Analysis of O^6-Methylguanine in Cancer Patient Blood during Administration of Cyclophosphamide Using Ultra High Performance Liquid Chromatography-Tandem Mass Spectrometry

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Authors' contributions

This work was carried out in collaboration among all authors. Author YH designed the study and wrote the protocol. Author RA managed the cancer patients. Author YC collected the blood, performed the DNA isolation, and analyzed the data using LC/MSMS. Author SAN did the optimization and method validation. Author Harmita supervised the analytical method. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Aims: To determine O^6-Methylguanine in Cancer Patient Blood during Administration of Cyclophosphamide using Ultra High Performance Liquid Chromatography-Tandem Mass Spectrometry.
Place and Duration of Study: Dharmais Cancer Hospital and Bioavailability/Bioequivalence

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Laboratory, Faculty of Pharmacy, University of Indonesia. Duration: Dec 2012 until May 2013.

Methodology Study Design: This study was approved by the Ethics Committee of Medical Faculty, University of Indonesia. Cross sectional design was conducted for this study, blood samples were collected from 72 cancer patients receiving four or more cycles of chemotherapy with regiment which contains cyclophosphamide. DNA adduct was analysed from isolated DNA after the fourth cycle chemotherapy or more by UPLC-MS/MS ESI+ and the analysis mode on value of m/z 166.10>149.10 and 166.10>134.10.

Results: The method was validated using a calibration curve with good linearity (r>0.999); the coefficient of variance was <6.54%; the recovery was in the range of 90.52-109.65% Among 72 analyzed samples, O^6-methylguanine was detected in 17 samples and could be quantified in 1 sample at a concentration of 5.87 ng/mL.

Conclusion: The results of this study showed that O^6-methylguanine is not always found in cancer patients treated with cyclophosphamide. The detected and quantified O^6-methylguanine can be a predictor of secondary cancer risk therefore, the dose administered should be monitored and set to the appropriate and safe levels, in order to reduce the risk of secondary cancer.

1. INTRODUCTION

Cyclophosphamide is an antineoplastic alkylating agent that alkylates DNA at nucleophilic sites (especially N^7-guanine, which is the major target) and produces cytotoxic effects that inhibit the development of cancer [1,2]. It is used in the treatment of Hodgkin's and non-Hodgkin's lymphoma, multiple myeloma, leukemia, neuroblastoma, sarcoma, ovary cancer, and breast cancer. One of the severe side effects of cyclophosphamide is secondary cancer, such as acute myeloid leukemia, non-Hodgkin's lymphoma, and bladder cancer [3]. It is classified as a Group 1 carcinogen by the IARC [4]. Cyclophosphamide has the highest number of secondary cancers observed after being used in primary cancer therapy [5]. Secondary cancers in patients after administering cyclophosphamide are not only found in breast cancer patients but also in other types of cancer, such as Hodgkin's and non-Hodgkin's lymphoma, Ewing's sarcoma, and ovarian cancer [6-8]. The secondary cancer, induced by the therapy, appears months or years after the therapy was stopped [9]. The secondary cancer is usually less responsive to chemotherapy than the primary cancer [10], therefore, therapy monitoring to minimize risk and to early detect secondary cancers will be more useful.

DNA has several nucleophilic sites that are vulnerable attacked by alkylating agents, such as O^6-guanine, which is one of the sites in guanine that forms hydrogen bonds with cytosine. Alkylation on O^6-guanine, which forms the DNA adduct O^6-alkylguanine, causes guanine to only form two hydrogen bonds, leading to mismatched DNA bases and mutations. This interference causes the mutagenicity of cyclophosphamide, leading to secondary cancers [11]. Detecting O^6-methylguanine can be one method to monitor the usage of chemotherapy and predict the risk of secondary cancers in patients who received the treatment [12]. A higher level of detected O^6-methylguanine in a patient indicates increased risk. The level of detected O^6-methylguanine depends on the method used [13]. A sensitive and selective analytical method for O^6-methylguanine is needed because it is found at low concentration in the biological samples. Many methods for the analysis of DNA adducts have been reported. Previous research has analyzed O^6-methylguanine *in vitro*, in rat blood, and in breast cancer patient blood (who had been administered with cyclophosphamide) using HPLC-fluorescence and strong cation exchange columns [14-16]. The analysis of DNA adducts using UPLC-MS/MS is the most sensitive and selective method compared to the others. The chemical structures of O^6-methylguanine and its isomers N7 methylguanine can be seen in Fig. 1. The goal of this research is to analyze and quantify of O^6-methylguanine formed in cancer patients who received cyclophosphamide during their chemotherapy.
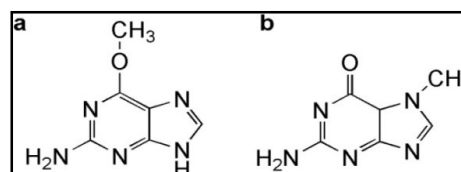
Fig. 1. Chemical structures of O6-methylguanine (a) and N7-methylguanine (b)

