ABSTRACT

Reactive oxygen species (ROS) are produced in cells by both endogenous and exogenous processes. Oxidative stress arises when the ROS are at high enough concentrations to overwhelm the cell's normal antioxidant defense mechanisms. The fact that DNA is modified by ROS has led to the suggestion that oxidative stress may be an important factor in the process of carcinogenesis.

Mammalian cells contain three main antioxidant enzymes that function to maintain ROS at very low concentrations. Catalase (CAT) acts to scavenge hydrogen peroxide. Glutathione peroxidase (GPX) detoxifies hydrogen peroxide as well as lipid-derived organic hydroperoxides. Superoxide dismutase (SOD) converts the superoxide anion radical to hydrogen peroxide. Decreased expression of CAT, GPX and SOD in cancer cells would be expected to result in elevated levels of hydrogen peroxide and superoxide. A number of studies have shown that the activities of these enzymes are decreased in tumor cells compared to adjacent normal tissue. Downregulation of antioxidant gene expression results in consistently elevated levels of ROS, a state referred to as persistent oxidative stress.

ROS cause several types of DNA damage, including base modification, strand breakage, and DNA-protein cross-links. One of the major oxidatively modified DNA bases in vivo is 8-hydroxy-2'-deoxyguanosine. This product, which is also referred to as 8-OH-dG or 7,8-dihydro-8-oxo-2'-deoxyguanosine, is mutation prone and results in a G:C to T:A transversion following DNA replication. Elevated levels of 8-OH-dG have been detected in cancers from several tissues (i.e., breast, lung and kidney). Human mutT homologue (hMTH1) prevents the formation of 8-OH-dG residues by
dephosphorylating 8-oxo-dGTP to 8-oxo-dGMP, thereby preventing misincorporation of the oxidized free nucleotide into DNA. Overexpression of hMTH1 mRNA has been detected in primary renal cell and breast carcinomas as well as in lung cancer cell lines. Human 8-oxoguanine DNA glycosylase (hOGG1) catalyzes the excision of 8-oxoguanine (8-oxoG) and 2,6-diamino-4-hydroxy-5-formamido-pyrimidine (Fapy) from DNA. To date, no studies have shown hOGG1 to be overexpressed in tumor tissues. However, expression of hOGG1 has been shown to be upregulated in vitro in response to oxidative stress. Human mutY homologue (hMYH) has an enzymatic activity that removes an adenine base from an A:8-oxoG base pair. No studies have examined the expression of hMYH in tumor tissues. If persistent oxidative stress is a feature of cancer cells, then it is predicted that hMTH1, hOGG1 and hMYH should be overexpressed in these cells.

By measuring protein levels of CAT, GPX, SOD, hMTH1, hOGG1 and hMYH in sputum samples from at-risk individuals (i.e., smokers), it should be possible to detect persistent oxidative stress in exfoliated cells from the lung. This may provide a new strategy for the early detection of lung cancer.
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CHAPTER I. THE ROLE OF OXIDATIVE STRESS IN CARCINOGENESIS

Reactive oxygen species (ROS) can be produced either by endogenous or exogenous processes. The family of ROS includes the superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and the hydroxyl radical (HO•) as well as lipid-derived oxygen-centered radicals (LO• and LOO•). Endogenous sources of ROS include cytochrome P450, inflammatory cells, mitochondria and peroxisomes. Exogenous sources of ROS include hyperoxia, ozone, radiation and xenobiotics. Oxidative stress arises when the level of these ROS overwhelm the cell’s normal antioxidant defense mechanisms (Sies, 1991). This cellular state may also occur if the antioxidant capacity of the cell is decreased. Cellular antioxidants include both non-enzymatic (e.g., glutathione, vitamin C and vitamin E) and enzymatic (e.g., catalase, glutathione peroxidase and superoxide dismutase) members. Thus, a state of oxidative stress may exist if dietary antioxidant levels are low or if enzyme expression is modified.

When excess ROS are present in the cell, either DNA, lipids or proteins may be attacked. The fact that DNA is modified by ROS has led to the suggestion that oxidative stress may be an important factor in the process of carcinogenesis. Indeed, it has been shown that antioxidant defenses are altered in pro-carcinogenic states (Cerutti et al., 1994). Further, epidemiological studies have shown that antioxidants protect against the development of cancer (Ames, 1983; Willet and MacMahon, 1984). Cerutti (1994) has stated that ROS possess three essential properties of carcinogens. These are the ability to: 1) elicit permanent structural changes in DNA (e.g., base-pair mutations and deletions); 2) activate both cytoplasmic and nuclear signal transduction pathways; and 3) modulate the activity of stress proteins and stress genes that function to regulate effector
genes related to growth, differentiation and cell death. Ames et al. (1993) have estimated that the number of oxidative hits to human DNA is approximately 10,000 per cell per day. Fortunately, DNA-repair enzymes efficiently remove the majority of the lesions formed; however, repair efficiency is not 100% (Ames and Shigenaga, 1992). The result is that oxidative DNA lesions accumulate with age. Mutations accumulate with age as well (Grist et al., 1992; Branda et al., 1993). It is for this reason that the odds of developing cancer increase with age.

ROS cause several types of DNA damage, including base modification, strand breakage, and DNA-protein cross-links (Halliwell and Aruoma, 1991). One of the major oxidatively modified DNA bases in vivo is 8-hydroxy-2'-deoxyguanosine (Toyokuni et al., 1994). This product, which is also referred to as 8-OH-dG or 7,8-dihydro-8-oxo-2'-deoxyguanosine, is mutation prone and results in a G:C → T:A transversion following DNA replication (Kuchino et al., 1987; Shibutani et al., 1991). A sensitive method for detection of 8-OH-dG has been described that employs high pressure liquid chromatography with electrochemical detection (HPLC/ECD; Floyd et al., 1986). Gas chromatography coupled with mass spectrometry (GC/MS) has also been used to detect this modified base (Dizdaroglu, 1992). Table 1 shows 8-OH-dG levels in control and tumor tissues for four different types of carcinomas in multiple case studies. It can be seen that in three out of the four types, 8-OH-dG levels are significantly elevated in tumor tissues compared to normal tissues from the same patients. The greatest difference in 8-OH-dG levels was observed in invasive ductal carcinomas of the breast where tumor levels were approximately ten times greater than in control tissue. The only type of cancer that didn’t exhibit a significant increase in 8-OH-dG levels was hepatocellular
Table 1. Content of 8-hydroxy-2'‐deoxyguanosine in human carcinoma (multiple case study)

<table>
<thead>
<tr>
<th>Organ</th>
<th>Histology</th>
<th>Control</th>
<th>Tumor</th>
<th>N; statistics</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>Invasive ductal carcinoma</td>
<td>4.13 ± 0.43</td>
<td>40.1 ± 11.1</td>
<td>(5; P &lt; 0.01)</td>
<td>GC/MS</td>
<td>Malins and Haimanot, 1991</td>
</tr>
<tr>
<td>Lung</td>
<td>Squamous cell carcinoma</td>
<td>11.2 ± 2.3</td>
<td>25.5 ± 7.0</td>
<td>(5; P &lt; 0.05)</td>
<td>GC/MS</td>
<td>Jaruga et al., 1994</td>
</tr>
<tr>
<td>Liver</td>
<td>Hepatocellular carcinoma</td>
<td>1.57 ± 0.21</td>
<td>2.29 ± 0.38</td>
<td>(11, 18; P = 0.174)</td>
<td>HPLC/ECD</td>
<td>Shimoda et al., 1994</td>
</tr>
<tr>
<td>Kidney</td>
<td>Renal cell carcinoma</td>
<td>3.60 ± 0.20</td>
<td>5.56 ± 0.41</td>
<td>(31; P &lt; 0.0005)</td>
<td>HPLC/ECD</td>
<td>Okamoto et al., 1994</td>
</tr>
</tbody>
</table>

The amount of 8-hydroxy-2'-deoxyguanosine per 10^3 deoxyguanosine in DNA is shown by means ± S.E.M. All of the samples except those from Shimoda et al., 1994 are paired samples from the same patient. GC/MS, gas chromatography with mass spectrometry; HPLC/ECD, high performance liquid chromatography with electrochemical detector. Table adapted from Toyokuni et al., 1995.

