ORIGINAL ARTICLE

Association of Oxidative Damage Measured by 8-hydroxyguanosine Formation with Altered Risks to Hepatocellular Carcinoma in Malaysian Study Population

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ABSTRACT

Introduction: Oxidative damage is an important factor contributing to ageing and many degenerative diseases. It can be detected by the DNA base damage, which is formation of 8-oxo-7,8-dihydro-2'deoxyguanosine (8-oxodG). The 8-oxodG is an important indicator of oxidative stress and has been competently specified as a recognized initiator of the carcinogenic process and premutagenic injury in mammalian cells. **Aims:** In this preliminary study, we investigated the possible association of oxidative DNA damage in hepatocellular carcinoma (HCC) patients in comparison with Malaysian healthy controls taking into account the different races and genders in both groups. **Method:** DNA of peripheral white blood cells was isolated from 91 HCC patients and 304 controls. The level of oxidative DNA damage was determined by ELISA procedure. **Results:** Quantitative measurement of 8-oxodG was higher in HCC patients at mean value of 3.30 ± 2.32 ng/ml. In controls, the average value is 1.57 ± 1.92 ng/ml. Comparison between gender showed that there was a significant difference observed in the level of 8-oxodG between male and female in controls, where $p = 0.003$. The level of 8-oxodG was higher in male than in female controls. There was a significant difference in the average value of 8-oxodG level between the controls and HCC patients where $p<0.001$. However, no significant difference in the level of 8-oxodG value was observed when compared between Malays and the non-Malays. **Conclusion:** HCC patients showed greater oxidative damage to DNA as compared to controls and this suggests oxidative DNA damage may contribute to the pathogenesis of HCC.

Keywords: 8-oxodG, Hepatocellular carcinoma, Oxidative DNA damage, Reactive oxygen species.

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INTRODUCTION

Reactive oxygen species (ROS) defined as a variety of molecules and free radicals derived from molecular oxygen (1). The ROS like superoxide radicals, hydrogen peroxide and other associated compounds are generated continuously in most tissues. ROS can react and harm many different macromolecules, such as proteins, lipids, and deoxyribonucleic acid (DNA) (2). Therefore, ROS play a vital role in many disease processes (3). Diseases like cancer, diabetes mellitus, insulin resistance, cardiovascular disease, atherosclerosis, and even aging are related to ROS (4). In the living cells, ROS can either be formed by the biochemical reactions occurring naturally or initiated by some external factors on a regular basis. Therefore, the intracellular antioxidant defensive system alone would not be able to prevent the damage done by ROS (5). There are many origins of ROS in human body (6, 7). During the process of mitochondrial respiration, 1 to 5% oxygen taken during respiration through electron transport formed superoxide anion radical (O2-), where 2 kg of these anions formed inside the human body annually (8, 9). Human are exposed to radiation through the environment, either naturally or through artificial sources such as radon gas, cosmic radiation and electromagnetic radiation to name a few.

Other source of exposure is through low wavelength ionizing radiation such as gamma rays, x-rays, and ultraviolet (uv) rays. Gamma rays separates water molecules in the body to form reactive hydroxyl radical meanwhile (uv) rays cleaved O-O covalent bonds on hydrogen peroxide to form two hydroxyl molecules (10). Deoxyribonucleic acid (DNA) is almost certainly the top biologically notable target of oxidative stress.

Continuous oxidative damage to DNA contributes largely to carcinogenesis as well as progression of many major cancers like breast, rectum, colon and prostate (11-13). The constant state of oxidative DNA damage in human cells is a predictive biomarker of cancer development later in life (15). Oxidative DNA damage involves the oxidation of purines and pyrimidines specifically and also alkaline labile sites as well as strand breaks, which is formed directly through DNA repair process (15-17). Almost all the damages are caused by ROS. ROS can attack any cell structures or molecules. Damage to (DNA is involved in at least two processes, which are cancer and aging (13). Disruption that occurs in cell metabolism can result in cell death while less lethal effects can lead to cancer induction and increase in tumour progression (18). There are more than one hundred classified oxidative DNA lesions (19). Some of the products formed from the purines and pyrimidines include 2-hydroxy adenine, 8-oxoadenine, fapiadenine, 5-hydroxymethyluracil, cysteine glycol, 5-hydroxycysteine and thymine glycol. There are also modifications in bases and pentose sugar groups. The modifications that occurred depend on the ROS involved, apart from the condition of the molecules that is being attacked such as oxygen tension, transition chelating metals as well as the presence of reducing agents (16). Hydroxyl radicals are able to produce almost all modification forms (15). Among all modifications, 8-oxo-7,8-dihydro-2-deoxyguanosine (8-oxodG/ 8OHdG) are the most ubiquitous product present in the body and the most studied (5, 20–22).

