C609T Polymorphism of NADPH Quinone Oxidoreductase 1 Correlates Clinical Hematological Toxicities in Lung Cancer Patients Treated with Amrubicin

Misato Nagata¹, Tatsuo Kimura¹, Tomohiro Suzumura¹, Yukimi Kira², Toshiyuki Nakai¹, Kanako Umekawa¹, Hidenori Tanaka¹, Kuniomi Matsuura¹, Shigeki Mitsuoka¹, Naruo Yoshimura¹, Takako Oka¹, Shinzoh Kudoh¹ and Kazuto Hirata¹

¹Department of Respiratory Medicine, Graduate School of Medicine, Osaka City University. ²Department of Central Laboratory, Graduate School of Medicine, Osaka City University.

Corresponding author email: kimutats@med.osaka-cu.ac.jp

Abstract

Background: Amrubicin hydrochloride (AMR) is a key agent for lung cancer. NADPH quinone oxidoreductase 1 (NQO1) metabolizes the quinone structures contained in both amrubicin (AMR) and amrubicinol (AMR-OH). We hypothesized that NQO1 C609T polymorphism may affect AMR-related pharmacokinetics and clinical outcomes.

Methods: Patients received AMR doses of 30 or 40 mg/m²/day on days 1–3. Plasma sampling was performed 24 hours after the first and third AMR injections. Concentrations of AMR and AMR-OH were determined by HPLC and the NQO1 C609T polymorphism was assayed by RT-PCR.

Results: A total of 35 patients were enrolled. At a dose of 40 mg/m², the T/T genotype exhibited a tendency toward a relationship with decrease concentrations of AMR-OH on days 2 and 4. The genotype also showed a significant decrease of hematological toxicities (P < 0.05).

Conclusions: NQO1 C609T polymorphism had a tendency of correlation with the plasma concentrations of AMR-OH, and thereby had significant correlations with hematologic toxicities.

Keywords: NQO1, amrubicin, lung cancer, SNP, hematological toxicity
Introduction

Lung cancer is the most commonly occurring cancer and represents the leading cause of malignancy-related death worldwide. Chemotherapy with a platinum-based two drug combination has been used as a standard treatment for SCLC since the development of cisplatin and etoposide in the 1980’s. Despite the excellent sensitivity of small cell lung cancer (SCLC) to chemotherapy and high response rates, tumor relapse is an often an unavoidable fact. Therefore, to develop a more attractive SCLC treatment, new drug cares regimens are needed which combine with cisplatin.

Anthracyclines such as daunorubicin and doxorubicin are widely used in the treatment of a variety of cancers. However, the cumulative dose-limiting cardiototoxicity of doxorubicin is a major obstacle to its use and great efforts have been made to discover means of ameliorating, preventing, and delaying this side-effect. Amrubicin hydrochloride (AMR) is a novel synthetic aminoanthracycline derivative with a structure similar to doxorubicin. AMR, with its predictable and manageable toxicities, is one of the most attractive agents for the treatment of SCLC and non-small cell lung cancer (NSCLC). Phase II studies of AMR monotherapy with relapsed SCLC resulted in an RR of the sensitive relapse patients of 50.0% to 53.0% and that of refractory relapse patients of 17.0% to 60.0%. In previously untreated SCLC, the combination chemotherapy with cisplatin yielded high overall RRs of over 80%. AMR is a promising agent for first and second line chemotherapies. In these reports, the most frequent toxicity was myelosuppression. Previous phase II studies of AMR monotherapy for treated SCLC found that treatment was associated with a high incidence of bone marrow suppression or grade 3 or 4 hematologic toxicities such as follows: neutropenia (83%), thrombocytopenia (20%), and anemia (33%) in Onoda’s report; and neutropenia (93%), thrombocytopenia (28%), and anemia (21%) in Inoue’s report. The patients receiving combined treatment with AMR and cisplatin experienced more profound myelotoxicity than those treated with AMR alone and the dose of AMR for combined treatment with cisplatin was less than that used for AMR monotherapy.