2. MATERIALS AND METHODS

This study was approved by the Ethics Committee of Medical Faculty, University of Indonesia. Cross-sectional design was conducted for this study. The samples were blood from 72 cancer patients, who administered chemotherapy with regiment which contains cyclophosphamide. The patients signed the informed consent prior participating in this study. The patients received 500-1000mg/m^2 of cyclophospamide. Then the DNA adduct was analysed from isolated DNA after the fourth cycle chemotherapy or more.

Blood samples from patients who fulfill the inclusion criteria were collected. Inclusion criteria were:

a. Patient of Dharmais Cancer Hospital.
b. Receive cyclophosphamide as cancer chemotherapy, single or combination.
c. Each patient does not receive any other alkylating agent in the therapy.
d. Patient has undergone 4 cycles of chemotherapy or more
e. Patient is willing to take part in the research and signed the Informed Consent.

2.1 Chemicals and Reagents

N^7-Methylguanine, O^6-methylguanine, guanine, and adenine were purchased from Sigma Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC grade), methanol (HPLC grade), acetic acid (HPLC grade), and formic acid (HPLC grade) were purchased from Merck (White House Station, USA). The reagents for the DNA Isolation are Proteinase K, Buffer AL, Buffer AW1, Buffer AW2, and Buffer AE (QIAamp DNA Mini Kits, QIAGEN), and ethanol absolute (Merck).

2.2 Preparation of the Stock and Working Standard Solutions

A stock solution of O^6-methylguanine was prepared at 1.0 mg/mL in methanol, and a stock solution of N^7-methylguanine was prepared at 1.0 mg/mL in methanol containing 10.0% (v/v) formic acid. A series of working standard solutions at appropriate concentration levels were obtained via diluting each standard solution with water containing 0.5% (v/v) formic acid. All solutions were stored at 4°C.

2.3 The UPLC-MS/MS Conditions

The UPLC-MS/MS system consisted of a binary pump, auto sampler, C$_{18}$ Acquity BEH column (1.7 µm, 100 mm × 2.1 mm, Waters, Milford, MA, USA), and mass spectrometry type quadrupole (Xevo TQD, Waters). The optimum chromatographic conditions were isocratic elution over 3 minutes, a mobile phase consisting of acetic acid (0.05%) in water-acetonitrile (95:5, v/v), a flow rate of 0.3 mL/min, and an ionization method of ESI+. The quantification was conducted using multiple reaction monitoring (MRM), and the quantification traces were 166.1>149.1 and 166.1>134.1 for O^6-methylguanine, 166.1>149.1 and 166.1>96.1 for N^7-methylguanine, 135.9>119.1 for adenine, and 152.1>110.2 for guanine. The injection volume was 10.0 µL. The mass spectrometry conditions are shown in Table 1. The data were processed using MassLynx, version 4.1 software (Waters, USA).

2.4 Method Validation

The validation parameters were the specificity, linearity, range, limit of detection, lower limit of quantification, accuracy, and precision. The method was validated in accordance with ICH guideline Validation of Analytical Procedures [17]. The specificity of the method was demonstrated by identifying 10 µL solutions of O^6-methylguanine and N^7-methylguanine based on their relative retention times. Calibration curves over the concentration ranges of 0.5-35.0 ng/mL for O^6-methylguanine. Weighted (1/x) linear regression analysis was used to determine the slopes, intercepts, and correlation coefficients (r). The limit of detection was the lowest concentration that was detectable but not necessarily quantifiable and have signal-to-noise ratio greater than 3. The lower limit of quantification was the lowest concentration that the method could measure with acceptable precision and accuracy and had signal-to-noise ratios greater than 5. The accuracy and precision were evaluated at four concentrations over three consecutive days. The accuracy of the method was expressed by the %recovery, whereas precision was expressed by the %CV.