8-oxodG stimulates the activation of codon 12 oncogene c-Ha-ras or K-ras in mammalian system (23). This adduct is chosen in this study because it is formed from the attack of hydroxyl radical on the position of C-8 deoxyguanosine. As a result, it is a specific marker for oxidative damage. Levels of 8-oxodG and other modified bases evaluated in isolated DNA indicate a dynamic equilibrium between oxidative damage and damage restoration rates (14, 24). 8-oxodG causes the transversion of G to T which can results in mutations when over accumulation occurs (24). In human tumour, transversions of T to G is the hot spot for mutation, the most occurring mutation in genes that are related with tumourigenesis such as tumour suppressor gene p53 (14). This modification involves the hydroxylation of C-8 guanine to DNA bases (17). The products of oxidative damage that have been repaired such as bases and deoxidized nucleic acid are unsuitable substrates for enzyme reaction during nucleotide synthesis. Furthermore, the structure of 8-oxodG is water soluble. Therefore, this product is generally excreted in urine without undergoing any metabolism and is suitable to be used as sample for determination of damage (25). Thus, its measurement could indicate whole-body DNA damage (26, 27). DNA damage determined through urine analysis can be combined with quantification of 8-oxodG in cellular DNA to study the rate of repair versus rate of damage and its implication (13).

Hepatocellular carcinoma (HCC) is the most common type of major liver cancer (28). This disease is the third most common cause of cancer deaths with a 5-year survival rate of 7% (29). Even though there are positive developments in diagnostic and therapeutic techniques, HCC incidents are increasing every year (24). There are more than 1 million deaths each year worldwide caused by HCC (30). There are distinct variation in geography and ethnicity in HCC incidents where the highest incidence are in China, Southeast Asia and South Africa (31) with the total that includes less than one incident per hundred thousand people in several area in Northern Europe to the total of more than one hundred people per hundred thousand people in several area in China and South Africa (32). The difference in the incident of liver cancer in various countries is caused by ethnical composition and environmental factors either happen naturally or triggered by socioeconomic, culture and lifestyle diversity (33). Main causing factor of HCC is infection of chronic hepatitis B virus (HBV), involving 50% to 80% cases. Other aetiological factors are infection of chronic hepatitis C virus (HCV), exposure to aflatoxin, male gender and various other chronic liver diseases (29). A research reported that most of the HCC incidents are higher in the area which has the exposure towards liver carcinogen such as aflatoxin (34). Nevertheless, activated immune response towards the host increases the formation of reactive oxygen species which plays a vital role in initiating the development of abnormal liver cells (18, 35). At the molecular level, a number of genetic occurrences have been linked with the development of HCC whereby major carcinogenic pathways related in HCC development and progression are contributed by the inactivation of the tumour suppressor p53, Wnt/wingless genes, predominantly through mutations of β-catenin, Ras/Raf/MEK/ERK and PI3K/Akt/ mTor (36).

MATERIALS AND METHODS

Determination of oxidative DNA damage level (8-oxodG)

Oxidative DNA damage level (8-oxodG) was determined by using a highly sensitive ELISA kit which was produced from Japan Institute for Control of Ageing (JaICA), Nikken Foods. Ltd.. The procedure for determination of oxidative DNA damage level involved pretreatment and ultrafiltration of samples before the ELISA assay was started. Pretreatment and ultrafiltration were done to isolate particles that might interrupt the ELISA assay procedure later. ELISA assay was conducted competitively in vitro and has the assay range of 0.125 ng/ml to 10 ng/ml.