Table 2. Content of 8-hydroxy-2'-deoxyguanosine in human neoplasm (single case study)

<table>
<thead>
<tr>
<th>Organ</th>
<th>Histology</th>
<th>Control</th>
<th>Tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>Fibrillary astrocytoma</td>
<td>1.36</td>
<td>2.49</td>
</tr>
<tr>
<td>Lung</td>
<td>Squamous cell carcinoma</td>
<td>9.68</td>
<td>10.1</td>
</tr>
<tr>
<td>Lung</td>
<td>Adenocarcinoma</td>
<td>7.33</td>
<td>23.0</td>
</tr>
<tr>
<td>Colon</td>
<td>Non-primary carcinoma</td>
<td>2.71</td>
<td>4.43</td>
</tr>
<tr>
<td>Stomach</td>
<td>Mucinous carcinoma</td>
<td>0.94</td>
<td>5.08</td>
</tr>
<tr>
<td>Ovary</td>
<td>Serous cystadenocarcinoma</td>
<td>3.11</td>
<td>9.20</td>
</tr>
</tbody>
</table>

Data based on GC/MS method are from Olinski et al., 1992. The amount of 8-hydroxy-2'-deoxyguanosine per 10^3 deoxyguanosine in DNA is shown. Table from Toyokuni et al., 1995.
carcinoma. Of the four studies, this was the only one that didn’t use paired control samples from the same patients. Instead, control liver samples were from patients with metastatic liver tumors; thus, the level of 8-OH-dG in these controls may have been higher than if paired control samples had been used. Data on 8-OH-dG levels from a single case study are shown in Table 2. Here, the greatest elevation of 8-OH-dG levels was observed in an adenocarcinoma of the lung. Together, these studies indicate that 8-OH-dG, a marker of oxidative stress, is elevated in a number of different types of cancer. Thus, it appears either that the rate of ROS generation is higher in tumor tissue compared to adjacent normal tissue or that the antioxidant capacity of the former is diminished.

Decreased expression of catalase, superoxide dismutase and glutathione peroxidase in cancer cells would be expected to result in elevated levels of hydrogen peroxide and superoxide (Figure 1). It is well established that these species are capable of inducing expression of the proto-oncogenes c-fos, c-jun, and c-myc (Crawford et al., 1988; Nose et al., 1991) via activation of the transcription factor NF-kB (Shreck et al., 1991). Thus, oncogene activation is one role of ROS in carcinogenesis. In the presence of trace metal ions such as iron and copper, hydrogen peroxide and superoxide react to produce hydroxyl radicals. These can, in turn, react directly with DNA to produce base modifications or strand breaks. Ultimately, such mutations can lead to a cellular state referred to as genomic instability, in which numerous chromosomal aberrations are typically present (Cheng and Loeb, 1993). This ability to induce genomic instability represents a second role of ROS in carcinogenesis. A third role proposed by Toyokuni et al. (1995) is the ability of ROS to induce chemotherapy resistance by upregulating other antioxidants such as adult T-cell leukemia-derived factor (ADF), glutathione S-
transferase (GST) and glutathione (GSH) (Figure 1). Finally, ROS may facilitate invasion and metastasis by causing damage to protease inhibitors (e.g., α1-protease inhibitor) by oxidizing methionine residues of the active site (Swaim and Pizzo, 1988; Johnson and Travis, 1979), thereby inducing enhanced action of proteases. Together, these functions of ROS indicate that these species are capable of participating in the initiation, promotion and progression phases of carcinogenesis. Thus, ROS may be considered to be complete carcinogens.

Figure 1. Schematic outline of the role of reactive oxygen species in cancer. From Toyokuni et al., 1995. SOD, superoxide dismutase; •OH, hydroxyl radical; ADF, adult T-cell leukemia-derived factor; GST, glutathione S-transferase; GSH, glutathione.
In this paper, the potential role of antioxidant enzymes and oxidative DNA damage repair enzymes as markers of oxidative stress in carcinogenesis will be addressed. The purpose of this study is to determine if catalase (CAT), glutathione peroxidase (GPX), superoxide dismutase (SOD), human MutT homologue (hMTH1), 8-oxoguanine DNA glycosylase (hOGG1) and human MutY homologue (hMYH) can be used as markers for the early detection of carcinomas of the lung.
CHAPTER II. CATALASE

Catalase (CAT) is the main cellular enzyme responsible for scavenging H₂O₂. CAT decomposes H₂O₂ to water according to the following reaction (Paniker and Iyer, 1965):

\[ 2 \text{H}_2\text{O}_2 \rightarrow 2 \text{H}_2\text{O} + \text{O}_2 \]

The CAT locus has been assigned to chromosome 11p13 (Junien et al., 1980). Ludwig et al. (1991) examined loss of heterozygosity (LOH) at the CAT locus using a cohort of 48 non-small cell lung cancer (NSCLC) samples and normal lung tissue from the same patients. The incidence of LOH at the CAT locus was 72% (21/29). Loss of the whole arm of chromosome 11p plays a minor role in generating 11p13 deletions in NSCLC. As a duplication of the remaining allele in samples with LOH at the CAT locus was rarely observed, interstitial deletion, and not mitotic recombination, seems to be the most common method for gene deletion.

Fong et al. (1994) examined loss of heterozygosity (LOH) at the CAT locus by means of restriction fragment length polymorphism (RFLP) analysis. Forty percent of the cases were squamous cell carcinomas (SCCs). The frequency of LOH at the CAT locus was 15% (6/40). This was similar to the 23% found by Weston et al. (1989). However, it was lower than the 72% found by Ludwig et al. (1991). This difference may be due to a higher percentage (66%) of SCCs in the latter study. With respect to survival, SCC cases with LOH at 11p13 had a significantly worse prognosis than SCCs without LOH at this locus \((p < 0.002)\). Adenocarcinomas (ACs), the other major histological subtype studied, showed no correlation between overall or regional LOH at 11p with
tumor stage, nodal progression, or survival. This indicates that LOH at 11p does not play a major role in determining tumor progression in this subtype.

Guner et al. (1996) determined the CAT activities in fifteen lung carcinoma tissue samples and corresponding normal samples from the same cases. These workers found 33.53 ± 6.09 U/mg protein (mean ± S.E.M.) of CAT in lung cancer tissue and 71.33 ± 14.38 U/mg protein in normal lung tissue. This difference was found to be significant at the level of \( p < 0.01 \). Jaruga et al. (1994) also determined the activity of lung cancer tissues to be lower than their surrounding cancer-free tissues; however, the difference between the two was not as great (Figure 2). Melloni et al. (1996) found no difference in lung epithelial lining fluid CAT levels between lung cancer patients and healthy non-smoking control subjects.

![Activity of catalase in human cancerous lung tissues and their surrounding cancer-free tissues. Values = mean ± S.D. (n = 3). From Jaruga et al., 1994.](image)

Due to the relative non-invasiveness of blood sampling, blood may be a good biological fluid for the application of oxidative stress biomarkers. Casado et al. (1995) have compared the activity of CAT in blood obtained from lung cancer patients to that of healthy age-matched controls. Without taking age into account, these workers found a significant decrease (\( p < 0.05 \)) in CAT activity in blood from the patients (186 ± 7 K/g of
hemoglobin, Hb) compared to that of the controls (214 ± 7 K/g of Hb). However, when the groups were stratified according to age, a significant increase in CAT activity was seen in the 28 – 37 yr age group comparing blood from patients (276 ± 21 K/g of Hb) to that of controls (234 ± 9 K/g of Hb). Conversely, the patients in the 58 – 67 yr age group exhibited decreased CAT activity (187 ± 9 K/g of hemoglobin) relative to controls (243 ± 9 K/g of hemoglobin). Thus, age must be considered as a potential confounding factor when investigating CAT activity in blood.
CHAPTER III. GLUTATHIONE PEROXIDASE

Glutathione peroxidase I (GPX) is a cytosolic selenium-dependent enzyme that acts to detoxify both organic hydroperoxides (ROOH) and H₂O₂ through a reaction coupled to the oxidation of reduced glutathione (Flohe et al., 1979) as follows:

ROOH + 2GSH → ROH + H₂O + GSSG

This reaction results in a reduction of the hydroperoxide to its corresponding alcohol and oxidation of GSH to GSSG.

