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GENOTOXICITY AND CARCINOGENICITY

High incidence of acute promyelocytic leukemia specifically induced by *N*-nitroso-*N*-methylurea (NMU) in Sprague–Dawley rats

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Abstract Carcinogenic agents such as *N*-methyl-*N*-nitrosourea can cause tumors. The aims of the present study were to evaluate and classify a subtype of AML (acute myeloid leukemia) that was induced by NMU. According to previous publications, NMU induces not only mammary cancer but also leukemia in Sprague–Dawley (S-D) rats. However, the subtype of leukemia involved in NMU-treated rats is unknown. We found that both organ weight and relative organ weights were significantly higher in NMU-exposed rats than in controls. Morphological changes of rat livers and spleens were assessed by histological evaluation (H&E staining), which found that these tissues were abnormal in appearance. Also, cytological examination of the blood showed immature white blood cells in a smear using Liu's and Papanicolaou stains, indicating that gross abnormalities and histopathological changes were pathologically observed. NMU leukemia incidence was 97.1%. In this study, immunohistochemical (IHC) analysis was valuable in classifying the leukemia of poorly differentiated blasts induced by NMU. Paraffin blocks were stained for MPO, CD3, CD15, CD20, and CD34 markers. The NMU-induced group was positive for MPO, but negative for CD3, CD15, CD20, and CD34. These CD markers suggest that they are useful in helping diagnose APL (M3) leukemia. The model of NMU-induced leukemogenesis in an S-D rat suggests a more definite way to classify APL. This APL will provide an important tool for chemical carcinogenesis and leukemia studies.

Keywords Acute promyelocytic leukemia · *N*-methyl-*N*-nitrosourea · CD marker · Histopathology · Alkylating agents

Abbreviations

NMU	*N*-methyl-*N*-nitrosourea
AML	Acute myeloid leukemia
S-D rats	Sprague–Dawley rats
H&E stain	Hematoxylin and eosin stain
IHC stain	Immunohistochemical stain
MPO stain	Myeloperoxidase stain
PAS stain	Periodic acid-Schiff stain
CBC	Complete blood count
WBC	White blood cell
RBC	Red blood cell
PLT	Platelet
Hb	Hemoglobin
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
CD marker	Cluster of differentiation marker
O^6-meG	O^6-methylguanine

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Introduction

Leukemia represents a group of malignant disorders characterized by a progressive and abnormal accumulation of blood cells, usually white blood cells. The cells that make up blood are produced in the bone marrow and the lymph system. Based on the disease progression and hematopoietic lineages involved, leukemia can be divided into 4 types: acute versus chronic and myeloid versus lymphoid (Huggins et al. 1982). Acute leukemia, such as acute myeloid leukemia (AML), is categorized according to the French-American-British (FAB) classification system. FAB divides AML into eight subtypes: undifferentiated AML (M0), myeloblastic leukemia without maturation (M1), myeloblastic leukemia with granulocytic maturation (M2), promyelocytic leukemia (M3), myelomonocytic leukemia (M4), monocytic leukemia (M5), erythroleukemia (M6), and megakaryoblastic leukemia (M7). The cause of leukemia is poorly understood in most cases, but it appears to involve some rearrangement of DNA. Leukemia develops in animals, either spontaneously or because of treatment with external or internal leukemogenic factors. External factors include alkylating drugs, ionizing radiation, or chemicals. Internal factors include chromosomal abnormalities leading to DNA changes.

The *N*-nitroso compounds are a large group of chemical molecules present in a number of environmental sources. Many of the *N*-nitroso compounds behave as mutagens as well as carcinogens in experimental animals and may play a role in causing some types of human cancer (Saffhill et al. 1985). Among the group, *N*-methyl-*N*-nitrosourea (NMU) is a nitrosourea compound with alkylating property and a highly potent direct-acting carcinogen that is capable of inducing tumor formation. The different organotropic effects of NMU depend upon the sex and age of the individual and the dosage and route of administration. Previous studies have shown that NMU-induced mammary tumors are a widely used animal model for breast cancer (Chan et al. 2007; Gullino et al. 1975; Russo et al. 1990; Russo and Russo 1996; Thompson and Singh 2000). However, it may cause (1) retinal degeneration (Yoshizawa et al. 2000), (2) colon cancer (Nauss et al. 1984), (3) prostate cancer (Boileau et al. 2003), (4) gastric cancer (Garcia-Gonzalez et al. 2000; Tatematsu et al. 1993), (5) brain cancer (Kokkinakis et al. 2001), (6) liver cancer (Kanduc 1995), (7) hematological disease (thymic lymphomas; da Silva Franchi et al. 2003), (8) and a variety of other cancers (urinary bladder cancer, intestinal tumors, odontomas, skin tumors, esophageal cancer) in animals (Beland et al. 1988; Berman 1988; Kunze et al. 1997; Lu et al. 1986; Zabezhinski et al. 1985). Several papers have indicated that NMU can induce leukemogenesis in thymic lymphoma and myelogenous leukemia (Drescher et al. 1982; Newcomb 1997; Seidel 1982; Seidel and Fey 1979; Seidel and Kreja 1984). Huggins et al. (1982; Huggins and Ueda 1984) found that a series of i.v. injections of NMU specifically elicited a high incidence of myelogenous leukemia in Sprague–Dawley rats. However, 8 kinds of AML subtypes (M0 through M7) that were involved in NMU-induced myelogenous leukemia have not been determined.

Identifying which leukemia subtype classification contributes most to tumor prognosis is important. Several reports in the literature have described unusual and rare hematopoietic tumors that expressed or did not express conventional lineage markers, such as CD3 (T-cell marker), CD20 (B-cell marker; Lewis et al. 2006), CD15 (monocyte marker; M4 and M5 positive; Akashi et al. 1991; Baer et al. 1998), and CD34 (pluripotent hemopoietic progenitors marker; M1 and M2 positive; Choi et al. 1998; Mesarosova et al. 1993). Because definite antibodies are available for immunophenotyping of acute leukemia and establishing a diagnosis of AML using H&E and immunohistochemical staining (Ramos-Vara 2005), we analyzed the biopsy sections that expressed these markers (CD3, CD15, CD20, and CD34) to determine which subtypes of myelogenous leukemia were induced by NMU. Stains were used and laboratory tests were performed on bone marrow, blood samples, and tissue to help diagnose the specific types of leukemia. The myeloperoxidase (MPO) stain distinguishes between immature cells in acute myeloblastic leukemia (cells stain positive) and those in acute lymphoblastic leukemia (cells stain negative; Elghetany et al. 1990; Linari et al. 1998). The periodic acid-Schiff stain (PAS) is primarily used to identify erythroleukemia, leukemia, of immature red blood cells (Iida et al. 1991; Roggli and Saleem 1982). In the present study, the primary goals were to characterize the histopathological changes involved in the progression of NMU-induced leukemia. Furthermore, these results should demonstrate the reliability of NMU specifically induced M3-acute promyelocytic leukemia in S-D rats.