Pretreatment of DNA

Before the determination of oxidative DNA damage level (8-oxodG) was carried out, DNA sample was

digested by the method suggested by Ravanant (1998) and Evans (1999) with some modification. First, 75 µg of DNA was pipetted into a final volume of 165 µl mixture of 15 unit of nuclease P1 enzyme, 15 µl of nuclease P1 enzyme buffer and autoclaved distilled water. The mixture was incubated for 2 hours at 37°C. Then, 1 U of alkaline phosphotase enzyme and 10 µl alkaline phosphotase enzyme buffer were added to the mixture and incubated at 37°C for 1 hour. Next, the digested DNA sample was ultrafiltrated to isolate any particles which carry molecular weight of more than 10000x by using microcon filtration tube by Milipore. All the DNA samples were then inserted into the filtration cup and the samples were centrifuged at 10000 xg for 15 minutes at 4°C. The samples that have been filtrated were used in the ELISA assay.

ELISA PROCEDURE

All the reagents inside the kit and samples were left at room temperature (20-25˚C) for 15 minutes prior usage. Then, antibody primer was mixed with antibody primer solvent and let to be dissolved before used. 50 µl of digested standard DNA sample (in duplicates) was pipetted into each well. For precision, the outermost well at column 1 and 8 were not used. Next, 50 µl of antibody primer that has been prepared beforehand was added to each of the 96 well microtiter plate that has been de-precoated with 8-oxodG. The 96 well microtiter plate (MTP) is capped with adhesive plastic microtiter plate cover and incubated overnight at 4˚C. After 18-20 hours, the contents inside the MTP were discarded into the wash basin and 250 µl of 1X washing solution was pipetted into each well. MTP was shook gently to allow the washing solution to cover all area inside the well. The washing solution was discarded by inverting the MTP on a napkin paper and the MTP well was then blotted several times with napkin papers. The washing step was repeated for three times. Then, secondary antibody was mixed with the solvent of secondary antibody and let dissolved thoroughly. 100 µl of secondary antibody solution was added to each of the wells and the surface of the MTP was tightly closed with adhesive plastic microtiter plate cover and incubated at room temperature for 1 hour. Meanwhile, the enzyme substrate solution was diluted with 100 times the volume of diluting solution. After 1 hour of incubation, the MTP contents were discarded into the wash basin and the washing steps that have been explained before was repeated once more. 100 µl of diluted enzyme substrate was added into each well. The MTP was incubated in room temperature for 15 minutes in the dark. MTP was wrapped with aluminum foil and placed on belly dancer at 400 rpm to mix well the contents of the MTP. Finally, 100 µl of end reaction solution was added into each well and after 3 minutes, the absorption reading has to be done within 30 minutes. The standard curve was used to determine the concentrations of 8-oxodG that was present in the samples. The standard curve was obtained by plotting the values of absorption against the log of standard concentrations of 8-oxodG which was provided by the kit.

CALCULATIONS

The determination of oxidative DNA damage level was carried out by measuring the level of 8-oxodG which was obtained by plotting the samples reading onto the standard curve of O.D absorption against the log of standard concentrations of 8-oxodG. This was provided by the kit. Before the standard curve was plotted, the average value of the O.D absorption was calculated by subtracting the average value of standard sample absorption or respondents samples ($O.D^S$) with the average value of standard blank ($O.D^B$) as shown.

$$\text{Absorption O.D} = (O.D^S) - (O.D^B)$$

Then, the value of the oxidative 8-oxodG DNA damage of subject samples were calculated and determined from the standard curve. The results were reported as ng 8-oxodG per 50 µl DNA sample or ng/ml.

QUALITY CONTROL

Each sample was assayed in duplicate at two different times. At least 5% of the overall samples were chosen to be repeated randomly in order to see the consistency of the data. To avoid side effect, the outermost well at column 1 and 8 were not used. In order to standardise the temperature inside the well, a volume of autoclaved distilled water was used to fill in the well at column 1 and 8.