The GPX gene has been mapped to chromosome 3p21 by in situ hybridization analysis of lymphocyte metaphase spreads (Moscow et al., 1994a). Karyotype analysis and loss of heterozygosity (LOH) studies have shown that this region of chromosome 3p is deleted in almost 100% of small cell lung cancers (SCLC) and 50% of non-small cell lung cancers (NSCLC) (Moscow et al., 1994b). Recently, the level of 8-OH-dG in lung tumors exhibiting LOH on chromosome 3p was found to be significantly higher ($p = 0.05$) than that of tumors with no loss on this arm (Figure 3a) (Hardie et al., 2000). Further, the activity of GPX was lower in tumors showing LOH on 3p, although this result was not statistically significant (Figure 3b) (Hardie et al., 2000).

![Figure 3](image.png)

Figure 3. (A) The association between 3p chromosomal loss and 8-OH-dG levels in lung tumors. Data presented are mean 8-OH-dG values/10⁶ dG bases ± S.E. (B) The effect of
1.OH on chromosome 3p on lung tumor glutathione peroxidase activity. Data presented are mean nmol GSH oxidized/min/mg cytosolic protein ± S.E. Figure from Hardie et al., 2000.

Genetic polymorphisms in detoxifying enzymes such as GPX are thought to play a role in determining the risk of developing cancer. Moscow et al. (1994b) have reported a nucleotide substitution at codon 198 of GPX; this substitution results in a change from proline to leucine. Recently, a significant association was found between this genotype and lung cancer risk (Ratnasinghe et al., 2000). In this study, 315 cases from the α-Tocopherol, β-Carotene Cancer Prevention Study cohort (Taylor et al., 1990) were matched to controls on age (± 5 years), intervention group, and study clinic. Odds ratios were calculated to be 1.8 (95% CI, 1.2-2.8) for heterozygotes and 2.3 (95% CI, 1.3-3.8) for homozygous variants compared to wild-type individuals. The prevalence of the polymorphism was found to be statistically different (p < 0.001) between controls (58%) and cases (70%).

Although a number of studies have shown highly variable amounts of GPX activity in tumor tissues, Jaruga et al. (1994) have demonstrated consistently lower levels of this enzyme in lung tumor tissues compared to that of surrounding cancer-free tissues (Figure 4).
Figure 4. Activity of glutathione peroxidase in human cancerous lung tissues and their surrounding cancer-free tissues. Values = mean ± S.D. (n = 3). From Jaruga et al., 1994.

Lower levels of GPX have also been detected in the lung epithelial lining fluid obtained from lung cancer patients as compared to healthy non-smoking control subjects (Figure 5) (Melloni et al., 1996).

![Graph showing ELF GPX activity](image)

**Figure 5.** Glutathione peroxidase activity in ELF from nonsmokers and smokers compared with patients with lung cancer. The GPX activity is expressed as U/ml of ELF. *P < 0.05 compared with nonsmokers; † P < 0.05 compared with smokers. From Melloni et al., 1996.

Scott et al. (1996) established that there is an acute-phase protein response (increased C-reactive protein concentration) at the time of diagnosis in patients with NSCLC. These workers (Sattar et al., 1997) then divided NSCLC patients into two groups depending on whether they had a C-reactive protein concentration < 35 mg/l (Group 1) or > 35 mg/l (Group 2) and measured GPX levels in controls and patients. Median C-reactive protein levels (in mg/l) were < 5, 15.0, and 144.0 for Controls, Group 1 and Group 2, respectively. Plasma median GPX activities (in U/l) were 166, 137, and 111 for Controls, Group 1 and Group 2, respectively. The GPX activity was significantly lower in Group 2 than in the Control Group (P < 0.01). These results suggest that GPX
levels may be affected by an acute-phase protein response. However, the authors caution that the changes in GPX may merely represent nutritional deficiencies (i.e., lowered selenium levels) since the majority of Group 2 patients had lost weight.

Crawford et al. (2000) measured hGPX1 mRNA levels in primary normal human bronchial epithelial (NHBE) cells from 23 non-lung cancer patients and 11 lung cancer patients. hGPX1 was expressed at significantly lower levels \( P = 0.01 \) in primary NHBE cells from patients with bronchogenic carcinoma compared to NHBE cells from non-lung cancer patients (Figure 6).

![Figure 6. hGPX1 expression for NHBE samples from subjects with cancer versus non-cancer control subjects. Individual NHBE samples are presented in two columns (cancer and control) along the X-axis. Individual gene expression values in molecules/10^3 \( \beta \)-actin molecules are plotted along the Y-axis. The cutoff line is positioned at a gene expression index value associated with \( \geq 90\% \) sensitivity. From Crawford et al., 2000.](image)

The majority of studies to date have shown a significant increase in GPX activity in tumor tissue compared to corresponding adjacent normal tissue. For example, Di Ilio et al. (1987) examined pulmonary tumors and normal adjacent tissues and found that in 19 cases out of 24, the GPX activity was higher in the tumor tissue. Determinations were performed using both \( H_2O_2 \) as substrate (to measure selenium-dependent GPX activity)
and cumene hydroperoxide (CHP, to measure total peroxidase activity). The mean value of total peroxidase activity was only slightly higher than that of selenium-dependent peroxidase activity (Table 3), indicating that human lung contains very little, if any, selenium-independent peroxidase activity.

Table 3. Selenium- and glutathione-related enzyme activities in human lung.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Non-Tumor</th>
<th>Tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cumene hydroperoxide</td>
<td>28 ± 15</td>
<td>42 ± 28*</td>
</tr>
<tr>
<td></td>
<td>(10 – 70)</td>
<td>(12 – 104)</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>23 ± 9</td>
<td>32 ± 20**</td>
</tr>
<tr>
<td></td>
<td>(10 – 43)</td>
<td>(9 – 93)</td>
</tr>
</tbody>
</table>

Table from Di Ilio et al., 1987

*a = nanomoles of NADPH oxidized/min/mg protein
Values are means ± S.D.
Values in parenthesis = ranges
n = 25 for all samples analyzed
* P < 0.025; ** P < 0.05

Carmichael et al. (1988) also investigated the difference in GPX activity between tumors and normal tissue taken from a site distal to the tumor. In this study, elevated levels of GPX activity was seen in six out of ten tumor samples using CHP as a substrate (Figure 7A) and nine out of ten tumor samples using H2O2 as a substrate (Figure 7B). Since CHP measures total peroxidase activity while H2O2 measures only GPX activity, it is surprising that the peroxidase activity was typically much higher when H2O2 was used as a substrate. In this report, the data are shown as a function of pathology of the tumor samples. Using H2O2 as a substrate, two adenocarcinoma samples had 18- to 30-fold higher GPX activity than in normal tissue (Figure 7B). A 3- to 6-fold elevation was also seen in 3 of the squamous cell carcinomas (Figure 7B). These data indicate that extremely elevated levels of GPX activity can be detected in certain tumor samples.
Figure 7. Glutathione peroxidase activity in human lung tumors. Values are expressed as fold changes in the activity relative to normal tissue from the same patient. The substrates used were: CHP (A) and H$_2$O$_2$ (B). The samples are ordered according to tumor type. 1, 5, 6, and 8, poorly differentiated squamous carcinoma; 2 and 7, poorly differentiated adenocarcinoma and adenosquamous carcinoma respectively; 3 and 10, large cell carcinomas; 4, small cell carcinoma. From Carmichael et al., 1998.

Howie et al. (1990) investigated GPX activity in 19 lung tumor samples. These samples were matched to normal tissue from the same patients. Selenium-dependent peroxidase activity, measured using H$_2$O$_2$ as a substrate, was similar in all normal samples. This activity was significantly increased (2- to 3-fold) over normal tissue levels in almost all of the tumors analyzed. Total peroxidase activity was also measured using CHP as a substrate. Tumor selenium-dependent peroxidase activity was highly correlated with total peroxidase activity ($r = 0.96, P < 0.01$). These data indicate that in these tissues, peroxidase activities are almost entirely mediated by the selenium-dependent GPX.