The FAB classification system divides AML into 8 subtypes, M0 through to M7, based on distinct stages of differentiation block associated with each lineage. Most patients diagnosed with AML belong to one of eight subtypes. The aim of the present study was to identify the subtype of AML induced by NMU. Identifying which subtype of leukemia contributes most to tumor prognosis is crucial, as treatment for AML varies accordingly. For example, acute promyelocytic leukemia (APL; M3) and acute monocytic leukemia are subtypes of AML that require treatment different from that of other subtypes. APL is unique among myeloid leukemias, due to its sensitivity to all-*trans* retinoic acid (ATRA), a derivative of vitamin A; however, ATRA cannot eliminate the leukemic clone (Warrell et al. 1993). Furthermore, ATRA therapy is associated with a unique side effect, retinoic acid syndrome

(RAS), characterized by fever and respiratory distress, weight gain, lower extremity edema, pleural or pericardial effusion, hypotension, and occasionally renal failure (Patatanian and Thompson 2008). Survival rates are higher following a combination of ATRA and chemotherapy than chemotherapy alone, among newly diagnosed cases of APL. A combination of drug types often strengthens the effects of the drugs, and many new combinations are being studied. The purpose of this study was to establish a series of methods for the evaluation of drugs in curing APL. This study shows that NMU-induced Sprague–Dawley rat leukemia is classified as leukemia M3 (APL, acute promyelocytic leukemia) and could be provided a specific model with which to assess the efficiency and safety of drugs in the treatment of that subtype.

Materials and methods

Reagents

N-methyl-*N*-nitrosourea was purchased from Sigma (St. Louis, MO, USA). NMU stored in a refrigerator at 4°C. NMU solution (0.5 g/100 ml) was freshly prepared, dissolved immediately before use in physiologic saline. CD3 (N1580), CD15 (clone C3D-1, M0733), CD20 (clone L26, N1502), CD34 (clone QBEnd, N1632), MPO (n1578), LSAB2 System-HRP, and liquid DAB substrate chromogen system were purchased from Dako Corp (Carpinteria, CA, USA); Eosin Y-solution (0.5% aqueous) was purchased from Merck KgaA (Darmstadt, Germany); Gill's Hematoxylin V, OG-6, EA-50, 0.5% periodic acid, and Schiff reagent were purchased from MUTO (Tokyo, Japan); Liu's A and Liu's B were purchased from BASO (Taipei, Taiwan).

Animal treatment

The experimental design is presented in Fig. 1. Male Sprague–Dawley rats (at 7 weeks old) were purchased from the National Science Council Animal Center, Taiwan. These animals were housed three per cage in an environmentally controlled animal room (at 25°C with a 12 h light–dark cycle). Animal food (Basal diet CE2) and water were provided ad libitum. The animals used in this study were cared for under protocols approved by the Instituted Animal Care and Use Committee of Chung Shan Medical University (IACUC, CSMU). Animals were randomly housed 3 to 4 per cage and divided into two experimental groups of 12 rats each: one control (untreated) and the other group which i.v. application of NMU. A total of 12 rats (NMU group) were given a series of six i.v. injection of NMU, 35 mg/kg of body weight, at biweekly intervals

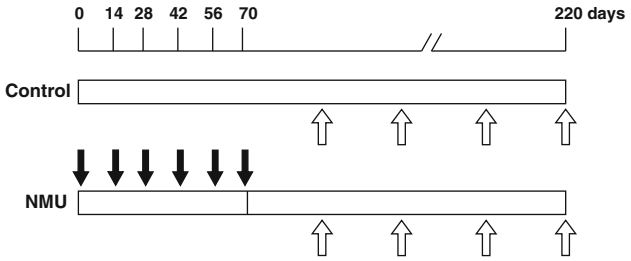

Fig. 1 The protocol of animal model (experimental design). Rats were injected NMU 2 weekly for 6 times. The day of the first injection is designated day 0. NMU 35 mg/kg i.v. (*closed arrow*), and animals after i.v. injection monitored blood data every 30–40 days from caudal vein (*open arrow*). The details were as described in "Materials and methods"

as described by Huggins et al. (Huggins et al. 1982) with some modification. NMU was injected in a caudal vein; the day of the first injection is designated day 0. After carcinogen treatment, they were kept on basal diet and water ad libitum until killing at the 220 days of the experiment.

Clinical observation and body weights

The animals were individually observed daily for mortality and weekly for clinical signs throughout the study period. Body weights were measured weekly.

Hematology and blood biochemistry

Blood was freshly obtained for hematological examination at intervals of 30–40 days after final NMU application; 0.5–1.0 ml of blood was drawn on occasion without foaming or hemolysis. Whole blood samples were collected with tubes coated with an anticoagulant, 0.3 M EDTA-2K (ethylenediamine tetraacetic acid). Complete blood count (CBC) analysis included WBC (white blood cell) count, RBC (red blood cell) count, PLT (platelet) count, Hb (hemoglobin) concentration, and WBC differential count, and all these were carried out by a fully automated hematology analyzer (Model XE-2100, Sysmex). The slides of blood smears prepared for Liu's and Papanicolaou stain were drawn from the files of the Department of Pathology, Chung Shan Medical University Hospital and were examined cytologically.

Biochemical data measurement

Analyses of all biochemical parameters in serum were examined with an autoanalyzer (Olympus AU2700, Olympus) using commercial reagent kits. The aspartate aminotransferase (AST, GOT), alanine aminotransferase (ALT, GPT), and uric acid were measured by enzymatic methods (AST, ALT: Kinetic UV test and UA: Enzymatic color test).

Pathological examination

Biopsies of liver and spleen were immediately obtained after the animals were killed (day 220). A portion of them were fixed in 10% formalin solution, processed using routine histology procedures, embedded in paraffin, cut into 5-μm sections, and placed on a glass microscope slide for analysis. The samples were stained with hematoxylin and eosin (H&E) for morphological evaluation and then examined microscopically. Other special staining procedures, such as periodic acid-Schiff (PAS) reaction by routine procedures, were drawn from the files of the Department of Pathology, Chung Shan Medical University Hospital.