NADPH quinone oxidoreductase 1 (NQO1) is a cytosolic flavoprotein that catalyzes the two-electron reduction of a variety of substrates. NQO1 reduces quinones to hydroquinones in a single two-electron step. This process bypasses the formation of potentially toxic, semiquinone radical intermediates. Not all hydroquinones are redox-stable, however, and in some cases metabolism by NQO1 yields a more active product. Redox-labile hydroquinones can react with molecular oxygen to form semiquinones and generate reactive oxygen species; alternatively, semiquinones can be generated via comproportionation reactions. In addition to potentially causing oxidative stress through this mechanism, the reduction of the quinone moiety can produce alkylate sites which injure DNA. In the case of mitomycin C, the generation of reactive intermediates by NQO1 has been proposed to result in alkylation and subsequent crosslinking of DNA. Doxorubicin and related anthracyclines are antitumor drugs with mechanisms of redox pathways that lead to the alkylation of nucleic acids by NQO1. The targets of induced oxidative stress include cell membrane receptors, cell membrane lipids, nucleic acids, and topoisomerase. NQO1 is one of the main enzymes that metabolizes the quinone structures contained in both AMR and amrubicinol (AMR-OH) and it induces AMR to produce a reduction-oxidation which causes oxidative stress. A point mutation (C-to-T base pair substitution) in exon 6 (position 609) of the human NQO1 cDNA has been detected that leads to a proline-to-serine substitution at position 187 in the amino acid sequence of the protein. This variant NQO1 genotype (known as NQO1*2) affects individual susceptibility to lung, bladder, breast, and colorectal cancers; the frequency of the homozygous mutant (T/T) genotype ranges from 4% in Caucasians to greater than 20% in Chinese populations. In the NCBI/NBI-single nucleotide polymorphism (SNP) databases, C609T SNP genotype frequencies of this NQO1 polymorphism are reported as follows: C/C, 39.5%–29.2%; C/T, 52.3%–50.0%; and T/T, 8.1%–20.8%, in Asian populations (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=1800566). Cell lines and tissues from organisms genotyped as homozygous for the NQO1*2 polymorphism are deficient in NQO1 activity. We hypothesized that genotypes at the NQO1 C609T polymorphic site may affect AMR-related pharmacokinetics and clinical outcomes. A recent report demonstrated that cisplatin did not...
affect the metabolism of AMR and AMR-OH.\textsuperscript{16} A pharmacogenomics study was thus performed on lung cancer patients treated with AMR alone or combination with cisplatin. This study was approved by the Institutional Review Board of Osaka City University. It was conducted according to the principles of the Declaration of Helsinki and good clinical practice guidelines.

**Patients and Methods**

**Patients**

This study was performed at Osaka City University Hospital. Patients were considered eligible for participation in this study if they were diagnosed with NSCLC or SCLC, histologically or cytologically evaluated as clinical stage IIIb or IV, and treated with AMR alone or with a combination of AMR and cisplatin. This study was part of other phase I/II studies conducted in our institute.\textsuperscript{8,24} Eligibility criteria also included the following: Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0–2; adequate organ function, including white blood cell count $\geq 4000 \times 10^6$/L; hemoglobin level $\geq 9.5$ g/dL; platelet count $\geq 100 \times 10^9$/L; aspartate aminotransferase and alanine aminotransferase levels $\leq 100$ IU/L; bilirubin level $\leq 1.5$ mg/dL; creatinine concentration $\leq 1.2$ mg/dL; electrocardiographic findings within normal range; and left ventricular ejection fraction on echocardiography $\geq 60\%$. Exclusion criteria were as follows: symptomatic brain metastasis requiring radiation treatment; accumulation of pleural fluid requiring treatment (eg, drainage); continuous serious infectious disease; additional serious medical conditions such as heart disease, interstitial pneumonitis, or uncontrollable diabetes; a history of drug allergy; other active, concurrent malignancies; or other problems judged by the investigators that made the patients ineligible for inclusion in this study. Patients who were pregnant or who wished to conceive were also considered ineligible. Written informed consent was obtained from all study patients. All patients underwent a medical history survey, physical and haematological examinations, and serum biochemistry tests more than once a week. The physical examination and biochemistry tests were repeated as part of normal clinical practice.

Toxic effects were assessed using National Cancer Institute Common Toxicity Criteria, Version 3.0.