RESULTS

Comparison of Oxidative DNA Damage by Measuring the Level of 8-oxo-dG Between Controls and HCC Patients

From 304 subjects in control group, an average of 8-oxodG level determined is 1.572 ± 1.9181 ng/ml whereas the average of 3.304 ± 2.3183 ng/ml is determined from 91 HCC patients. In this study, it was assumed that both population variants from controls and HCC patients are not the same. This is obtained from F test. Furthermore, when carrying out the t-test, there is a significant difference in the average value of 8-oxodG level between the controls and HCC patients with a statistical value of $t = -6.493$, $p<0.001$. This indicated that HCC patients have higher oxidative DNA damage compared to normal subjects in controls (Fig. 1).

Comparison of Oxidative DNA Damage by Measuring the Level of 8-oxo-dG Between Malays and Non-Malays in Controls

From 99 subjects of Malay in control group, an average of 8-oxodG level determined is 1.335 ± 1.6836 ng/ml. An average value of 1.686 ± 2.0155 ng/ml is determined from 205 Non-Malay subjects of the control group. The t-test analysis indicates that there is no significant difference in the average value of 8-oxodG level

between the Malays and Non-Malays in control group with a statistical value of t = -1.60, p>0.05. This value indicates that both population variants of Malays and Non-Malays from the control group is not the same. This result is obtained from the F test. This indicates that races do not contribute towards oxidative DNA damage by the formation of 8-oxodG (Fig. 2).

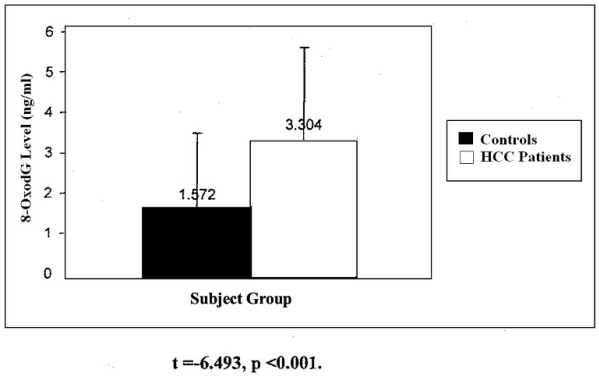


Fig. 1 : Comparison of oxidative DNA damage by measuring the Level of 8-oxo-dG between controls and HCC patients.

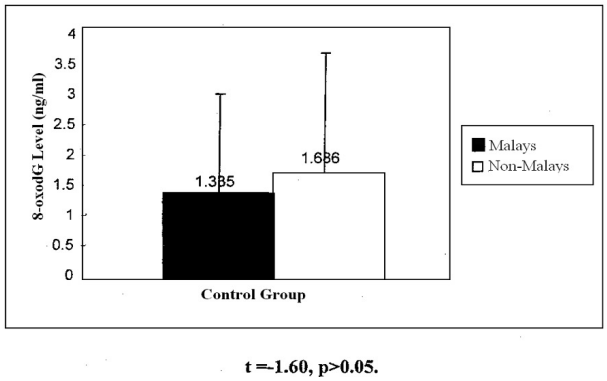


Fig. 2 : Comparison of oxidative DNA damage by measuring the level of 8-oxo-dG between Malays and Non-Malays in controls

Comparison of Oxidative DNA Damage by Measuring the Level of 8-oxo-dG Between Malays and Non-Malays in HCC Patients

From 39 subjects of Malay races in HCC patients, an average of 8-oxodG level determined are 3.592 ± 2.5984 ng/ml. An average value of 3.029 ± 2.0601 ng/ml is determined from 52 Non-Malay subjects HCC patients. Based on the F test, it is assumed that both populations of Malays and Non-Malays in HCC patients are the same. The t-test analysis indicates that there is no significant different in the average value of 8-oxodG level between the Malay s and Non-Malays in HCC patients with a statistical value of t = 1.173, p>0.05 (Fig. 3).