Immunohistochemistry

Immunohistochemical investigations were carried out on paraffin-embedded sections. Sections were dried in oven at 50°C overnight, deparaffinized in xylene, rehydrated in graded alcohol series, immersed in citrate buffer (pH 6.0), and incubated in autoclave for 25 min. Sections were then removed and allowed to cool at room temperature for 20 min and rinsed with running water and distilled water for a total of 10 min, and blocked for the endogenous peroxidase by incubating in a solution of 3% hydrogen peroxide (H2O2) for 10 min. Tissue sections were washed with PBS and then immunostained with primary antibodies for 30 min. The antigen–antibody complex was visualized by avidin–biotin-peroxidase complex method (Stirling 1994) followed by diaminobenzidine tetrahydrochloride (DAB) as a chromogen. After washing, sections were counterstained with Gill's hematoxylin, washed with tap water, mounted with Permount (Merck, Darmstadt, Germany) and examined by light microscopy. Immunohistochemical analysis demonstrated that the latter was performed by applying antibodies against CD3, CD15, CD20, CD34, and MPO.

Statistical analysis

All data are presented as means ± SD. The statistical significant differences compared with untreated group were calculated by Student's *t*-test. The differences with probability value less than 0.05 or 0.005 were considered significant.

Results

The effect of NMU on body weight and the weights of visceral organs

Leukemia was induced in Sprague–Dawley rats with the carcinogen NMU. We first investigated the body weight of rats in NMU-treated groups. Each group had 12 individuals. Although the body weight of all rats increased by the end of the experiment, the NMU-treated group presented a mean body weight that was 13.8% less than the control rats (Fig. 2).

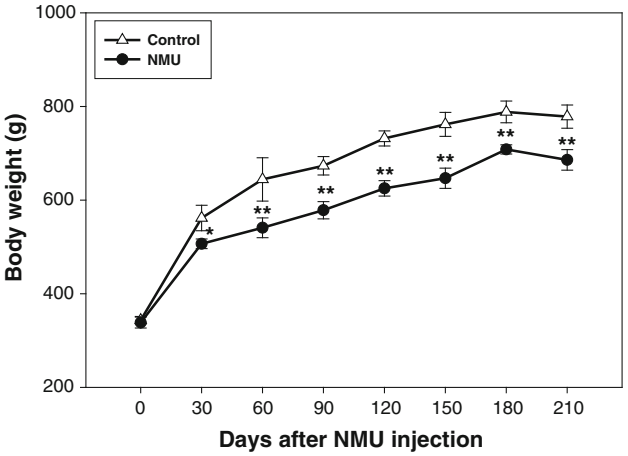

Fig. 2 Body weight changes in NMU-treated or untreated rats were shown. Body weights of animals in two groups were recorded once in every 30 days. The body weight values were expressed as mean ± SD, $n = 12$. *$P < 0.05$; **$P < 0.005$, compared with control group

During the experimental period, the NMU-administered group gained body weight gradually until the end of study (220th days). Nevertheless, the body weights of NMU-administered group were still lower than control group. In addition, the liver and spleen weights in the NMU-treated group were significantly increased (47.09 g ± 6.22 and 9.65 g ± 4.24, respectively) when compared to the normal group (19.32 g ± 1.95 and 1.25 g ± 0.18, respectively; Table 1). Furthermore, in the rats exposed to NMU alone, the relative liver weight (0.068 ± 0.011) and relative spleen weight (0.0137 ± 0.00719) obtained from the organ/body weight ratios were increased significantly when compared with the untreated rats (0.025 ± 0.003, 0.0016 ± 0.00023, respectively; Table 1). Gross evaluation of the livers and spleens from the NMU-exposed rats revealed that these organs were abnormally enlarged in appearance (data not shown). Normal spleens appeared dark brown, but the enlarged spleens with infiltrative tumors induced by NMU appeared paler. This finding suggested that NMU contributed to an aggressive malignancy.

The effect of NMU on complete blood count

In the above-mentioned indices of visceral organ results, significant differences were found between the two groups. Venous blood samples were taken once from the tails of the mice in the control and experimental groups before

Table 1 NMU affected the weight of liver and spleen in S-D rats

Group	Liver weight (g/rat)[a]	Related weight (LW/BW)[b]	Spleen weight (g/rat)[a]	Related weight (SW/BW)[b]
Treatment				
Control	19.32 ± 1.95	0.025 ± 0.003	1.25 ± 0.18	0.0016 ± 0.00023
NMU	47.09 ± 6.22**[,c]	0.068 ± 0.011	9.65 ± 4.24*[,c]	0.0137 ± 0.00719

[a] Values are the average of twelve rats ($n = 12$)

[b] Data represented as net weight of liver or spleen/body weight (LW or SW/BW)

[c] Statistically with mean ± SD, * $P < 0.05$; ** $P < 0.005$, compared with control group

treatment. WBC, RBC, PLT, and Hb levels were tested. The WBC count was significantly higher in the NMU-induced group (13×10^3/μl vs. 10×10^3/μl; Fig. 3a). In contrast, other hematological data of NMU-treated rats were lower (mean of 7×10^6/μl for RBC counts; 600×10^3/μl for PLT counts; 12 g/dl for Hb concentration at 35 mg/ml) when compared to control rats (9×10^6/μl, $1{,}100 \times 10^3$/μl, 16 g/dl, respectively; Fig. 3b–d). The values of RBC and Hb decreased in the NMU-treated group, indicating a tendency to anemia. Next, the effects of NMU on the WBC differential count were examined. The differential count measures the percentages of each type of leukocyte present. Table 2 shows that the percentage of neutrophils in the NMU-induced group decreased more than in the normal group. These results suggest that the administration of NMU to rats induces an increase in the WBC count without affecting the neutrophil index. The increased production of leukemia blasts led to a reduction in the percentage of normal neutrophils and implied a direct correlation between leukemia blasts and the number of WBCs.

