Association of *CYP3A4/5* genotypes and expression with the survival of patients with neuroblastoma

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Abstract. Neuroblastoma (NB) is a rare pediatric disease in Lebanon for which poor prognosis remains a major challenge. Genetic polymorphism of genes coding for drug-metabolizing enzymes may influence the response of a patient to chemotherapy. This study investigates a possible association between *CYP3A4/5* polymorphism and expression levels and survival in NB patients. All patients with stage III and IV NB diagnosed between 1993 and 2012 in three major hospitals in Beirut were included (n=27). Demographic information and survival time were obtained from medical records. *CYP3A4* and *CYP3A5* genotypes and expression levels were determined in archival tumors by polymerase chain reaction (PCR) and restriction fragment length polymorphism and quantitative PCR, respectively. Additionally, *MYCN* amplification was assessed. A Cox proportional hazards model was used to evaluate potential associations, adjusting for *MYCN* amplification. A statistically significant increase in the risk of mortality was observed in patients with *MYCN* amplification [hazard ratio (HR) 4.11, 95% confidence interval (CI) 1.14-14.80]. Patients with *CYP3A5* expression levels above the median had a lower risk of mortality (HR 0.61, 95% CI 0.21-1.74) and patients with *CYP3A4* expression levels above the median had a higher risk of mortality (HR 2.00, 95% CI 0.67-5.90). *CYP3A5**3/*3 homozygote mutants had a 4.3-fold increase in the risk of mortality compared with that of homozygote wild-type or heterozygote mutants (HR 4.30, 95% CI 0.56-33.30). Carriers of the *CYP3A4**1B mutant allele had a 52% lower risk of mortality compared with that of non-carriers (HR 0.48, 95% CI 0.06-3.76). Although the results of the present study did not achieve statistical significance, associations were observed, which indicates that *CYP3A4* and *CYP3A5* may modulate the clinical outcome of NB. Further studies with larger sample sizes are required to characterize the effects of the polymorphism and expression levels of *CYP3A4/5* on the survival of patients with NB.

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Introduction

Neuroblastoma (NB) is a solid embryonal tumor of the autonomic nervous system, derived from primitive sympathetic neurons. In >50% of all cases of NB, tumors arise in the adrenal medulla, while the rest originate in the paraspinal sympathetic ganglia, with a highly variable clinical presentation (1). Between 1975 and 2011, NB occurred in 1 in 7,000 live births worldwide and accounted for 7-10% of all childhood cancers (2). The annual age-standardized incidence rate (ASR) of NB in the Children Oncology Group between 1986 and 2001 was estimated to be 10.53 cases per million individuals in North American populations (3). In Lebanon, the National Cancer Registry (NCR) reports NB as a very rare malignancy, with an ASR of 2 cases per million individuals in 2007 (4).

According to the Surveillance Epidemiology and End Results (SEER) databases, the clinical outcome for patients with NB has improved in the last 40 years (5). However, this improvement is primarily attributed to an increase in cure rates among patients with the benign form of the disease. The cure rates among children with malignant high-risk tumors have shown only modest improvement, despite marked escalations in the intensity of therapy provided (6). Despite recent therapeutic advances, no salvage treatment modalities exist to date, and high-risk NB is still characterized by a low survival rate, with 50-60% of patients showing a relapse (7).

A number of previous studies indicated that genetic factors may be involved in the clinical outcome and the response to treatment of NB patients (8). Tumor-derived genomic information has been used since the 1980s to predict prognosis, particularly since the *MYCN* oncogene was first discovered

to be the target of high-level amplifications at chromosome band 2p24. Due to the influence of *MYCN* amplification on the clinical outcome of NB, it is currently used as a standard biomarker for treatment stratification (9). In 1985, NB patients with an *MYCN* gene amplification of 1, 3 and ≥10 gene copies were estimated to have an 18-month survival rate of 70, 30 and 5%, respectively (10). In addition to *MYCN* amplification, NB stage III or IV, abnormal chromosomal deletion/translocation and an older age at diagnosis have all been identified as being associated with poor prognosis (11).