Comparison of Oxidative DNA Damage by Measuring the Level of 8-oxo-dG Between Genders in Controls

From 304 subjects, there are 227 males and 77 females in the control group. In 227 male subjects in the control group, an average value of 8-oxodG level determined is 1.736 ± 2.0334 ng/ml. In 77 female subjects, an average value of 1.087 ± 1.4330 ng/ml. based on the F test, both population variants are assumed different. The t-test analysis indicates that there is a significant difference in the average value of 8-oxodG level between the males and female in control group with a statistical value of t = 3.063, p<0.005. This indicates that males have higher oxidative DNA damage compared to females in control group (Fig. 4).

Comparison of Oxidative DNA Damage by Measuring the Level of 8-oxo-dG Between Genders in HCC Patients. From 91 subjects, there are 73 males and 18 females in HCC patients. In 73 male subjects, an average of 8-oxodG level determined is 3.374 ± 2.2559 ng/ml. In 18 female subjects in HCC patients, an average value of 2.823 ± 2.5378 ng/ml is determined. The t-test analysis indicates that there is no significant difference in the average value of 8-oxodG level between males and females in HCC patients with a statistical value of t = 0.906, p>0.05. This result is obtained by assuming both of the population variants of males and females in HCC patients are the same (Fig. 5).

DISCUSSION

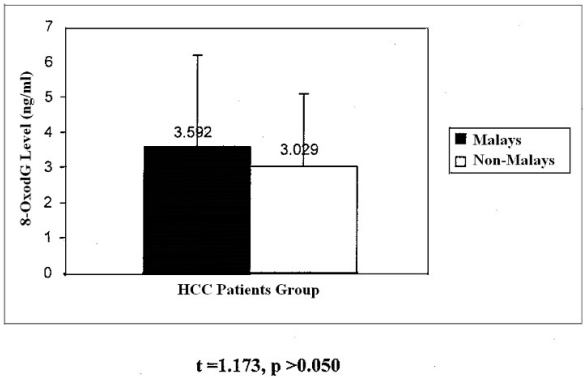


Fig. 3 : Comparison of oxidative DNA damage by measuring the level of 8-oxo-dG between Malays and Non-Malays in HCC patients.

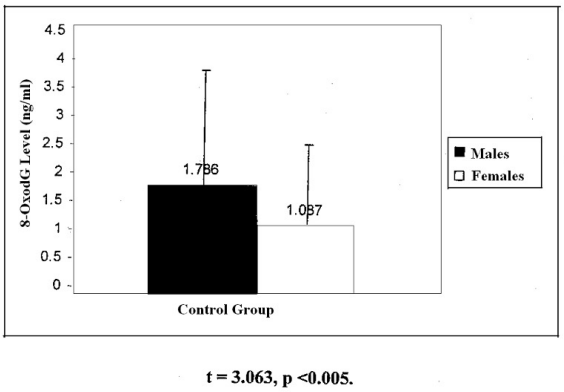
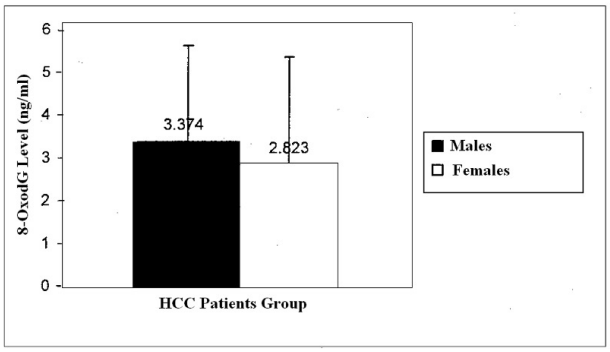


Fig. 4 : Comparison of oxidative DNA damage by measuring the level of 8-oxo-dG between genders in controls.



t = 0.906, p>0.05

Fig. 5 : Comparison of oxidative DNA damage by measuring the level of 8-oxo-dG between genders in HCC patients.

The relation of oxidative DNA damage level with increase risks of HCC

In this study, 8-oxodG is chosen instead of 8-oxoG because the mutagenic lesion of 8-oxoG is excreted out of the body faster in the presence of glycosylase (37). The average oxidative DNA damage level for the control group is 1.5719 ± 1.9181 ng/ml whereas the average of 3.3041 ± 2.3183 ng/ml is determined from HCC patients. These clearly indicate that HCC patients have higher oxidative damage level compared to subjects in control groups. There is a possibility that this oxidative DNA damage plays a role in initiating hepatocarcinogenesis. Previous study showed that 8-oxodG base pairs are easily combined with A instead of C and this can result in huge amount of transversion from G→T. The transversion from G→T are common in tumour genes such as p53 gene (19).