2.5 Samples Preparation

The extraction of DNA from 200 µL blood samples was performed according to the procedures of the QIAamp Mini Kits from QIAGEN [18] as followed:

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- Blood in K3EDTA tube was added to tube which contain proteinase K and Buffer AL and incubated on 56°C to destroy blood cell and release the DNA.
- Added absolute ethanol binded released DNA and separated it from sample matrix. QIAamp Spin column which used in this extraction consisted of a designed silica layer can trap the DNA on it when centrifuged.
- Then added buffer AW1 and AW2 to separate protein from DNA, therefore the purity of DNA was increased.
- The DNA on silica layer was eluted by using buffer AE, this buffer consisted of 10 mM Tris.Cl, 0,5 mM EDTA, pH 9,0, therefore it can store DNA and avoid DNA degradation in low pH.

The concentration of the isolated DNA in Buffer AE was determined using GeneQuant DNA-RNA Calculator. The DNA was stored in -20°C before analysis. The DNA solution (200 µL) was mixed with HPLC grade water and formic acid (90%) in equal volume [19]. The solution was mixed and heated at 80°C for 60 minutes using a Thermomixer (Eppendorf) [20]. The resulting hydrolyte was injected into the UPLC-MS/MS.

3. RESULTS AND DISCUSSION

A new simple, rapid, accurate, and precise method for the analysis of O^6-methylguanine and N^7-methylguanine using UPLC-MS/MS has been developed. Analysis using UPLC-MS/MS produces sensitive, selective and rapid results within 3 minutes. Before analyzing the compounds with MS, we have to transfer them to gas phase. ESI+ was chosen as the ionization method because it provides mild ionization, and it is simple and suitable for analytes. O^6-methylguanine and N^7-methylguanine are isomers and they have the same precursor ion (m/z 166.1) and a sensitive product ion (m/z 149.1). O^6-methylguanine was detected at m/z 166.10>149.10 and 166.10>134.10, while N^7-methylguanine at m/z 166.10>149.10 and 166.10>96.10. The precursor ion (m/z 166.1) showed that the compounds were protonated, and their molecular weight was increased by one amu. The most sensitive produced ion (m/z 149.1) showed that the amine group (-NH$_2$) detached from the structure. The produced ion at 134.10 showed that the methoxy group (CH$_3$O-) detached from the structure of O^6-methylguanine, whereas the produced ion at 96.10 showed that N^7-methylguanine has lost the –CONHCNH$_2$ group from its structure. This fragmentation can be seen in Fig. 2.

Various combinations of solvents were investigated to find the most suitable mobile phase that produces rapid, sharp, and sensitive peaks. The analysis showed that the combination of 0.05% acetic acid-acetonitrile (95:5, v/v) using isocratic elution and a flow rate of 0.3 mL/min produced the best peak.

The method showed specificity for O^6-methylguanine and N^7-methylguanine with retention time of O^6-methylguanine was 1.44 min, while 1.09 min for N^7-methylguanine. The peaks are also sharp and free from other interferences (Fig. 3).