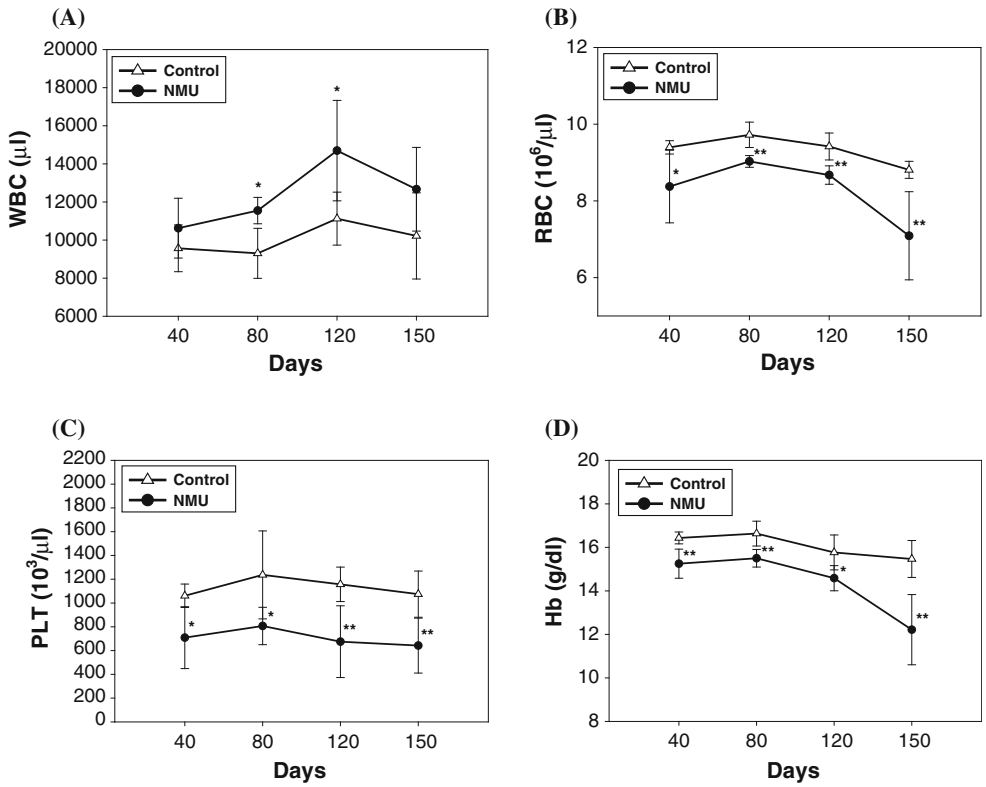

Fig. 3 Effect of NMU on the complete blood count in Sprague–Dawley rats. **a** The count of white blood cell (WBC); **b** the count of red blood cell (RBC); **c** the count of platelet (PLT); **d** the count of hemoglobin (Hb). HAs were given orally for at least 150 days, and 35 mg/kg NMU (in saline) was injected 2 weekly for 7 times as described in "Materials and methods". The indicated days are counted after iv. NMU and recorded once about 30–40 days. These values were expressed as mean ± SD, $n = 12$. *$P < 0.05$; **$P < 0.005$, compared with control group

Table 2 NMU affected the differential count of blood in S-D rats

D.C. (%)	Group	
	Control	NMU only
Neutrophil	72.84 ± 8.17	NA
Lymphocyte	11.12 ± 1.93	1.15 ± 1.62
Monocyte	4.1 ± 2.89	0 ± NA
Eosinophil	10.42 ± 6.48	0.33 ± 0.32
Basophil	0.94 ± 1.08	0 ± NA

NA not applicable

DC The examination on white blood cell differential count (DC) of blood smears

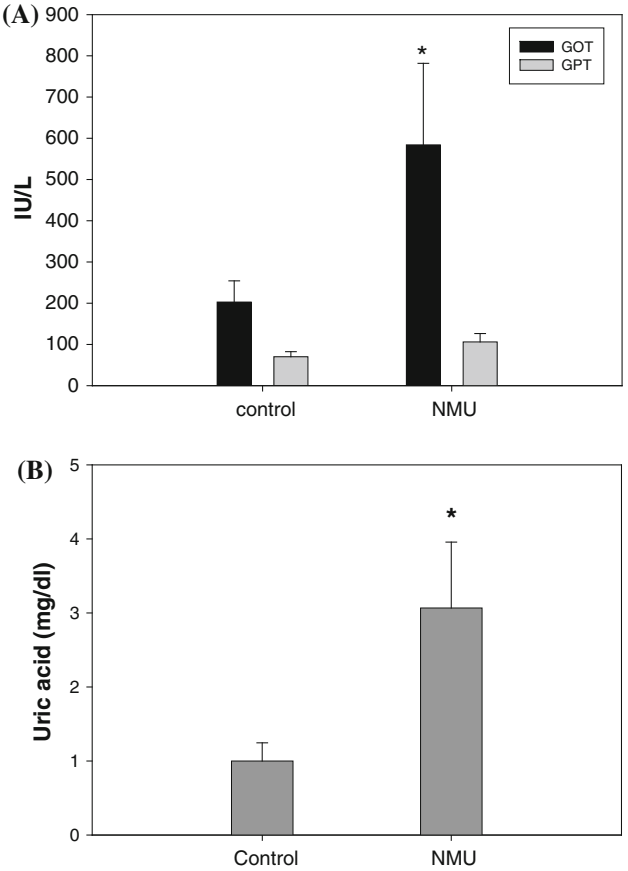

Fig. 4 Effect of NMU on serum biochemical analysis in Sprague–Dawley rats. **a** The activities of glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT); **b** the level of uric acid. HAs were given orally for at least 150 days, and 35 mg/kg NMU (in saline) was injected 2 weekly for 7 times as described in "Materials and methods". The indicated days are counted after iv. NMU and recorded once about 30–40 days. These values were expressed as mean ± SD, $n = 12$. *$P < 0.05$, compared with control group

The effect of NMU on serum biochemical analysis

The hepatic function test is used to evaluate the liver for injury, infection, inflammation, or abdominal swelling. Based on degeneration or changes in the permeability of the liver cell membrane, a release of a large amount of transaminase (GOT and GPT) into the blood stream from the damaged liver cells will occur. Thus, the degree of liver cell injury can be estimated from the level of serum GOT and GPT. NMU was found to induce a significant increase in the levels of serum GOT (approximately 600 IU/l) and serum GPT (approximately 110 IU/l; Fig. 4a).

Uric acid (UA) is the end product of purine nucleotide metabolism in humans. Determining the level of serum uric acid is useful in detecting hyperuricemia and in diagnosing leukemia (Hafiz and Islam 2009), gout, polycythemia, and renal dysfunction. The serum uric acid level in NMU-treated rats was higher than the normal values of the control group (Fig. 4b).

The effect of NMU on cytological morphology features

High numbers of white blood cells were apparent when a blood sample from the NMU-induced group was viewed under a microscope. Smears were prepared for cytological evaluation and stained using Papaniclaou and Liu's methods. The Papaniclaou staining method found more atypical single cells in the NMU group than in the control group. These atypical cells were round, dark blue in color, and approximately two to three times the size of lymphocytes (Fig. 5a, b). Distinguishing lymphoblasts from myeloblasts in a Papanicolaou stained smear was difficult. Air-dried smears using Liu's stain method may be more useful in the precise classification of lymphoma/leukemia. Figure 5c, d show that Liu-stained smears in the NMU-induced group were cellular and revealed a markedly polymorphous mixture of small to large cells. A greater proportion of large, dark staining, blast-like promyelocytes and metamyelocytes and dysplastic granulocytes (arrows in Fig. 5d) were present in NMU-treated rats. The smaller cells were mature neutrophils. Most neutrophils were normal or occasionally dysplastic in the progression of the leukemia. The large cells were primitive and had abnormal nuclear maturation. The overall cytomorphological picture posed serious difficulties in differentiating this condition from those of the peripheral blood smear. Abnormal leukemia cells equivalent to immature white blood cells were verified in smears from the NMU-induced group.