Comparison of oxidative DNA damage level between Malays and Non-Malays in Malaysian population

The comparison of oxidative DNA damage levels does not show any significant difference. This indicates that either Malays or Non-Malays (race) does not influence oxidative DNA levels directly respectively. As discussed before, Malay and Non-Malay subjects are staying in the same population, they were exposed to similar environment. Apart from that, nutritional intake and smoking habit are also similar, thus resulting in indifference oxidative DNA damage level.

Comparison of oxidative DNA damage levels between particular race done in different countries through meta-analysis or collaborative research showed ambiguous data. There is a vast difference in the average value of 8-oxodG obtained from previous studies compared to this present one. Therefore, the value comparison is irrelevant because it all depends on the measurement platform used. For example, the level of 8-oxodG levels obtained from GC-MS method are higher than the levels obtained from HPLC-EC method. Apart from that, the DNA isolation method chosen for oxidative damage analysis plays an important role in determining the reading. Using phenol for this purpose was reported to cause depletion in metal ions which naturally present in biological extracts. These ions participated in the Haber-Wiess/Fenton reaction and in turn will generate free hydroxyl radicals which can oxidise large amount of DNA (19).

According to Helbrock et al. (1998), the comparisons of oxidative 8-oxodG levels can be categorised from DNA isolation methods that the researchers used. DNA isolation method using Sodium Iodide is reported to contribute to less than ten percent of the level obtained through other DNA isolation method including the method that used phenol (38).

Having subjects from different population with probably different lifestyles and nutritional habit may also cause the difference in the oxidative damage level. There is not much similar research done in a multiracial population anyway. If it was carried out the sample size for most researches is too small to produce a conclusive finding.

Comparison of oxidative DNA damage level between genders in Malaysia

The average 8-oxodG levels determined in male subjects is 1.7364 ± 2.0334 ng/ml whereas the average value of 8-oxodG levels in female subjects is 1.0874 ± 1.4330 ng/ml. The t-test analysis indicates that there is a significant difference in the average value of 8-oxodG level between the males and female in control group with a statistical value of t = 3.063, p>0.005. The exact causes of this situation are still unclear. However, these trends could be best explained by the fact that male subjects are seen to lead more unhealthy lifestyles compared to female subjects. According to Huang et al. (2000), a few aspects in lifestyles such as smoking habit, alcohol consumption and bad sleeping pattern can contribute easily towards the production of high oxidative DNA damage (39).

A research done in Europe showed that 8-oxodG levels are higher in males compared to the female subjects. The research was trying to relate the association of 8-oxodG levels with the content of antioxidant diet intake in the blood stream. However, the analysis could not relate nor explain the relation of antioxidant diet with high level of 8-oxodg readings in males. The level of intrinsic antioxidant defense such as glutathione, superoxide dismutase and glutathione peroxidase measured does not show any significant correlation between genders (40).

CONCLUSION

In conclusion, in this research, HCC patients showed greater oxidative DNA damage as compared to controls and the findings of the study suggests that oxidative DNA damage may contribute to the pathogenesis of HCC. Comparison between gender showed that there is

a significant difference observed in the level of 8-oxodG between males and females in controls. However, no significant difference was observed in Malays and non-Malays in the level of 8-oxodG.

ACKNOWLEDGEMENT

We would like to thank Prof. Datuk Dr. Aminuddin Ahmad, Prof. Datuk Dr. Harun Ahmad, and Prof. Emeritus Datuk Dr. Wan Zurinah Wan Ngah for their help in facilitating blood samples collection from HCC patients of Hospital Kuala Lumpur and Hospital Universiti Kebangsaan Malaysia. We would also like to thank all the staff in National Blood Bank of Malaysia for their support during the blood collection of control samples.

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