The calibration curve was linear over the concentration range of 0.5-35.0 ng/mL for O^6-methylguanine and 1.0-40.0 ng/mL for N^7-methylguanine with a regression coefficient (r) of 0.9999 for both O^6-methylguanine and N^7-methylguanine. The calibration curves are shown in Fig. 4.
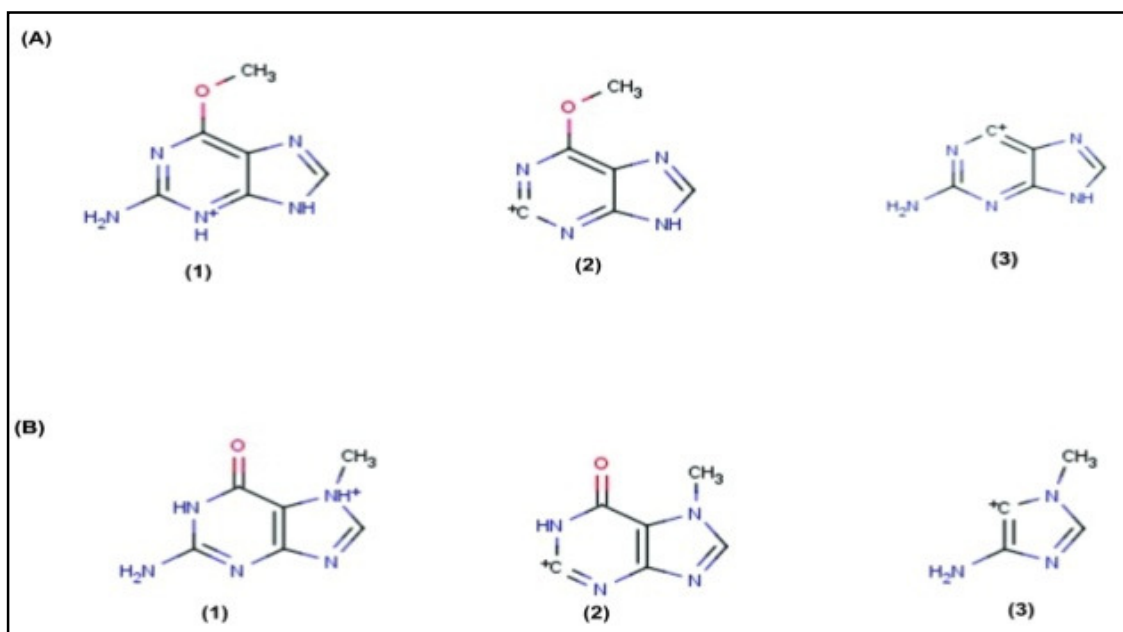
The limits of detection and lower limits of quantification were determined from the signal-to-noise ratios and were found to be 0.1 ng/mL and 0.2 ng/mL for O^6-methylguanine and 0.5 ng/mL and 1 ng/mL for N^7-methylguanine. The intraday and interday accuracy and precission values are shown in Table 2. These parameters were observed at four concentrations: the LLOQ, low, medium, and high concentration. The intraday accuracy value of O^6-methylguanine ranged from 92.63% to 109.65% with %CV values ≤6.54%. N^7-methylguanine ranged from 98.64% to 106.65% with %CV value ≤3.12%. The interday accuracy values for O^6-methylguanine were 90.52%-109.65% with %CV values ≤2.68% and for N^7-methylguanine were 93.77%-106.65% with %CV values ≤ 1.67%.

## 3.1 Analysis of O6-methylguanine in Samples

This research only determined O6-methylguanine because it has carcinogenic effect thus can trigger secondary cancer on patients administered with alkylating agent. The formation of N7-methylguanine will form cross-linking with DNA and will give cytotoxic effect, while the O6-methylguanine compound does not form cross-linking with DNA and the compound is more stable than N7- methylguanine so that it can accumulate in the human body.

Table 1. Analytical condition for the mass spectrometry

| Compound	Ion fragment (m/z)	Ionization mode	Capillary voltage (kV)	Temperature of gas desolvation (℃)	Flow rate of desolvation gas (L/hour)	Orifice voltage (V)	Collision voltage (V)
O^6-MeG	166.10	ESI +	3,5	500	1000	40	-
	149.10					40	17
	134.10					40	23
N^7-MeG	166.10					42	-
	149.10					42	17
	96.10					42	26
Adenine	135.97					40	-
	119.18					40	33
Guanine	152.16					37	-
	110.28					37	31

Fig. 2. Fragmentation of O^6-methylguanine (A): (1) m/z 166.1; (2) m/z 149.1; (3) m/z 134.1 and N^7-methylguanine (B): (A) m/z 166.1; (2) m/z 149.1; (3) m/z 96.1
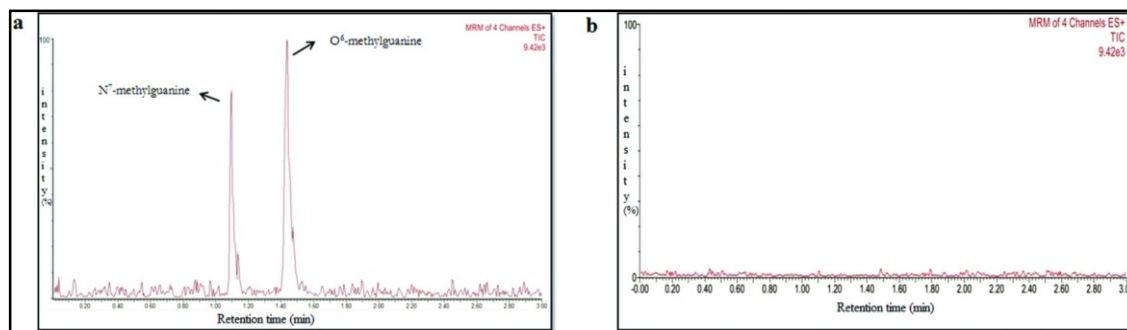
Fig. 3. Chromatogram of O6-methylguanine and N7-methylguanine (a). Chromatogram of the DNA hydrolyte of a healthy subject (b)