The effect of NMU on histopathological features

Liver and spleen samples of NMU-injected mice were submitted for routine histological analysis to characterize their morphological aspects. Figure 6 shows the histological findings using H&E stain on the different organs. The spleen and liver were frequently enlarged in all cases and

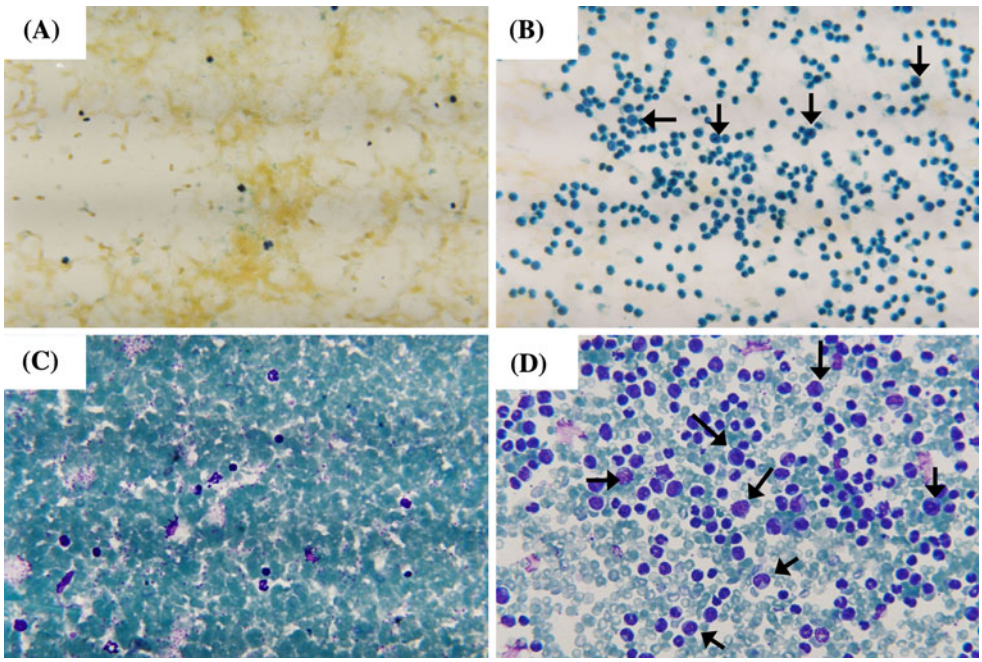

Fig. 5 Hematology analysis for whole blood cells. Representative pictures of normal control were shown in **a** and **c**. After animals were treated with NMU alone (35 mg/kg) for 220 days, animals were killed and blood was collected **b** and **d**, which showed monotonous malignant cells. The *panels* were illustrated by Papanicolaou staining (*top*) and Liu's staining (*bottom*). The *arrows* indicated immature white blood cells at a higher magnification (×400)

showed leukemic cell infiltration. Since the portal vein brought much more blood to the liver, cancer cells infiltrated the portal area and sinusoids in the NMU-induced group (Fig. 6b). The spleen showed diffusely leukemic cell infiltration induced by NMU (Fig. 6d). In the spleen, the normal architecture of red and white pulp was destroyed in NMU-treated rats with a massive spread of undifferentiated cells (compare Fig. 6c, d). Histopathological examination revealed 97.1% leukemia incidence (Table 3). Of the 12 NMU-induced rats, six developed leukemia, one did not develop leukemia, and five died. The histopathological examination confirmed that the lesion represented a malignancy.

The effect of NMU on immunohistochemical analysis

Immunohistochemistry can be helpful in the diagnosis and classification of these leukemia blasts. NMU was the most potent agent to elicit myelogenous leukemia (Huggins et al. 1982). Chuang and Li demonstrated that the malignant cells can be derived from the myeloid or lymphoid lineage by immunohistochemical methods (Chuang and Li 1997).

Immunohistochemical staining for CD3 and CD20 can help reach a decisive estimation. CD3 generated an activation signal in T lymphocytes, and CD20 was active in all B-cell lymphomas and leukemia. CD3 and CD20 expressions were negative in malignant cells (Fig. 7). Further, MPO was used in the diagnosis of acute myeloid leukemia to demonstrate that the leukemic cells were derived from the myeloid lineage. The results showed that MPO staining was strongly positive in neoplastic cells (Fig. 8b, d). These results indicated that NMU-induced blast cells were derived from the myeloid lineage.

The criteria established by the FAB Co-operative Group include eight categories for the classification of acute myeloid leukemia (AML; M0 through to M7 (Bennett et al. 1985)). The blast cells of M0, M5, M6, and M7 leukemia of the FAB classification are MPO-negative (Bennett et al. 1991; Elghetany et al. 1990; Linari et al. 1998). The presences of MPO-positive leukemia blasts are in FAB M1 to M4 and M6 (Domingo-Claros et al. 2002). Therefore, periodic acid-Schiff stain was used to distinguish between acute myeloid leukemia and acute erythroleukemia. PAS-positive erythroblasts were found in the majority of FAB M6 (Iida et al. 1991; Roggli and Saleem 1982). Figure 9 shows that the blasts induced by NMU were cytochemically negative for PAS staining in both liver and spleen tissues. Thus, this result implied that the blasts induced by NMU did not belong to FAB M6.

The expression of CD15 was associated with AMLs with the monocytic component-FAB M4 and M5 (Akashi et al. 1991; Baer et al. 1998). In addition, CD34 was expressed by the early hematopoietic progenitor cells-FAB M1 and M2, but belonged to FAB M3 and was CD34-negative (Choi et al. 1998; Mesarosova et al. 1993). Antigens, identified by antibodies on the blasts, induced by NMU as neither CD15 nor CD34 were expressed as negative (Fig. 10). The NMU-treated tissues express MPO (Fig. 8) and not CD3, CD15, CD20, or CD34 (Figs. 7, 10) Taken together, these results suggest that tumors induced by NMU were of the AML FAB subtype M3-APL.