**Pharmacokinetic analysis**

AMR was dissolved in 20 mL physiological saline and administered once intravenously as a 5 minute injection at a dose of 30 mg/m\textsuperscript{2}/day or 40 mg/m\textsuperscript{2}/day, on days 1 to 3, every 3 weeks. Blood sampling was performed during the initial cycle of AMR treatment. Blood samples (7 mL) were collected in tubes containing EDTA as an anticoagulant 24 hours after the first and third AMR injection (days 2 and 4). Blood samples were immediately centrifuged and aliquots of plasma frozen at $-80^\circ$C until they were analyzed. The plasma concentrations of AMR and AMR-OH were measured by high-performance liquid chromatography (HPLC) as described previously.\textsuperscript{25} The components were separated using HPLC on two reverse-phase columns linked with a connector (Onyx Monolithic C18, 100 $\times$ 4.6 mm) using 4 mM sodium 1-octanesulfonate, 2.3 mM acetic acid: tetrahydrofuran: dioxane (15:2:6, v/v/v) as an eluent. AMR and AMR-OH were measured using a fluorescence detector set at an excitation wavelength of 480 nm and a detection wavelength of 550 nm. Standard AMR and AMR-OH powders with purities $\geq 99\%$ were supplied by Dainippon Sumitomo Pharmaceuticals Company Limited (Osaka, Japan). The limit of quantitation was 8.00 ng/mL using a sample volume of 100 µL. Values beneath the lower limit were extrapolated using HPLC data.

**DNA extraction and genotyping**

Genomic DNA was extracted from the peripheral blood samples and 3 human lung cancer cell lines with known NQO1 C609T genotypes (SBC3: C/T, PC9: C/C, and A549: C/C) as positive and negative controls by using the QIAamp DNA Mini Kit (Qiagen K. K, Tokyo, Japan) according to the manufacturer’s instructions. Extracted DNA samples were stored at $-80^\circ$C until examination. DNA concentrations were checked by optical density at 260–280 nm (Nano Drop ND-1000; Thermo Fisher Scientific Inc, Wilmington, DE, USA). Real-time polymerase chain reaction (PCR) genotyping of the C609T polymorphism was used to determine phenotypes of the NQO1 protein. Genotyping was performed using
a Taqman® Drug Metabolism Genotyping Assay™ (Assay ID: C_2091255_30; Applied Biosystems Japan Ltd, Tokyo, Japan) according to the manufacturer’s instructions. All assays were conducted in 96-well plates. Plates were read on an Applied Biosystems 7500 Real-time PCR system using the Sequence Detection System Software (Applied Biosystems Japan Ltd, Tokyo, Japan).

**Statistical analyses**

Differences between plasma concentration levels of AMR and AMR-OH were evaluated by Student’s t-test. Correlations between the plasma concentration of AMR-OH and each genotype were compared using a one-way ANOVA. All analyses were two-sided, and P-values less than 0.05 were considered statistically significant. Statistical analyses were performed with PRISM software (GraphPad Software Inc, California, USA).

**Results**

**Patient characteristics**

Between November 2004 and January 2007, 35 patients were enrolled in this study. The patient population profile is provided in Table 1. Ten patients had SCLC, 23 patients had NSCLC, and 2 patients had other tumors (thymic cancer and neuroblastoma). First-line treatment was performed for 20 patients, 15 of which had NSCLC. Second-line treatment was performed for 13 patients, 6 of which had NSCLC. AMR treatment alone was administered to 16 patients, including 6 patients with SCLC, 9 patients with NSCLC, and 1 patient with thymic cancer. Combined treatment with both cisplatin and AMR was administered to 19 patients, including 4 patients with SCLC, 14 patients with NSCLC, and 1 patient with neuroblastoma. The regimen for SCLC was cisplatin (20 mg/m²) and AMR (30 mg/m²) on days 1 to 3, whereas that for NSCLC was cisplatin (80 mg/m²) on day 1 and AMR (30 mg/m²) on days 1 to 3. These schedules were used for phase I/II studies in our hospital and have been previously reported.8,24 Plasma concentration levels of AMR and AMR-OH are shown in Table 2. One toxicity-related death was reported in a patient with SCLC who received combination chemotherapy with cisplatin (20 mg/m²) and AMR (30 mg/m²) on days 1 to 3. The plasma concentrations of AMR and AMR-OH on days 2 and 4 in this patient were 7.1 ng/mL and 7.2 ng/mL on day 2, and 17.4 ng/mL and 10.8 ng/mL on day 4, respectively.