Table 2. Intraday and interday accuracy and precission values for O^6-methylguanine and N^7-methylguanine

| Compound	Intraday				Interday			
	Concentration (ng/mL)	Mean (ng/mL)	% recovery	%CV	Concentration (ng/mL)	Mean (ng/mL)	% recovery	%CV
O^6-methylguanine	0.50	0.5076	100.12	6.54	0.50	0.5085	100.29	2.68
	2.00	2.0129	99.45	3.18	2.00	2.0327	100.43	1.05
	20.00	20.3118	100.16	0.80	20.00	20.1880	99.55	0.59
	30.00	30.4902	100.23	1.78	30.00	30.5936	100.57	0.39
N^7-methylguanine	1.00	1.03069	101.85	3.12	1.00	1.01530	100.33	1.64
	5.00	5.08892	100.57	1.34	5.00	5.07656	100.33	0.38
	20.00	20.17063	99.66	0.53	20.00	20.20513	99.83	0.21
	30.00	30.35096	99.97	1.31	30.00	30.49916	100.46	0.47
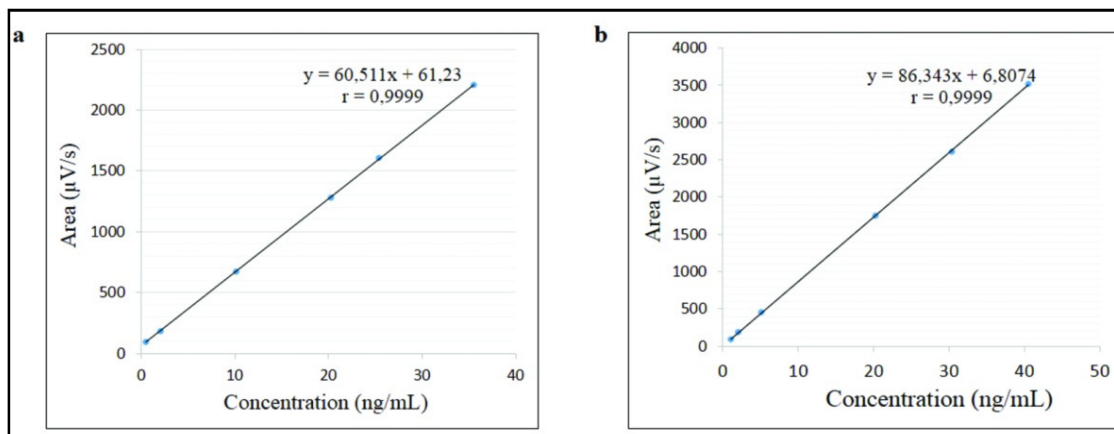
Fig. 4. Calibration curve of O6-methylguanine (a) and N7-methylguanine (b)

The analysis was conducted on 72 hydrolysate samples. The samples consisted of 50 breast cancer patients, 18 non-Hodgkin's lymphoma patients, 2 acute lymphoblastic leukemia patients, 1 lymphoma Burkitt patient, and 1 primitive neuroectodermal tumor patient. The patients received different doses of cyclophosphamide based on the area of the body surface, cancer type, protocol of chemotherapy, and level of toxicity. The doses ranged from 500-1600 mg/mm^2. Among the 72 samples, 24 patients received 4 cycles of chemotherapy, 23 patients received 5 cycles, and 25 patients received 6 cycles. One cycle is 21 or 29 days.

Before running the samples, the hydrolysate of the healthy subjects' DNA was injected. The chromatograms of the healthy subject showed that there were no peaks of O^6-methylguanine and N^7-methylguanine, which means that the formation of O^6-methylguanine and N^7-methylguanine as alkylated adducts did not occur in patients who were not exposed to the alkylating agents. Among the 72 analyzed samples, O^6-methylguanine was detected in 17