Zachara et al. (1997) analyzed 57 lung tumor samples and their corresponding adjacent normal tissues for GPX activity. These workers found the mean GPX activity of
the tumor samples (91.8 ± 44.2 units/g protein) to be significantly higher ($p < 0.0001$) than that of the adjacent normal tissue (61.4 ± 23.1 units/g protein). No differences in GPX activities were found between the tumor types examined (i.e., squamous cell carcinoma, adenocarcinoma, large cell carcinoma and small cell carcinoma). Although no statistically significant differences were found for GPX activity with respect to the stage of disease, Stage III tumors did have a lower mean GPX activity (79.4 ± 35.1 units/g protein) than Stage I tumors (101.3 ± 48.6 units/g protein). Although only one Stage IV tumor was examined, this had an extremely low GPX activity (20.13 units/g protein). These results indicate that GPX activity is increased in lung tumors compared to adjacent normal tissue. However, they also suggest that GPX activity may decrease with stage of the disease. These workers also compared red cell and plasma GPX activities of lung cancer patients to that of healthy control subjects. Red cell GPX activity was significantly ($p < 0.004$) decreased in cases (13.4 ± 3.3 units/g Hb) compared to controls (16.8 ± 6.3 units/g Hb). Likewise, plasma GPX activity was significantly ($p < 0.05$) decreased in cases (196 ± 71 units/liter) compared to controls (222 ± 61 units/liter).

Iwasaki et al. (1998) examined immunohistochemical staining for GPX in both Stage I and Stage III lung adenocarcinomas to determine if there was a difference in enzyme expression between patients who suffered no relapse within a four year period after surgery and those who relapsed. Staining was graded as follows: Grade 1, diffuse, strong staining in $\leq 20\%$ of the cells; Grade 2, between 20% and 80% of the cells exhibit strong staining; and Grade 3, more than 80% of the cells exhibit strong staining. The results of this study are shown in Tables 4 and 5 below.
Table 4. Staining intensity of Stage I lung adenocarcinoma with glutathione peroxidase antibody.

<table>
<thead>
<tr>
<th>Grade</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relapse (n = 8)</td>
<td>0*</td>
<td>3 (38%)</td>
<td>5 (62%)</td>
</tr>
<tr>
<td>No relapse (n = 22)</td>
<td>14 (64%)*</td>
<td>8 (36%)</td>
<td>0</td>
</tr>
</tbody>
</table>

*Table adapted from Iwasaki et al., 1998.
*p < 0.01

Table 5. Staining intensity of Stage III lung adenocarcinoma with glutathione peroxidase antibody.

<table>
<thead>
<tr>
<th>Grade</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relapse (n = 22)</td>
<td>0*</td>
<td>16 (73%)</td>
<td>6 (27%)</td>
</tr>
<tr>
<td>No relapse (n = 9)</td>
<td>3 (33%)*</td>
<td>6 (67%)</td>
<td>0</td>
</tr>
</tbody>
</table>

*Table adapted from Iwasaki et al., 1998.
*p < 0.01

These data indicate that GPX overexpression occurs in a higher frequency of cells in lung adenocarcinomas from patients who relapsed compared to those patients who didn’t relapse.
CHAPTER IV. SUPEROXIDE DISMUTASE

Superoxide dismutase (SOD) is an enzyme that catalyzes the dismutation of superoxide anion radicals to hydrogen peroxide according to the following reaction:

\[ 2 \text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \]

Two forms of SOD exist. SOD1 is the cytoplasmic form that contains copper and zinc at its active site while SOD2 is the mitochondrial form that contains manganese at its active site (Fridovich, 1989). Under normal conditions, these enzymes act to keep superoxide concentrations below \(10^{-11}\) M.

SOD1 has been mapped to chromosome 21q22.1 (Wulfsberg et al., 1983) while SOD2 has been mapped to chromosome 6q25.3 (Church et al., 1992). To date, there are no reports on LOH at chromosome 21q22 in lung cancer. However, a study by Virmani et al. (1998) has demonstrated LOH at chromosome 6q25.3 in 22% (2/9) of SCLC cell lines and 73% (8/11) of NSCLC cell lines. Recently, Sanchez-Cespedes et al. (2001) reported chromosomal alterations (i.e., either allelic losses or gains) in 50% (9/18) of primary adenocarcinomas of the lung using a marker for chromosome 6q25-27. The fact that the chromosomal region harboring the SOD2 gene is apparently deleted in a significant fraction of NSCLC cases provides support for the notion that SOD2 may be a tumor suppressor gene. This idea is supported by in vitro studies that have shown that overexpression of SOD2 results in a reversion in phenotype. Church et al. (1993) showed that increased expression of SOD2 caused a suppression of the malignant phenotype of human melanoma cells. Yan et al. (1996) found that SOD2 overexpression caused phenotypic reversion in SV40-transformed human lung fibroblasts. These results
suggest that decreased expression of SOD2 may be involved in the development of the malignant phenotype during carcinogenesis.

The SOD activities of fifteen lung carcinoma tissue samples and corresponding normal samples from the same cases have been determined by Guner et al. (1996). These workers found $3.13 \pm 0.51 \text{ U/mg protein}$ in normal lung tissue and $1.42 \pm 0.24 \text{ U/mg protein}$ (mean $\pm$ SEM) of SOD in lung cancer tissue. This difference was found to be significant at the level of $p < 0.01$. Jaruga et al. (1994) also detected a decrease in SOD activity in lung tumors compared to their surrounding cancer-free tissues (Figure 8). This type of depression in activity has also been seen in lung epithelial lining fluid (ELF) when cancer patients were compared with healthy non-smoking controls (Figure 9) (Melloni et al., 1996).

Figure 8. Activity of superoxide dismutase in human cancerous lung tissues and their surrounding cancer-free tissues. Hatched bars = normal tissue. Solid bars = cancerous tissue. Values = mean $\pm$ S.D. ($n = 3$). From Jaruga et al., 1994.
Figure 9. Superoxide dismutase activity in ELF from nonsmokers and smokers as compared with patients with lung cancer. The Cu-Zn SOD activity is expressed as U/ml of ELF. *$p < 0.05$ compared with nonsmokers; † $p < 0.05$ compared with smokers. From Melloni et al., 1996.

Iwasaki et al. (1998) examined immunohistochemical staining for SOD in both Stage I and Stage III lung adenocarcinomas to determine if there was a difference in enzyme expression between patients who suffered no relapse within a four year period after surgery and those who relapsed. Staining was graded as follows: Grade 1, diffuse, strong staining in ≤ 20% of the cells; Grade 2, between 20% and 80% of the cells exhibit strong staining; and Grade 3, more than 80% of the cells exhibit strong staining. The results of this study are shown in Tables 6 and 7 below.

Table 6. Staining intensity of Stage I lung adenocarcinoma with superoxide dismutase antibody.

<table>
<thead>
<tr>
<th>Grade</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relapse (n = 8)</td>
<td>3 (38%)*</td>
<td>4 (50%)</td>
<td>1 (12%)</td>
</tr>
<tr>
<td>No relapse (n = 22)</td>
<td>18 (82%)*</td>
<td>4 (18%)</td>
<td>0</td>
</tr>
</tbody>
</table>

Table adapted from Iwasaki et al., 1998.
* $p < 0.05$

Table 7. Staining intensity of Stage III lung adenocarcinoma with superoxide dismutase antibody.

<table>
<thead>
<tr>
<th>Grade</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relapse (n = 22)</td>
<td>10 (45%)*</td>
<td>12 (55%)</td>
<td>0</td>
</tr>
<tr>
<td>No relapse (n = 9)</td>
<td>8 (89%)*</td>
<td>1 (11%)</td>
<td>0</td>
</tr>
</tbody>
</table>
These data indicate that SOD overexpression occurs in a higher frequency of cells in adenocarcinomas from patients who relapsed compared to those patients who didn’t relapse. Similar results were obtained with both Stage I and Stage III adenocarcinomas.