**Clinical outcome and toxicities due to amrubicin treatment**

The most frequent toxicities were leucopenia and neutropenia. Grade 4 hematological toxicities included leucopenia in 7 patients (20%), neutropenia in 16 patients (46%), thrombocytopenia in 1 patient (3%), and anemia in 1 patient (3%). Grade 3 and 4 non-hematological toxicities included nausea/vomiting in 4 patients (11%), loss of appetite in 6 patients (17%), and febrile neutropenia in 5 patients (14%). No cardiac toxicity was observed during this study.

**NQO1 C609T polymorphisms and pharmacokinetics**

Genotypes at the NQO1 C609T polymorphism were determined for all 35 patients (100%).
Genotypes observed were C/C, C/T, and T/T in 12 (34.3%), 16 (45.7%), and 7 (20.0%) patients, respectively. These genotype distributions were in Hardy-Weinberg equilibrium.

In the 19 patients receiving AMR at a dose of 30 mg/m², the genotypes observed were C/C, C/T, and T/T in 7 (36.8%), 9 (47.4%), and 3 (15.8%) of patients, respectively. The plasma concentrations of AMR in the respective genotypes on days 2 and 4 were as follows: C/C, 7.84 ± 3.62, 18.96 ± 12.56; C/T, 6.36 ± 6.49, 11.76 ± 3.86; and T/T, 6.40 ± 0.99, 9.33 ± 7.14 ng/mL. There are no significant differences between NQO1 genotypes (P = 0.8532, P = 0.1488, respectively). The plasma concentrations of AMR-OH in the respective genotypes on days 2 and 4 were as follows: C/C, 7.84 ± 3.62, 18.96 ± 12.56; C/T, 6.36 ± 6.49, 11.76 ± 3.86; and T/T, 6.40 ± 0.99, 9.33 ± 7.14 ng/mL. There are no significant differences between NQO1 genotypes (P = 0.5314, P = 0.5685, respectively).

In 16 patients receiving AMR at a dose of 40 mg/m², the genotypes C/C, C/T, and T/T were observed in 5 (31.2%), 7 (43.8%), and 4 (25.0%) patients, respectively. The plasma concentrations of AMR in the respective genotypes on days 2 and 4 were as follows: C/C, 10.22 ± 2.42, 22.48 ± 11.15; C/T, 11.04 ± 4.07, 19.03 ± 10.07; and T/T, 9.87 ± 5.77, 12.00 ± 6.54 ng/mL. There are no significant differences between NQO1 genotypes (P = 0.8904, P = 0.3020, respectively). Splendidly, the plasma concentrations of AMR-OH on days 2 and 4 in the respective genotypes were C/C, 9.46 ± 2.08, 20.5 ± 5.89; C/T, 8.11 ± 1.97, 15.9 ± 5.44; and T/T, 6.10 ± 2.38, 11.2 ± 4.47 ng/mL, and exhibited tendencies toward a relationship with NQO1 genotypes (P = 0.0947, P = 0.066, respectively). Figure 1 showed the plasma concentration of AMR and AMR-OH at a dose of 30 mg/m² (A,C), and AMR and AMR-OH at a dose of 40 mg/m² (B,D).

### NQO1 C609T polymorphisms and pharmacodynamics

Correlations between toxicities and NQO1 genotypes in patients receiving AMR at a dose of 40 mg/m² was determined because statistical significance between drug concentration and NQO1 genotypes was observed from an increase in a dose of 30 mg/m² to 40 mg/m². Figure 2 illustrates hematological toxicities according to genotype (C/C, C/T, or T/T) in patients receiving AMR at a dose of 40 mg/m². Decreases in WBC, neutropenia, hemoglobin, and platelet counts were related to NQO1 C609T genotypes (P = 0.013, P = 0.105, P = 0.027, and P = 0.0005, respectively) (Fig. 2A–D).

### Discussion

We demonstrated that the T/T genotype at the NQO1 C609T polymorphism exhibited a tendency toward a relationship with decreased concentrations of AMR-OH on days 2 and 4; it was also related to a significant decrease of hematological toxicities in patients receiving AMR at a dose of 40 mg/m². These findings suggest that the NQO1 genotype may be a candidate biomarker for hematological toxicities related to AMR treatment at a dose of 40 mg/m².