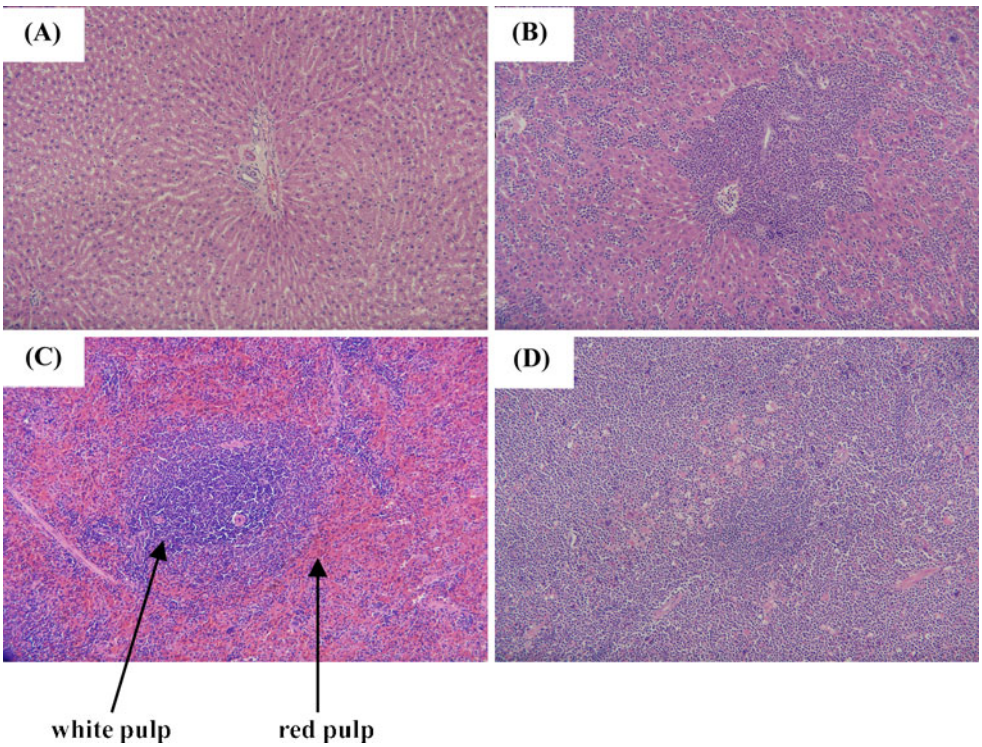

Fig. 6 Histopathological examinations of tumors in NMU-treated or untreated rats were shown. Paraffin-embedded sections of representative liver and spleen tissues were stained by H&E staining. Representative pictures of normal control were shown in **a** and **c**. Animals were treated with NMU alone (35 mg/kg), which showed marked infiltrative cells in **b** and **d**. The *panels* were illustrated in liver (*top*) and spleen (*bottom*) tissues. **a**–**d** ×100. Some relevant structures and elements are illustrated

Table 3 Effects of NMU-induced rat's tumorigenesis

Treatment	Leukemia Tumor incidence[a] (%)	Rate of death
Control	0/12 (0%)	0/12 (0%)
NMU only	11/12 (97.1%)	5/12 (41.6%)

[a] Tumor incidence is equal to the number of dead rat which had leukemia combined with the number of tumor rat

Discussion

The presence of cachexia—as defined by a series of clinical symptoms, such as anorexia, weight loss, muscular atrophy, tissue wasting, and altered organ function—is frequently observed in cancer and makes a decisive contribution to morbidity and mortality (Torelli et al. 1997). Figure 2 showed a slight gain of body weight in NMU-treated groups throughout the experiment. However, the average body weight of NMU-treated rats was less than that of the control rats. We hypothesized that gain of body weight in the NMU-induced group did not reach control group because S-D rats were suffering from a lack of appetite (Fig. 2). As regards viscera, large numbers of blast cells occurred in the bloodstream that may have accumulated in other sites producing swollen glands. This may have led to the development of an enlarged spleen or liver (data not shown). In other words, the liver and spleen were enlarged due to an invasion of leukemia cells. In this study, the gross characteristics of visceral organs have shown evidence of cancer induced by NMU.

AML is a malignant disorder of the blood that is characterized by blocked or impaired differentiation of hemopoietic stem cells. This results in an abnormal accumulation of immature precursors and a suppression of growth and maturation of cells involved in normal hemopoiesis (Lowenberg et al. 1999; Schiffer 2003; Smith et al. 2004). As a result, AML is sometimes discovered during a routine blood test in an asymptomatic individual. More often, patients have constitutional complaints arising from anemia, leukocytosis (or neutropenia), or thrombocytopenia. In this study, we observed a significant decrease in the RBC count, platelet count, and hemoglobin concentration in the NMU-induced group. In contrast, the WBC count increased during the experimental period (Fig. 3). However, the percentage of neutrophils lowered in those rats receiving NMU (Table 2). The condition of raise WBC count corresponded with the reduction of these neutrophils because mature neutrophils were undetectable. In a word, we observed a significant increase in the WBC level together with a decrease in the relative content of neutrophils. The cytological examination

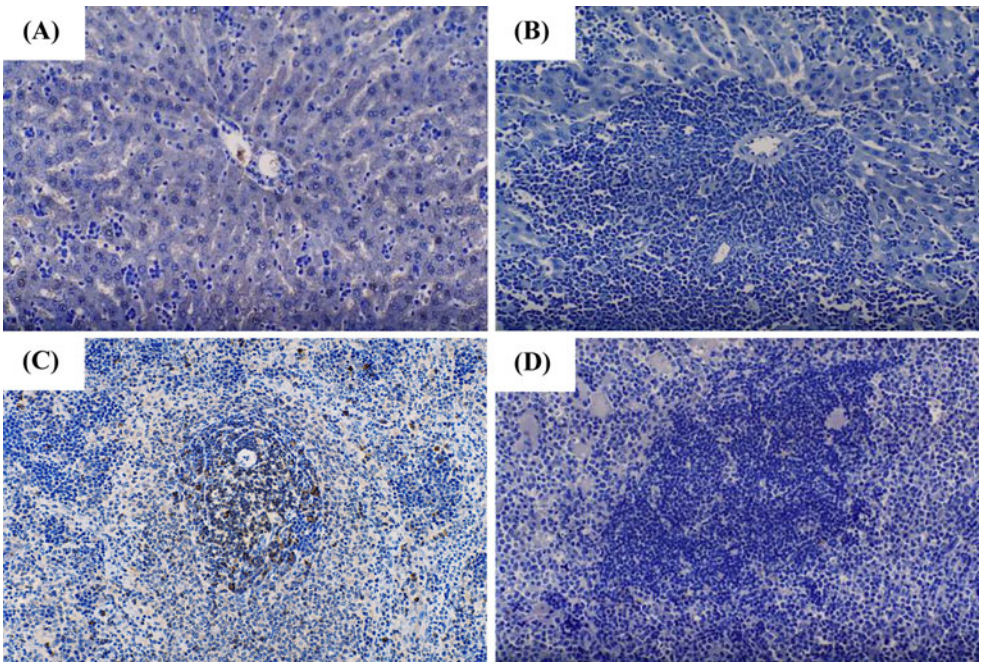

Fig. 7 Immunohistochemical analysis for anti-CD3 and anti-CD20 antibodies. Paraffin-embedded sections of representative liver and spleen tissues were stained with the antibodies against CD3 and CD20. Representative pictures of CD3 expression were shown in **a** and **c**, as well as CD20 expression in **b** and **d**. Animals were treated with NMU alone (35 mg/kg), which showed uniformly negative. The *panels* were illustrated in liver (*top*) and spleen (*bottom*) tissues. **a**–**d** ×200