As mentioned previously, blood may be an important fluid with respect to the development of biomarkers for persistent oxidative stress. Casado et al. (1995) have compared the activity of SOD in blood obtained from healthy controls to that of lung cancer patients. When the data were stratified according to sex, these workers found a significant decrease ($p < 0.05$) in SOD activity in blood from the male patients ($2.9 \pm 0.3$ U/ml of blood) compared to that of the controls ($4.1 \pm 0.1$ U/ml of blood). No significant difference was seen between female patients and controls.

Cigarette smoke is known to contain oxidants capable of inducing cellular oxidative stress (Pryor, 1997). Hulea et al. (1995) examined the activity of SOD in red blood cells from non-smokers and smokers in different age groups. Their data are summarized in Table 8.

Table 8. Red blood cell superoxide dismutase activity (IU/L).

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Non-smokers</th>
<th>Smokers</th>
<th>$p$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 – 25 yrs</td>
<td>908 ± 14.5</td>
<td>1205 ± 140</td>
<td>0.0005</td>
</tr>
<tr>
<td>26 – 45 yrs</td>
<td>951 ± 16.2</td>
<td>1418 ± 156</td>
<td>0.0005</td>
</tr>
<tr>
<td>46 – 65 yrs</td>
<td>1085 ± 71.4</td>
<td>840 ± 40.4</td>
<td>0.0005</td>
</tr>
<tr>
<td>66 – 80 yrs</td>
<td>724 ± 50.8</td>
<td>612 ± 28.5</td>
<td>0.0005</td>
</tr>
</tbody>
</table>

Table adapted from Hulea et al., 1995. Values shown are means ± S.E.M.
These data indicate that the response of red blood cells to cigarette smoke is dependent on age. In smokers up to 45 yrs of age, SOD activity is elevated, indicative of an adaptive response to persistent oxidative stress. In older people, this adaptive response no longer occurs, resulting in increased oxidative stress in the red cells. This finding is important because it suggests that cellular overexpression of antioxidant enzymes varies with age. Therefore, age must be taken into consideration when attempting to use these enzymes as biomarkers of oxidative stress in carcinogenesis.
CHAPTER V. FORMATION AND REPAIR OF 8-OH-dG

8-Hydroxy-2'-deoxyguanosine (8-OH-dG) is an oxidatively modified base in DNA that results from the attack of either a hydroxyl radical or singlet oxygen on deoxyguanosine (Cadet et al., 1997). 8-OH-dG is mutation prone and results in a G:C → T:A transversion following DNA replication (Kuchino et al., 1987; Shibutani et al., 1991). The repair of 8-OH-dG was first elucidated in E. coli cells (Figure 10). 8-OH-dG is recognized by the MutM protein and excised. If DNA replication occurs before MutM repair, then dATP is inserted opposite the mutagenic lesion. MutY represents a second line of defense. This enzyme acts to remove the adenine base from the 8-OH-dG:A mispair, which reverses this mispair back to a 8-OH-dG:C pairing. If both of these defenses fail, as in cells defective in mutM and mutY, then the result is a G:C → T:A transversion. This dual defense mechanism involving MutM and MutY was first proposed by Michaels et al. (1992).

8-OH-dG can also be produced via oxidation of dGTP to form 8-oxo-dGTP followed by incorporation of the oxidized nucleotide into DNA (Maki and Sekiguchi, 1992; Grollman and Moriya, 1993; Pavlov et al., 1994; Kamath-Loeb et al., 1997). Misincorporation of 8-oxo-dGTP into DNA is prevented by the repair enzyme MutT which dephosphorylates this nucleotide to yield 8-oxo-dGMP (Maki and Sekiguchi, 1992). In cells deficient in mutT, 8-oxo-dGTP is incorporated into DNA to yield 8-OH-dG lesions opposite either cytosine or adenine bases (Figure 10). In the case of the A:8-oxo-dG mispairing, the actions of MutY and MutM actually help fix the mutation instead of repairing it. Thus, incorporation of 8-oxo-dGTP opposite adenine will always lead to
an A:T \rightarrow C:G transversion. Therefore, MutT is the only line of defense against this particular mutation.

![Diagram](image)

Figure 10. 8-Oxoguanine-related mutagenesis and its avoidance mechanism. \( \text{G} \) represents 8-oxo-guanine. From Sekiguchi, 1996.

MutT exhibits 8-oxo-dGTPase activity and thereby acts to convert 8-oxo-dGTP to 8-oxo-dGMP (Figure 11), which cannot be incorporated into DNA (Maki and Sekiguchi, 1992). Phosphorylation of both GMP and dGMP to their corresponding nucleoside diphosphates is catalyzed by human guanylate kinase. However, this enzyme is inactive for 8-oxo-dGMP (Hayakawa et al., 1995). Once formed, 8-oxo-dGMP is dephosphorylated by the action of 8-oxo-dGMPase to yield the corresponding nucleoside, 8-oxo-deoxyguanosine (Hayakawa et al., 1995). This nucleoside can then be transported through the cell membrane and excreted into urine.
Figure 11. Metabolic pathways for normal and oxidized guanine nucleotides. (1) nucleotide diphosphate reductase. (2) nucleoside diphosphate kinase. (3) DNA polymerase. (4) guanylate kinase. From Sekiguchi, 1996.
CHAPTER VI. HUMAN MutT HOMOLOGUE (hMTH1)

Like *E. Coli*, humans also utilize a number of repair enzymes to deal with oxidative DNA damage. Human MutT homologue (hMTH1) is the human equivalent of the *E. Coli* MutT enzyme described in chapter 4. hMTH1 was first identified by Mo *et al.* (1992) who showed that it is an 18-kD protein which acts to hydrolyze 8-oxo-dGTP to 8-oxo-dGMP. The hMTH1 gene has been mapped to chromosome 7p22 (Furuichi *et al.*, 1994). Very few studies have examined LOH at 7p22 in lung tumors. Benachenhou *et al.* (1998) found that this region was deleted in only 10.7% (3/28) of the NSCLC cases examined.

The first study examining expression levels of hMTH1 in cancer was performed by Okamoto *et al.* (1996). These workers measured hMTH1 mRNA levels in forty primary renal-cell carcinoma samples and their corresponding adjacent normal tissues. Figure 12 shows the results of quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis of representative samples from this cohort. They found that levels of hMTH1 were significantly higher in renal-cell carcinomas than those in adjacent normal tissue (*p* < 0.0001). The relationship between hMTH1 mRNA levels and clinical stage of the disease was also examined. Cases were divided into two groups according to the clinical stage (Table 9). Significantly higher hMTH1 mRNA expression was detected in the advanced-stage group compared to the early-stage group (*p* = 0.039). These results indicate that persistent oxidative stress occurs in renal-cell carcinoma. Further, these results suggest that the level of oxidative stress increases with the clinical stage of the disease.
Figure 12. Representative quantitative RT-PCR of hMTH1. Renal-cell carcinoma and adjacent non-tumorous tissue of 9 paired cases. hMTH1 mRNA expression (top, 255 bp) is shown with β2-microglobulin (β2m) mRNA expression as a control (bottom, 120 bp). N, non-tumor; T, Tumor. Clinicopathological features are indicated on the top of each case number. The upper panel consists of early stage tumors while the lower panel shows advanced stage tumors. From Okamoto et al., 1996.
Table 9. mRNA expression of the hMTH1 gene in each clinical stage of renal-cell carcinoma.

