result in accumulation of 8-OH-Gua thereby overwhelming the DNA repair enzymes causing deleterious mutations that could potentially increase the risk of pancreatic cancer. Indeed, pancreatic cancer and breast cancer tissues have significantly higher levels of 8-OH-dG compared to nonmalignant tissues of the pancreas or the breast [5,13]. Importantly, DNA repair of oxidatively induced DNA lesions has been associated with a variety of cancers, further underscoring the importance of this DNA repair pathway in the etiology of cancer, reviewed in [14]. To this end, previous studies have revealed that DU-145 and PC-3 human prostate cancer cell lines and human breast cancer cell lines are defective in the cellular repair of oxidatively induced DNA lesions [15–18]. In addition, deficient repair of 8-OH-Gua has recently been reported in lung cancer cell lines exhibiting reduced *hOGG1* mRNA and low levels of hOGG1 [19,20]. Furthermore, Parker and co-workers reported deficient repair of 8-OH-Gua in colorectal cancer cell lines [21]. Altogether, these studies suggest an association between deficient repair of oxidatively induced DNA damage and the etiology of various forms of cancer including pancreatic cancer.

Altered expression of the mismatch repair proteins, hMLH1 and hMSH2 and DNA ligases has been reported in the pancreas of pancreatic cancer patients [22]. Additionally, increased expression of DNA ligases has been reported in the pancreatic cancer cell line, MIAPaCa-2, in response to DNA damaging agents [23–25]. These studies support our findings and suggest that altered levels of DNA repair enzymes could constitute risk of pancreatic cancer.

Genetic mutations of the *hOGG1* that alter the amino acid sequence have been shown to result in reduced hOGG1 activity [26]. The defective repair activity of BxPC-3 cells is not associated with mutations in the hOGG1 since sequence analysis did not reveal *hOGG1* mutations.

The stimulation of 8-OH-Gua repair by overexpressing hOGG1 or by addition of recombinant hOGG1 indicates that the deficient repair in BxPC-3 cells is associated with the level and activity of endogenous hOGG1. These findings further suggest that hOGG1 is essential for efficient repair of 8-OH-Gua in BxPC-3 cells. These results are consistent with previous studies that complemented defective mitochondrial repair in HeLa cells and in pulmonary artery endothelial cells by transfection with a plasmid carrying the mitochondrial *hOGG1* [27,28].

Thus, we have shown that BxPC-3 is deficient in BER of 8-OH-Gua. This deficiency correlates with defective expression of the hOGG1 and the corresponding mRNA. To our knowledge, this is the first direct evidence of defective repair of 8-OH-Gua in a pancreatic cancer cell line.

These studies suggest that deficient repair of 8-OH-Gua may be a biomarker for the risk of pancreatic cancer in some individuals.

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Fig. 1.

BER of 8-OH-Gua. Nuclear extracts (50 μ g) were incubated at 37 °C with a duplex oligonucleotide containing a single 8-OH-Gua. After removing the aliquots the reactions were stopped and the products separated by electrophoresis. (A) Gel showing qualitative repair of 8-OH-Gua by AG11134, (B) gel showing qualitative repair of 8-OH-Gua by BxPC-3, and (C) quantified products of 8-OH-Gua repair by AG11134 (clear bars), and BxPC-3 (black bars). Lane 1: oligonucleotide size markers (8–32 bp), lanes 2–5: repair kinetics (0 h, 2 h, and 4 h, respectively), lane 6: hOGG1, and lane 7: undamaged duplex oligonucleotide plus extracts. At least three independent experiments were performed for each cell line and the error bars represent $\pm$ SEM. The asterisks denote a statistically significant difference ($p < 0.05$).

Fig. 2.

Expression of hOGG1 protein and *hOGG1* mRNA. (A) Western blot analysis of hOGG1 using 50 μ g of nuclear extracts. Beta actin was used as a loading control. The gel shown is representative of at least three independent repeats. (B) Analysis of *hOGG1* mRNA levels by quantitative real-time RT-PCR. The clear bars represent AG11134 cell line and the black bars represent BxPC-3 cell line. The first two bars represent *GAPDH* mRNAs while the last two represent *hOGG1* mRNAs. The results represent at least six independent repeats and the error bars represent $\pm$ SEM. The asterisks denote a statistically significant difference ($p < 0.05$).

Fig. 3.

Complementation of 8-OH-Gua-repair by transient transfection of the *hOGG1*. BxPC-3 cells were transfected with a plasmid carrying the *hOGG1* gene or the vector alone. Nuclear extracts were subsequently prepared and the BER assay performed. (A) Expression of hOGG1, (B) repair of 8-OH-Gua by nuclear extracts from cells transfected with vector alone, (C) repair of 8-OH-Gua by nuclear extracts from cells transfected with plasmid carrying the *hOGG1*, and (D) quantified results. Lane 1: oligonucleotide size markers (8–32 bp), lanes 2–6: repair kinetics (0 h, 1 h, 2 h, 4 h, and 6 h, respectively), lane 7: hOGG1, and lane 8: undamaged duplex oligonucleotide plus extracts. The gray bars represent repair of 8-OH-Gua by extracts from *hOGG1*-transfected cells and black bars represent repair of 8-OH-Gua by extracts from empty vector-transfected cells. At least three independent experiments were performed and the error bars represent $\pm$ SEM. The asterisks denote a statistically significant difference ($p < 0.05$).

Fig. 4. Stimulation of 8-OH-Gua-repair by recombinant hOGG1. Recombinant hOGG1 protein was added to increasing amounts of BxPC-3 nuclear extracts and repair reactions performed. (A) Gel showing qualitative repair products, and (B) quantified results. Gray bars represent BxPC-3 reactions supplemented with recombinant hOGG1 and black bars represent BxPC-3 alone. At least three independent reactions were performed and the error bars represent $\pm$ SEM. The asterisks denote a statistically significant difference ($p < 0.05$).