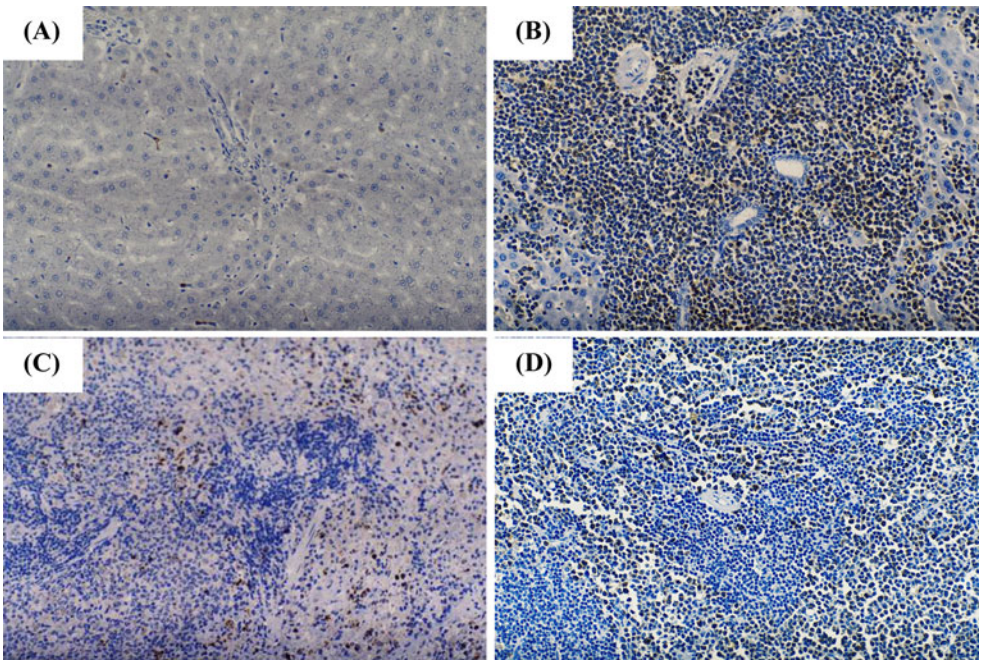

Fig. 8 Immunohistochemical analysis for MPO antibodies. Paraffin-embedded sections of representative liver and spleen tissues were stained by MPO staining. Representative pictures of normal control were shown in **a** and **c**. Animals were treated with NMU alone (35 mg/kg), which showed prominent MPO-positive cells in **b** and **d**. The *panels* were illustrated in liver (*top*) and spleen (*bottom*) tissues. **a**–**d** ×200

of the blood sample revealed marked leukocytosis with a left shift demonstrating that immature neutrophils were released into the peripheral blood (Fig. 5). More than 3% of these blasts present in the peripheral blood were found MPO positive on staining (Anand et al. 2005). MPO immunohistochemistry was used to confirm that the blasts were of

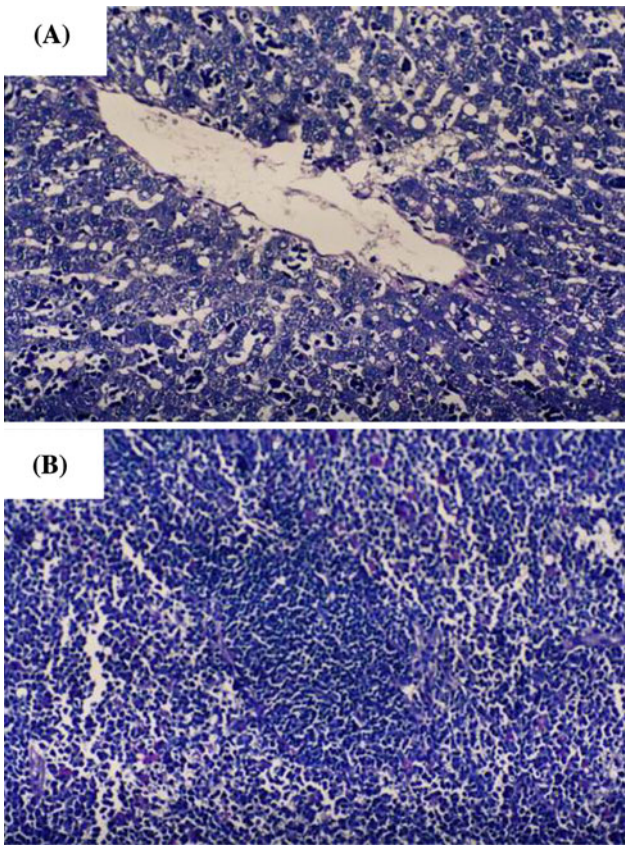

Fig. 9 PAS staining for liver and spleen tissues in NMU-treated or untreated rats was shown. Paraffin-embedded sections of representative liver and spleen tissues were stained by PAS staining. Representative pictures were uniformly negative in **a** liver and **b** spleen. Tissues were treated with NMU alone (35 mg/kg) at original magnification ×200

myeloid lineage (Fig. 8). Over an observation period of 220 days, no rats in the control group developed leukemia. The incidence rate of tumors induced by NMU was 91.7%, and the death rate among these was 41.6% (Table 3). These results suggest that cancer-associated cachexia occurred in these animal models.

Usually, the classification of leukemic cells can be determined from their appearance under the microscope, but sometimes special chemical tests are needed for validation. Immunophenotyping of AML by immunohistochemistry is of value in the utilization of monoclonal and polyclonal antibodies that are specific in determining different paths of differentiation and different stages of maturation and, thus, the differentiation and maturation of the AML subclass. Using a large series of antibodies, APLs less frequently expressed the myelomonocytic antigens, CD3, CD15, CD20, and CD34, whereas expression of several pan-myeloid markers, such as anti-MPO, were more frequent. Table 4 summarizes the results. The immunohistochemical data show that S-D rats had acute myelogenous leukemia after NMU treatment.