<table>
<thead>
<tr>
<th>Clinical Stage</th>
<th>Na</th>
<th>Means ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Kidney</td>
<td>–</td>
<td>0.14 ± 0.08b</td>
</tr>
<tr>
<td>Renal-cell carcinoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>0.34 ± 0.04</td>
</tr>
<tr>
<td>T1N0M0</td>
<td>4</td>
<td>0.15 ± 0.06</td>
</tr>
<tr>
<td>T2N0M0</td>
<td>25</td>
<td>0.31 ± 0.04</td>
</tr>
<tr>
<td>T3aN0M0</td>
<td>1</td>
<td>0.53</td>
</tr>
<tr>
<td>T3bN0M0</td>
<td>3</td>
<td>0.37 ± 0.08</td>
</tr>
<tr>
<td>T2N0M1</td>
<td>3</td>
<td>0.39 ± 0.22</td>
</tr>
<tr>
<td>T3aN0M1</td>
<td>2</td>
<td>0.53 ± 0.19</td>
</tr>
<tr>
<td>T3bN0M1</td>
<td>1</td>
<td>0.58</td>
</tr>
<tr>
<td>T4N0M1</td>
<td>1</td>
<td>0.90</td>
</tr>
</tbody>
</table>

aNumber of cases in which gene expression was determined. bThe values of hMTH1 mRNA expression represent relative expression of this gene to β2-microglobulin. ES, early stage; AS, advanced stage. Table adapted from Okamoto et al., 1996.

Only one study has been performed to date examining the expression of hMTH1 in lung cancer. Kennedy et al. (1998) measured hMTH1 mRNA levels by Northern analysis in normal human bronchial epithelial (NHBE) cells, SV-40-immortalized non-tumorigenic HBE cells (BEAS-2B) and twelve lung cancer cell lines. The results of this analysis are shown in Figure 13. The BEAS-2B cells and eleven out of twelve lung cancer cell lines exhibited overexpression of hMTH1 mRNA relative to that of NHBE cells. These workers also measured levels of 8-oxo-deoxyguanosine in DNA prepared from these cell lines in order to examine the relationship between oxidative DNA damage and hMTH1 mRNA levels (Table 10). BEAS-2B cells and all ten of the lung cancer cell lines examined showed lower levels of 8-oxo-deoxyguanosine than either of the two NHBE strains. The average value for NHBE cells was 5.60 ± 0.75 (mean ± absolute deviation) 8-oxo-deoxyguanosine residues per 10^5 normal deoxyguanosine (dG) residues. The average value for the lung cancer cell lines was 3.02 ± 0.85 (mean ± SD) 8-oxo-
Figure 13. Northern analyses of \textit{hMTH1} mRNA expression in NHBE, BEAS-2B, and human lung cancer cells. Relative Ratio = ratio of \textit{hMTH1} mRNA to \textit{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. From Kennedy et al., 1998.

Table 10.

\begin{tabular}{lll}
\hline
Cell line & Pathology$^a$ & 8-oxo-dG/10$^4$ dG$^b$ \\
\hline
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 \\
\hline
\end{tabular}

$^a$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$^4$ normal deoxyguanosine residues was determined by high pressure liquid chromatography with electrochemical detection (HPLC-ECD).

From Kennedy et al., 1998.
deoxyguanosine residues per $10^5$ normal deoxyguanosine (dG) residues. Values of 8-oxo-dG/$10^5$ dG (Table 10) were plotted against hMTH1 to GAPDH mRNA ratios (Figure 13) to determine whether there is a relationship between total 8-oxo-dG levels and hMTH1 mRNA expression. Figure 14 shows that an inverse linear relationship ($r = -0.75$) exists between these parameters. Together, these results suggest that persistent oxidative stress is a feature of both BEAS-2B cells and lung cancer cell lines. Overexpression of hMTH1 in these cells acts to protect them from oxidative DNA damage, thereby keeping levels of 8-oxo-dG lower than in NHBE cells.

![Figure 14. Relationship between 8-oxo-deoxyguanosine levels and hMTH1 expression for human lung cancer cells. From Kennedy et al., 1998.](image)

Wani et al. (1998) examined the expression of the 8-OH-7,8-dihydroguanosine triphosphatase (hMTH1) gene in normal human breast ductal cells and human breast tumor cells. By in situ hybridization, they found that hMTH1 mRNA levels were not detectable in normal human breast ductal cells. Conversely, staining was detected in 30-85% of the tumor cells in individual tumors (Table 11). Increased expression of hMTH1 was not found to be associated with tumor grade or metastatic malignancy (Table 11).
Table 11.
Expression of the 8-OH-7,8-dihydroxyantamine triphosphatase gene and the surgical/pathological characteristics of breast tumors

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Surgical Pathological Report of Tumors</th>
<th>E-oxo-GTPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>006</td>
<td>Grade 3, ductal carcinoma, one of 18 axillary lymph nodes are involved by metastatic carcinoma</td>
<td>30.0 ± 2.0</td>
</tr>
<tr>
<td>089</td>
<td>Grade 3, invasive ductal carcinoma, 10 of 18 lymph nodes contain metastatic carcinoma</td>
<td>40.0 ± 3.4</td>
</tr>
<tr>
<td>119</td>
<td>Grade 2, infiltrating ductal carcinoma, with lymph nodes identified</td>
<td>61.0 ± 1.5</td>
</tr>
<tr>
<td>003</td>
<td>Grade 3, invasive poorly differentiated infiltrating carcinoma, 16 designated level I and II lymph nodes negative for metastatic tumor</td>
<td>70.0 ± 3.0</td>
</tr>
<tr>
<td>065</td>
<td>Grade 3, infiltrating ductal carcinoma</td>
<td>72.2 ± 3.0</td>
</tr>
<tr>
<td>118</td>
<td>Grade 3, invasive carcinoma</td>
<td>75.0 ± 3.1</td>
</tr>
<tr>
<td>062</td>
<td>Grade 1, poorly differentiated infiltrating ductal carcinoma, eight of 11 axillary nodes contain metastatic carcinoma</td>
<td>78.0 ± 2.4</td>
</tr>
<tr>
<td>011</td>
<td>Grade 3, ductal carcinoma, no definite lymphatic invasion</td>
<td>78.0 ± 2.6</td>
</tr>
<tr>
<td>337</td>
<td>Grade 3, infiltrating ductal carcinoma, with left axillary lymph nodes</td>
<td>80.0 ± 1.5</td>
</tr>
<tr>
<td>195</td>
<td>Grade 2, invasive ductal carcinoma, metastatic carcinoma in four of 10 lymph nodes</td>
<td>82.0 ± 2.8</td>
</tr>
<tr>
<td>037</td>
<td>Grade 2, infiltrating mammary carcinoma, features of variant lobular carcinoma, 15 reactive lymph nodes free of metastatic malignancy</td>
<td>85.0 ± 3.3</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td></td>
<td>68.3 ± 4.2</td>
</tr>
</tbody>
</table>

*The surgical pathological reports for each case were anonymously supplied by the Ohio State University Tissue Procurement Service. Tumor grade was used as an indicator of pleomorphism and mitotic activity of the tumor. Lymph node involvement provided an indication of metastatic malignancy.

*Percentage of ductal cells ± SEM of four different areas exhibiting moderate to strong staining for antibody.

From Wani *et al.*, 1998

These workers also examined the relationship between expression of molecular biomarkers of tumor growth and progression (*i.e.*, cyclin D1, cyclin D3, estrogen receptor, p53, Ki-67, and c-erbB-2) and *hMTIHI* expression. No association was found for any of these markers. The results of this study suggest that persistent oxidative stress is a feature of breast tumors. However, unlike renal-cell cancer (Okamoto *et al.*, 1996), there does not appear to be an increasing level of oxidative stress with clinical stage of the disease in breast cancer.

No studies have been performed to date to determine the mechanism of upregulation of *hMTIHI* expression in cancer. One possibility is that there may be more copies of *hMTIHI* in cancer cells. A number of studies have shown gains of chromosome 7p, either via polysomy 7 or partial gains of 7p (Testa and Siegfried, 1992; Balsara *et al.*, 1997; Bjorkqvist *et al.*, 1998). Another possibility is that *hMTIHI* expression may be upregulated by ROS. Narayanan *et al.* (1997) have shown that exposure of human cells to α-particles results in the production of both superoxide and hydrogen peroxide. Thus,
if ROS do, in fact, mediate expression of *hMTH1*, then exposure of cells to α-particles would be expected to result in an upregulation of gene expression. Kennedy, C.H. (unpublished results) exposed NHBE cells to 0.67 Gy α-particles and measured changes in *hMTH1* mRNA levels by Northern analysis (Figure 15). These results show that *hMTH1* mRNA levels do increase in NHBE cells following exposure to α-particles and that maximum expression occurs four hours after exposure.