In our study of 35 patients, genotypes C/C, C/T, and T/T were observed in 12 (34.3%), 16 (45.7%), and 7 (20%) patients, respectively. These frequencies are comparable to those reported in the NCBI/NBI-SNP databases.

A recent report demonstrated that cisplatin did not affect the metabolism of AMR and AMR-OH. In general, the toxic effects of cisplatin include nephrotoxicity, nausea and vomiting, ototoxicity, neurotoxicity, and relatively minor hematological toxicities. Other reports have noted that the pharmacokinetic parameters of cisplatin were significantly higher in patients with nausea and vomiting or nephrotoxicity than in those without these conditions. In addition, in a
PK/PD study of combined treatment with cisplatin and docetaxel, neutropenia was positively correlated with area under the time curve for docetaxel, but not for cisplatin.\(^28\) In our study, of the 19 patients receiving 30 mg/m\(^2\) of AMR, 4 patients were co-administered cisplatin (20 mg/m\(^2\)) on days 1 to 3, whereas 14 were co-administered cisplatin (80 mg/m\(^2\)) on day 1. No significant difference in plasma concentrations of AMR-OH was observed between the patient groups receiving combination treatment with cisplatin at doses of 20 mg/m\(^2\) and 80 mg/m\(^2\) \((P = 0.407)\). In our study, no relationship between plasma level of cisplatin and hematological toxicity was apparent (data not shown). Therefore, the influence of cisplatin on hematological toxicity may be negligible.

The impact of the NQO1*2 polymorphism has also been demonstrated using excised human tumors in vitro; tumor tissues genotyped as NQO1 *2/*2 were more resistant to mitomycin C than those genotyped as wild type.\(^{29,30}\) In 117 patients genotyped for the NQO1*2 polymorphisms, individuals with the low activity NQO1 genotypes (homozygotes or homozygous NQO1*2) had reduced survival compared with individuals with the wild-type NQO1 genotype. Genotype-phenotype relationships were also confirmed in this study, with individuals with NQO1*1/*2 heterozygotes having significantly less tumor NQO1 activity than those with the homozygous wild-type NQO1 genotype.\(^31\) In our study, significant relationships were observed between NQO1 genotypes and hematological toxicities. For patients receiving AMR at a dose of 40 mg/m\(^2\)/day, it may be possible to predict hematological toxicities by genotyping the NQO1 polymorphism ahead of the treatment.

Takakuwa et al identified a relationship between this SNP and amrubicinol cytotoxicity in vitro; cell lines genotyped T/T had significantly lower NQO1 expression.

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**Figure 1. NQO1 C609T polymorphisms and pharmacokinetics.** The plasma concentration with mean ± SD, ng/mL. (A) AMR on day 4 in patients receiving AMR at a dose of 30 mg/m\(^2\). (B) AMR on day 4 in patients receiving AMR at a dose of 40 mg/m\(^2\). (C) AMR-OH on day 4 in patients receiving AMR at a dose of 30 mg/m\(^2\). (D) AMR-OH on day 4 in patients receiving AMR at a dose of 40 mg/m\(^2\). *Exhibited a tendency toward a relationship with NQO1 genotypes (B) \(P = 0.0947\), (D) \(P = 0.066\), respectively.

Note: *\(P < 0.1\).
and greater sensitivity to amrubicinol than cell lines (both NSCLC and SCLC) with other genotypes. However, no information about the generation and metabolism of amrubicinol was provided in this report. Interestingly, plasma concentrations of AMR-OH in patients with a T/T genotype at NQO1 exhibited a reduced tendency toward a relationship with AMR at a dose of 40 mg/m² than those in patients with other genotypes; this was related to decreases in WBC, hemoglobin, and platelet counts. Clinically, it may be interesting to explore the relationship between drug concentration and AMR metabolism. These plasma levels will probably also be significantly correlated with the polymorphism if the authors run this study with more patients in the future.