samples and was quantified in 1 sample. In the other 54 samples, O^6-methylguanine could not be quantified or detected, which could be caused by several factors. First, the doses of cyclophosphamide given to the patients were appropriate, therefore the metabolites of cyclophosphamide did not attack the O^6-guanine site, which is less nucloeophilic than the N^7-guanine site. Second, O^6-methylguanine was formed, but O^6-methylguanine-DNA-methyltransferase (MGMT) removed the alkyl group from the DNA. Among the 18 samples containing O^6-methylguanine, 14 samples (77.78%) were breast cancer patients, and the other 4 samples (22.22%) were non-Hodgkin's lymphoma patients. Compared to the other samples, 14 of the 50 samples of breast cancer patients were positive for O^6-methylguanine (28%), and 4 of 18 samples of non-Hodgkin's lymphoma patients were also positive (21.05%). The doses of cyclophosphamide for the positive samples were in the range of 665 mg/m^2-1600 mg/m^2. 77.78% of these samples had doses more than 800 mg/m^2. The dose depended on the chemotherapy protocol that was adjusted in
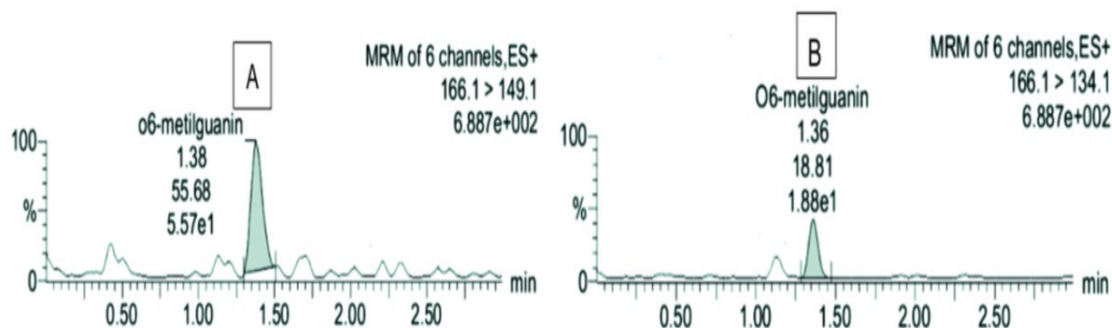
Fig. 5. Chromatograms of the sample that could be quantified: (A) Peak of O6-methylguanine with m/z 166.1>149.1; (B) Peak of O6-methylguanine with m/z 166.1>134.1

accordance with the area of the body surface of each patient [19]. The chemotherapy protocol for the 18 positive samples consisted of cyclophosphamide-methotrexate-5-fluorouracil (3 patients), cyclophosphamide-vincristine-prednisone (1 patient), 5-fluorouracil-doxorubicin-cyclophosphamide (5 patients), cyclophosphamide-docetaxel (3 patients), 5-fluorouracil-cyclophosphamide (1 patient), doxorubicin-cyclophosphamide (1 patient), and rituximab-cyclophosphamide-doxorubicin-vincristine-prednisone (2 patients). A patient for whom the O^6-methylguanine concentration could be quantified was a non-Hodgkin's lymphoma patient. The chemotherapy agent was 5-fluorouracil-cyclophosphamide and the concentration of O^6-methylguanine in the sample was 5.868 ng/mL. The chromatogram of the sample can be seen in Fig. 5.

This patient received 4 cycles of chemotherapy with a 29 day interval. In one cycle, he took 1400 mg of cyclophosphamide, which divided into 3 doses: 500 mg, 500 mg, and 400 mg.

The highest doses (1500 mg to 1600 mg) were correlated to the accumulation of O^6-methylguanine in the patient's blood. The higher doses caused a higher level of accumulated O^6-methylguanine [21]. The accumulation of O^6-methylguanine was also related to the activity of the repair enzyme O^6-methylguanine-DNA-methyltransferase. The level and activity of the enzyme was not the same for each individual because of inter-individual variations.

Inter-individual variations in the level MGMT enzyme activity, caused by factors such as age, gender, and lifestyle (exposure to carcinogens), altered the level of O^6-methylguanine in the patient's blood [22]. This difference could be a reason for the different levels of O^6-methylguanine between patients with the same treatment. The analysis could not conclude that all patients who received higher doses of cyclophosphamide in their chemotherapy had higher levels of O^6-methylguanine and a higher risk of secondary cancer because of the variations in enzyme activity. The detected and quantified O^6-methylguanine can be a predictor of secondary cancer risk, therefore, the therapeutic doses can be monitored and set to appropriate and safe levels, reducing the risk of secondary cancer. To determine the relationship between secondary cancer risk and cancer type and chemotherapy doses, there should be a continuation of this study for several years by checking for the appearance of secondary cancers in the patients with positive O^6-methylguanine results.

4. CONCLUSION

The results of the study showed that O^6-methylguanine is not always found in patients suffering from cancer treated with cyclophosphamide. The detected and quantified O^6-methylguanine can be a predictor of secondary cancer risk, therefore, the therapeutic dose given can be monitored and set to appropriate and safe levels to reduce the risk of secondary cancer.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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