![Figure 15](image)

Figure 15. mRNA levels of *hMTH1* in NHBE cells following exposure to 0.67 Gy α-particles. Values indicate hours after exposure that cells were treated for mRNA preparation. Upper band = *hMTH1*. Lower band = *GAPDH*. From Kennedy, C.H. (unpublished results).

Cellular damage resulting from inhalation of asbestos may be caused by the production of ROS (Mossman and Marsh, 1989). Kim *et al.* (2001) examined the expression of *hMTH1* in human lung alveolar epithelial cells after exposure to crocidolite asbestos. These workers found that levels of *hMTH1* mRNA increased up to 18 hr post-exposure and then decreased to control levels at 27 hr (Figure 16).
Figure 16. Time course of expression of hMTH1 mRNA in A549 cells exposed to crocidolite asbestos. (a) Ethidium bromide staining of PCR products. (b) Quantitated results are expressed as the proportion of hMTH1 to β-actin. Bars show the standard error. *, P < 0.05; **, P < 0.01. From Kim et al. (2001).

Together, the results of the α-particle study (Kennedy, C.H., unpublished results) and the crocidolite asbestos study (Kim et al., 2001) suggest that cellular production of ROS does result in the upregulation of hMTH1 expression. However, further work is needed to understand the mechanism of hMTH1 regulation in cells.
CHAPTER VII. 8-OXOGUANINE DNA GLYCOSYLASE (hOGGI)

8-Oxoguanine DNA glycosylase (hOGGI) is the human homologue of the OGG1 gene found in the yeast Saccharomyces cerevisiae. The OGG1 protein catalyzes the excision of 8-oxo-guanine (8-oxoG) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy) from DNA (van der Kemp et al., 1996; Nash et al., 1996). OGG1-deficient strains of S. cerevisiae exhibit a mutator phenotype and specifically accumulate G:C → T:A transversions (Thomas et al., 1996). The OGG1 protein exhibits the same enzymatic activity as the E. coli protein mutM. E. Coli mutators lacking the MutM protein also have an increase specifically in G:C → T:A transversions (Cabrera et al., 1998).

The hOGGI gene has been cloned and sequenced (Aburatani et al., 1997; Arai et al., 1997; Lu et al., 1997; Radicella et al., 1997; Roldan-Arjona et al., 1997; Rosenquist et al., 1997). hOGGI has been localized to chromosome 3p25-26 by fluorescent in situ hybridization analysis of metaphase spreads (Arai et al., 1997; Lu et al., 1997; Radicella et al., 1997; Roldan-Arjona et al., 1997). This region commonly exhibits LOH in lung cancer cells (Hibi et al., 1992).

Arai et al. (1997) have shown that hOGGI is ubiquitously expressed in human tissues (i.e., heart, brain, placenta, liver, lung, skeletal muscle, kidney, and pancreas). However, the expression level in lung was lower than most of the other tissues analyzed. These workers also examined 28 human lung cancer cell lines by Northern and Southern blot analysis. hOGGI transcripts were detected in all cell lines examined by Northern blot analysis. Southern blot analysis was performed with EcoRI digests of genomic DNA from these cell lines. In all of these cell lines, the hOGGI probe detected a single band of
11 kbp. No genetic alterations such as homozygous deletions or rearrangements were detected.

Kohno et al. (1998) performed polymerase chain reaction single-stranded conformational polymorphism (PCR-SSCP) analysis on 45 primary lung tumors in order to detect somatic mutations in hOGG1. Electrophoretic patterns of tumor DNA were compared with that of corresponding normal DNA. No mutations were detected. However, 23 of 45 individuals (51%) were heterozygotes for polymorphisms of the hOGG1 gene. LOH was detected in 15 of the 23 cases (65%). PCR-SSCP analysis was also carried out in 52 lung cancer cell lines. Only one mutation was found. In NCI-H526 cells, a G → T transition was detected at the last nucleotide of exon one. This mutation resulted in a truncated protein. These results validate those of Arai et al. (1997) showing that the mutation rate of hOGG1 is extremely low in lung cancer.

Chevillard et al. (1998) analyzed 25 small cell lung carcinomas by RT-PCR to measure hOGG1 expression levels. hOGG1 expression was detected in all of the samples analyzed. The cDNAs were further analyzed by denaturing gradient gel electrophoresis (DGGE). Two of the tumors had abnormal electrophoretic patterns by DGGE as compared to wild-type hOGG1. The domains showing alterations were amplified by RT-PCR and sequenced. The mutations are shown in Table 12. Both of them are missense mutations, resulting in an amino acid change in the protein sequence. By means of site directed mutagenesis, it was shown that the Arg131Gln mutation completely abolishes the capacity of the hOGG1 protein to repair 8-oxoG. Both of the tumors showing mutations were homozygous mutants, suggesting a hemizygosity due to loss of the wild-type allele. The fact that mutations were only detected in 2 out of 25 tumors (8%)
provides further evidence for the idea that the rate of \textit{hOGG1} mutations in lung cancer is low.

Table 12. Characteristics of the \textit{hOGG1} mutations.

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Codon</th>
<th>Sequence Mutation</th>
<th>Nucleotide Position</th>
<th>Protein Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>131</td>
<td>CGA $\rightarrow$ CAA</td>
<td>730</td>
<td>arg $\rightarrow$ gln</td>
</tr>
<tr>
<td>19</td>
<td>85</td>
<td>GCT $\rightarrow$ TCT</td>
<td>591</td>
<td>ala $\rightarrow$ ser</td>
</tr>
</tbody>
</table>

From Chevillard \textit{et al.}, 1998.

Sugimura \textit{et al.} (1999) examined the role of a common polymorphism in the \textit{hOGG1} gene (Ser326Cys) with respect to lung cancer susceptibility. Repair activity of the Ser326 protein has been shown to higher than that of the Cys326 protein in complementation of \textit{E. coli} that is defective in the repair of 8-hydroxyguanine (Kohno \textit{et al.}, 1998). In the Sugimura \textit{et al.} (1999) study, allele distributions of the Ser326Cys polymorphism were examined in a case-control study of male lung cancer in Okinawa. These workers found that individuals with the Cys/Cys genotype were at an increased risk of squamous cell carcinoma (OR adjusted for age and smoking history = 3.01; 95% CI = 1.33-6.83) and nonadenocarcinoma (adjusted OR = 2.18; 95% CI = 1.05-4.54). The odds ratios for other histological subtypes of lung cancer were not significant. The distribution of this polymorphism was measured in Chinese, Japanese, Micronesians, Melanesians, Hungarians, and Australian Caucasians. The Cys allele was much less prevalent in the latter three populations. These results indicate that the Ser326Cys polymorphism may be a risk factor for susceptibility to the development of lung cancer.

Wikman \textit{et al.} (2000) examined the role of the \textit{hOGG1} Ser326Cys polymorphism in lung cancer susceptibility by means of a nested case-control study. The prevalence of the Ser/Cys genotype was 41.0% in controls and 30.5% in cases. The prevalence of the Cys/Cys genotype was 1.9% in controls and 4.8% in cases. Although the prevalence of
the Cys/Cys genotype was higher in cases than in controls, the calculated odds ratio was not statistically significant (OR = 2.2; 95% CI = 0.41-11.79). These workers also measured LOH at the *hOGG1* locus in 16 lung tumor samples from individuals heterozygous for the Ser326Cys polymorphism. Seven out of the 16 samples (43.8%) exhibited clear LOH. The variant Cys326 allele was lost in 5 out of 7 samples. Tumor stage, smoking (pack years), asbestos (fibers/g lung tissue) and occupational exposure had no influence on the LOH results. LOH was found to be less common in adenocarcinoma cases (2/7, 29%) than among small cell carcinoma cases (4/8, 50%).

The results of this study do not provide support for a role of the Ser326Cys polymorphism in lung cancer susceptibility. They do, however, show that LOH at the *hOGG1* locus is a frequent event in lung cancer. This may lead to increased mutational damage due to ROS in smokers.