Leukemia is like other cancers that result from mutations in the DNA, inactivate tumor suppressor genes, or activate oncogenes. These actions destroy the regulation of cell division and differentiation or lead to death. These mutations are likely to be influenced by genetic factors and may take place spontaneously or as a result of exposure to radiation or carcinogenic agents. NMU is a nitrosourea chemical with alkylating property. No information is available on environmental exposure to NMU in prior years (TRI 2001). Nevertheless, occupational exposure

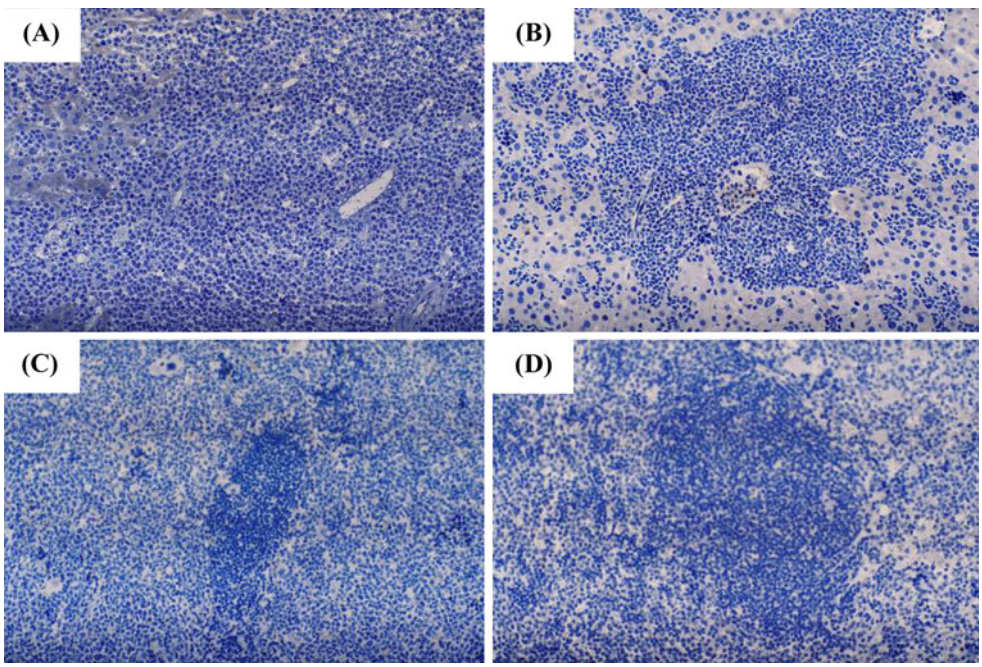

Fig. 10 Immunohistochemical analysis for anti-CD15 and anti-CD34 antibodies. Paraffin-embedded sections of representative liver and spleen tissues were stained with the antibodies against CD15 and CD34. Representative pictures of CD15 expression were shown in **a** and **c**, as well as CD34 expression in **b** and **d**. Animals were treated with NMU alone (35 mg/kg), which showed uniformly negative. The *panels* were illustrated in liver (*top*) and spleen (*bottom*) tissues. **a–d** ×200

Table 4 Expression of different marker on APL induced with NMU in S-D rats

Stain	Marker	Expression
CD3	T-cell lymphocytes	−
CD20	B-cell lymphocytes	−
MPO	Myeloblastic leukemia (M1, 2, 3, 4 and 6)	+
PAS	Erythroleukemia, M6	−
CD15	M4 and M5	−
CD34	M1 and M2	−

may occur through inhalation or dermal contact with a small number of individuals, primarily those who use the chemical in research laboratories (IARC 1978). NMU is also used for laboratory synthesis of diazomethane and has been studied for use as an antineoplastic agent. Natural occurrence, a NMU derivative, streptozotocin, has been isolated from *Streptomyces achromogenes* (HSDB 2001). It is a broad-spectrum carcinogen capable of inducing tumors in various organs (Swenberg et al. 1975; Tsuda et al. 1983). It is an alkylating agent that can modify guanine bases and add methyl groups to both oxygen and nitrogen atom sites in DNA. Among the oxygen adducts formed by the agent, O^6-methylguanine (O^6-meG) is regarded as being most responsible for the induction of mutagenic lesions. This methylated nucleotide pair with thymine (T), instead of cytosine (C), leads to G:C → A:T transition mutation during DNA replication (Christmann and Kaina 2000). Hence, O^6-methylguanine plays a major role in mutagenesis, carcinogenesis, and cytotoxicity (Burns et al. 1988; Pegg 1984; Pegg et al. 1995; Richardson et al. 1987; Sato et al. 2003). This promutagenic lesion appears to have an important role in chemical carcinogenesis since G to A mutations activates the *ras* proto-oncogene observed in NMU-induced animal tumors. Previous studies have documented Ki-*ras* mutations as well as chromosomal anomalies in thymocytes from 67% of NMU-treated C57BL/6 J mice with an early preleukemic phase of the disease (Newcomb et al. 1995). Chemically induced rodent tumor models help us understand the series of genetic changes during carcinogenic development. However, which genes are involved in NMU-induced APL in the S-D strain has not yet been determined.

NMU alone is not a mammary carcinogen in mice, but it reportedly induces leukemia (Dexter et al. 1974; Huggins et al. 1982), lymphomas (Joshi and Frei 1970a, b), and other cancers. The hemopoietic disease occurs after NMU-exposed in rats that the type remains unclassified. The rat model of NMU-induced leukemogenesis was used to determine which subtype was involved. In any acute leukemia, determining the subtype is necessary because subcategorizing leukemia may lead to more specific and effective chemotherapeutic regimens and, hopefully, more cures in AML patients. Among acute leukemia, APL is characterized by typical morphology with blast cells and abnormal promyelocytes. *All-trans*-retinoic acid (ATRA) was derived by the intracellular oxidation of plasma retinol (vitamin A) and represented the first example of clinically successful differentiating agent (Warrell et al. 1993). For treatment purpose, the distinction between myeloid and lymphoid leukemia has been solved, since APL (M3) benefits from treatment with ATRA (Degos and Wang 2001; Fenaux et al. 2001). However, a few major side effects of ATRA therapy have the risk of rapid overproduction of white blood cells (so-called ATRA syndrome) and the rapid development of drug resistance. Thus, chemotherapy is still being used in conjunction with ATRA therapy. Current available clinical trials show that combination of ATRA with several agents, for instance, anthracycline, idarubicin, cytosine arabinoside (Ara-C), and arsenic trioxide (As_2O_3), has a higher rate of complete remission in APL (Petrie et al. 2009). These could limit the use of ATRA as post-remission treatment, and therefore future works should be applied to the search of new agent with comparable current these strategies, which were still incomplete. These aforementioned interpretations have led to the use of the NMU model maybe as a platform for development of cancer therapy investigations.

In conclusion, our results might serve as evidence that the relevance of some key positive markers (such as MPO) may confirm the diagnosis and subclassification of AML. These data support the conclusion that NMU induced specifically acute promyelocytic leukemia in Sprague–Dawley rats. This information may be useful in combination with the analysis of tissue and blood morphology for the classification of APL.

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Conflict of interest The authors declare that there are no conflicts of interest.

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