NADPH quinone oxidoreductase and carbonyl reductase play major roles in the metabolism of amrubicin, with the generation of the active metabolite amrubicinol mainly catalyzed by carbonyl reductase (CBR) exclusively in the liver cytosol in the presence of NADPH. In our study, there was no significant difference between NQO1 genotypes in the ratio of plasma concentration of AMR to AMR-OH at a dose of 40 mg/m² (data not shown), suggesting that there is no relationship between this SNP and CBR enzyme activity. Protein turnover studies in NQO1 wild type and mutant cell lines demonstrated that the half-life of wild-type NQO1 was greater than 18 hours, whereas that of mutant NQO1 was 1.2 hours. The C609T SNP of NQO1 results in a proline-to-serine substitution, leading to ubiquitination and rapid degradation of the variant protein. In patients treated with AMR at a dose of 40 mg/m², it is possible that the generation of AMR-OH increased in response to increased levels of NQO1 protein. In addition to NQO1, NADPH: cytochrome P450 reductase (POR) is another enzyme known to metabolize amrubicinol to an inactive metabolite. NQO1 plays a role under both aerobic and hypoxic conditions.
conditions, whereas POR is the key enzyme under hypoxic conditions.\textsuperscript{13,14} In an investigation of human metabolism of amrubicin in vitro using liver microsomes, amrubicin was shown to be metabolized by 54\% under aerobic conditions.\textsuperscript{16} There was no relationship between \textit{POR} gene expression levels and amrubincinol cytotoxicity in vitro.\textsuperscript{32} Accordingly, we speculate that POR is also a key enzyme in this process and future studies will include an assessment of the role of POR in amrubincinol cytotoxicity in vivo in specific microenvironments, such as in the liver cytosol or microsomes and under anaerobic or aerobic conditions.

Previously, we reported the relationships between AMR-OH and hematological toxicity during treatment with AMR alone, as well as during co-administration with CDDP, using a sigmoid Emax model for pharmacodynamics analysis.\textsuperscript{33} Based on our previous study, this pharmacogenomics study was performed and revealed that \textit{NQO1} genotype has a significant correlation with hematologic toxicities in patients receiving AMR. Further clinical studies are warranted to examine the \textit{NQO1} protein activity, the relationship of POR and \textit{NQO1}, and the influence of tumor tissue microenvironment—especially the hypoxic condition—on AMR metabolites.

Our study has some limitations. Firstly, several types of lung cancer patients were enrolled and as such variations in tumor properties may have influenced the study findings. Secondly, the population included in our study was small. Finally, the \textit{NQO1} activity and generation of reactivated oxygen intermediates by redox cycling were not examined. In order to confirm our findings and to identify factors influencing inter-individual variability of AMR, further studies and a larger number of patients would be highly desirable.

**Conclusion**

We found that the T/T genotype at the \textit{NQO1} C609T polymorphism exhibited a tendency toward a relationship with decrease concentrations of AMR-OH on days 2 and 4. We also found it to be related to a significant decrease of hematological toxicities in patients receiving AMR at a dose of 40 mg/m\textsuperscript{2}. Avoiding or reducing severe hematological toxicities associated with AMR therapy is of great importance. Genotype at this \textit{NQO1} SNP has potential use in the prediction of hematological toxicities in individual patients treated with AMR at a dose of 40 mg/m\textsuperscript{2}.

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**Competing Interests**

Author(s) disclose no potential conflicts of interest.

**Author Contributions**

MN and TK contributed equally to this study. MN participated in the design of the study, acquisition of data, statistical analysis, drafting of the manuscript, and revision of the manuscript. TK participated in the design of the study, acquisition of data, statistical analysis, drafting of the manuscript, and revision of the manuscript. SK conceived the study, participated in the design of the study, participated in data acquisition, and helped revise the manuscript. TS, TN, KU, HT, KM, SM, NY, TO, and KH were all involved in revising the manuscript. YK performed the real-time PCR experiments. All authors read and approved the final manuscript.

**Disclosures and Ethics**

As a requirement of publication authors have provided the publisher signed confirmation of compliance with legal and ethical obligations, including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality, and protection of human research subject. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclosures are made in this section. The external blind peer reviewers report no conflicts of interest.
In this paper we describe the NQO1 C609T polymorphism had correlate with hematologic toxicities in patients receiving amrubicin to avoid or reduce severe hematological toxicities associated with AMR therapy. Genotype at this NQO1 SNP has potential use in the prediction of hematological toxicities in individual patients treated with AMR at a dose of 40 mg/m².

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