Ishida *et al.* (1999) identified two new polymorphisms in exon one of the *hOGG1* gene. Allele two was an A → G transition at base -23 (relative to the start codon) and allele three was a G → T transversion at base -18. These polymorphisms were examined in a population-based study to examine a possible association with lung-cancer risk. The prevalence of allele three was significant among Japanese patients with adenocarcinoma of the lung (OR = 3.152; 95% CI = 1.266-7.845). No significant association was found with the risk of squamous cell carcinoma (OR = 0.861; 95% CI = 0.098-7.585). It was suggested that the allele 3 polymorphism might affect translation efficiency. The results of this study indicate that decreased excision repair of 8-hydroxyguanine by *hOGG1* may play a role in predisposition to the development of lung cancer.
Cellular damage resulting from inhalation of asbestos may be caused by the production of ROS (Mossman and Marsh, 1989). Kim et al. (2001) examined the expression of hOGG1 in human lung alveolar epithelial cells after exposure to crocidolite asbestos. These workers found that levels of hOGG1 mRNA rose significantly in a time-dependent manner (Figure 17). Maximum hOGG1 expression was detected 27 hours after exposure. At this time point, mRNA levels were 7.5-fold above that of the control. These results suggest that hOGG1 expression is upregulated in response to oxidative stress.

Figure 17. Time course of expression of hOGG1 mRNA in A549 cells exposed to crocidolite asbestos. (a) Ethidium bromide staining of PCR products. (b) Quantitated results are expressed as the proportion of hOGG1 to β-actin. Bars show the standard error. *, P < 0.05; **, P < 0.01. From Kim et al. (2001).
CHAPTER VIII. HUMAN MutY HOMOLOGUE (hMYH)

The MutY protein of *Escherichia coli* has an enzymatic activity that removes an
adenine base from an A:8-oxoG base pair (Michaelas *et al*., 1992). *E. coli* mutators
lacking the MutY protein have an increase specifically in G:C → T:A transversions
(Ngheim *et al*., 1988). It has been demonstrated that polymerases responsible for DNA
replication incorporate A across from 8-oxoG up to 200-fold more frequently than they
incorporate C (Shibutani *et al*., 1991). However, polymerases involved in repair
preferentially insert C across from 8-oxoG. Thus, the action of MutY allows MutM
another opportunity to repair the lesion.

The human homologue of the *mutY* gene (*hMYH*) has been cloned, sequenced and
mapped to chromosome 1p32.1-1p34.3 (Slupska *et al*., 1996). This region of
chromosome one has been reported to be frequently deleted in human NSCLC
(Gasparanian *et al*., 1998; Chizhikov *et al*., 2001). Losses in 1p32-pter have been
significantly correlated with advanced stage of the disease as well as post-operative
metastasis and relapse. On this basis, it has been suggested that this region may harbor
tumor suppressor genes involved in the progression of NSCLC. Because of its anti-
mutator activity, *hMYH* is a good candidate for being a tumor suppressor gene.

To date, no studies have examined LOH specifically at the *hMYH* locus. No work
has been done to compare expression levels of *hMYH* mRNA in tumor tissue to that of
corresponding normal tissue. Finally, no studies have investigated the possibility of
*hMYH* mutations in lung cancer samples. Thus, a significant amount of work needs to be
done before the role of *hMYH* in lung cancer is elucidated.
CHAPTER IX. SUMMARY AND RECOMMENDATIONS FOR FUTURE RESEARCH

The literature reviewed in this paper is summarized in Tables 13 and 14.

Table 13. Antioxidant enzyme activities in lung cancer.

<table>
<thead>
<tr>
<th>Antioxidant Enzyme</th>
<th>Activity in Lung Tumors$^a$</th>
<th>Activity in ELF Fluid$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase</td>
<td>↓</td>
<td>No Difference</td>
</tr>
<tr>
<td>Glutathione Peroxidase</td>
<td>↓↑</td>
<td>↓</td>
</tr>
<tr>
<td>Superoxide Dismutase</td>
<td>↓</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Enzymatic activity in tumors relative to normal tissues.

$^b$Enzymatic activity in ELF fluid from lung cancer patients relative to healthy controls.

$\downarrow =$ decreased activity; $\downarrow \uparrow =$ some studies show decreased activity and some show increased activity.

Table 14. Oxidative DNA damage repair gene mRNA levels in lung cancer.

<table>
<thead>
<tr>
<th>Repair Gene</th>
<th>Expression in Lung Tumors$^a$</th>
<th>Response to ROS$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>hMTH1</td>
<td>↑$^c$</td>
<td>↑</td>
</tr>
<tr>
<td>hOGG1</td>
<td>No Difference</td>
<td>↑</td>
</tr>
<tr>
<td>hMYH</td>
<td>?</td>
<td>?</td>
</tr>
</tbody>
</table>

$^a$mRNA levels in tumors relative to normal tissues.

$^b$mRNA levels in exposed cells relative to unexposed control cells.

$^c$mRNA levels in lung cancer cell lines relative to NHBE cells.

$\uparrow =$ increased mRNA levels. $? =$ no data available.

The results of the MEDLINE searches done to prepare this paper indicate that, in general, antioxidant enzyme activity is decreased in lung cancer. Conversely, at least one oxidative DNA damage repair gene (hMTH1) appears to be upregulated in lung cancer.

To date, no studies have been performed to look at either gene expression or enzymatic activity of the six genes studied in either sputum samples or bronchoscopy samples.

Together with ELF, these are the samples that must be examined to ascertain whether the genes examined in this study can be utilized as biomarkers for the early detection of lung cancer. Although Melloni et al. (1996) examined the activities of CAT, GPX and SOD in ELF fluid, these workers had only a very small sample size (i.e., 5 non-smokers, 10 smokers and 10 lung cancer patients). Due to interindividual variability, it is not
surprising that no significant differences in CAT activity were observed between the three experimental groups. Clearly, this study must be repeated with a larger cohort of individuals.

The major limitation of doing studies with ELF is the invasiveness and cost of the sample collection procedure. Bronchoalveolar lavage using fiberoptic bronchoscopy must be performed in order to collect ELF. Therefore, this method does not seem to be practical for early detection of cancer. Conversely, the collection of sputum samples is non-invasive and inexpensive. In an early study, Saccomanno et al. (1974) showed that the progression of lung cancer in a smoker could be detected by means of sputum cytology. Normal sputum contains epithelial cells from the lining of the respiratory tract. Tumors within the respiratory tract may slough cells into the sputum. Further, pre-malignant cells may also be present in sputum samples. For these reasons, sputum is the sample of choice for detecting early genetic changes in lung carcinogenesis.

If pre-malignant or malignant cells are present in sputum, it is predicted that protein levels of CAT, GPX and SOD should be lower in these cells relative to normal cells. Protein levels of hMTH1, hOGG1 and hMYH should be higher in pre-malignant or malignant cells relative to normal cells. In order to test this hypothesis, sputum samples from patients with untreated lung cancer could be collected and stained with antibodies for these six proteins. Through the use of antibodies conjugated to different fluorescent labels, it should be possible to identify cells that underexpress antioxidant enzymes and overexpress oxidative DNA damage repair enzymes. If cells of this type are identified, the sample could then be read by a pathologist to determine if the cells exhibit morphological changes characteristic of cancer cells. If the method can be validated
using samples from patients with untreated lung cancer, then the method could be applied to at-risk individuals \(i.e.,\) smokers with no known history of lung cancer. Detection of pre-malignant or malignant cells by this method would necessitate follow-up by bronchoscopy and/or chest X-ray. If bronchoscopy is recommended, bronchoalveolar lavage could be performed to collect ELF fluid, facilitating the analysis of a greater number of cells. This would serve to confirm the results of the sputum analysis.

It is concluded that antioxidant enzymes and oxidative DNA damage repair enzymes may be good markers for the early detection of lung cancer. However, technological hurdles \(e.g.,\) the production of labeled antibodies) must be overcome before analyses can be undertaken.
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