Current treatments for patients with high-risk NB include induction of remission, consolidation of the remission, and eradication of the minimal residual disease. The most commonly used induction chemotherapy includes dose-intensive cycles of cisplatin and etoposide, alternated with vincristine, doxorubicin, cyclophosphamide or ifosfamide and topotecan (12,13). A number of drug-metabolizing enzymes (DMEs) appear to be involved in the activation or inactivation of these drugs, including cytochrome P450 (CYP), *N*-acetyltransferase (NAT) and glutathione S-transferase (GST) (14). Therefore, individuals possessing a modified ability to metabolize these drugs may have an increased risk of relapse or mortality. In a large cohort of Australian individuals with NB studied over 15 years (n=209), carriers of the *NAT1*11* allele variant were significantly less likely to relapse or succumb to the disease, while children who were GSTM1 null were more likely to relapse or succumb to the disease, after adjusting for *MYCN* amplification, stage of the disease and age at diagnosis [hazard ratio (HR), 1.6, P=0.04] (15). The phase I oxidation enzymes CYP3A4 and CYP3A5 were also found to metabolize a number of the drugs used in NB treatment (16-22) (Table I). Single nucleotide polymorphisms (SNPs) in these CYP genes may alter their enzymatic activity, hence affecting clinical outcome. However, despite these observations, there are no studies on the role of cytochrome P450 in NB clinical outcome to date. In the present study, the potential association between the genotype and expression levels of *CYP3A4* and *CYP3A5* and mortality were examined in a group of Lebanese patients with NB.

Materials and methods

Study design and participants. The current study employed a retrospective cohort design. Three major medical centers in the Lebanese capital city of Beirut participated. Specimens from children with histologically confirmed stage III or IV NB, diagnosed between 1993 and 2012, were obtained from the medical archives of Saint George Hospital University Medical Center, Hotel Dieu De France Hospital and the University Medical Center - Rizk Hospital (all Beirut, Lebanon) and provided to the current study anonymously. Patients excluded from the study were those with stage I or II NB (n=2) and those with missing archival tumors (n=9). Of the 38 patients identified, 27 were included in the study.

Approval was obtained from the Institutional Review Board of the University of Balamand prior to conducting the study. A review of the medical records of all patients was performed anonymously to obtain the following information: Age at diagnosis, gender, NB stage, date of diagnosis, relapse state, date of mortality and administered chemotherapeutic drugs.

Nucleic acid extraction. Sections (10 μm thick) of identified paraffin-embedded NB tumors were obtained. These were prepared by a trained pathologist, avoiding necrotic areas to ensure effective nucleic acid extraction. Sections were subjected to deparaffinization by xylene and protein digestion by proteinase K (Qiagen, Valencia, CA, USA). Tumor DNA was extracted from 50% of the sections using a QIAamp DNA FFPE Tissue kit (Qiagen), while RNA was extracted from the remaining 50% of sections using an RNeasy FFPE kit (Qiagen), both according to the manufacturer's instructions.

CYPs genotyping analysis. *CYP3A4**1B and *CYP3A4**1A alleles were detected by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) using 100-150 ng of extracted DNA and the appropriate primers (Table II) at an annealing temperature of 55˚C as previously described (23). The resulting 334-bp PCR product was digested by the *Pst*I restriction enzyme (Thermo Scientific, Waltham, MA, USA) overnight at 37˚C. *CYP3A5**1 and *CYP3A5**3 alleles were detected using 100-150 ng of tumor DNA and the appropriate primers (Table II) at an annealing temperature of 55˚C as previously described (24). The resulting 293-bp PCR product was digested by the *Ssp*1 restriction enzyme (Thermo Scientific) overnight at 37˚C. Digested fragments were then analyzed using ethidium bromide staining and agarose gel electrophoresis, and examined under UV light.

MYCN amplification. *MYCN* amplification was assessed for each NB tumor sample using extracted DNA in a Real-Time PCR TaqMan Detection assay (Applied Biosystems, Foster City, CA, USA). The degree of *MYCN* amplification was derived from the ratio of the number of copies of the *MYCN* oncogene to the number of copies of the reference gene *β-actin* (*MYCN*:*β-actin* ratio). The 50-μl PCR mixture contained 10 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 10 mmol/l EDTA, 60 nmol/l passive reference dye ROX, 3.5 mmol $MgCl_2$, 0.2 mmol/l of each dNTP, 300 nmol/l of each primer, 200 nmol/l of each probe, 0.5 U of AmpliTaq Gold, and 1 U of AmpErase UNG (Applied Biosystems) (Table II). The two genes were amplified as follows: 120 sec at 95˚C followed by 45 cycles of: 30 sec at 95˚C, 30 sec at 60˚C, and 30 sec at 72˚C.

The concentrations of *MYCN* and *β-actin* were estimated in the same assay via extrapolation on external calibration curves. The threshold cycle value (C_T) for each sample was used to estimate the target concentration on the curves. Calibration curves were constructed with reference DNA extracted from the peripheral blood of two healthy male donors (aged >50) with no chronic diseases. The DNA concentration was determined by spectrophotometric measurement (Nanodrop 1000; Thermo Scientific) and expressed as number of molecules per microliter as previously described (25). The DNA was serially diluted to plot the calibration curves and the degree of amplification of each sample was derived from the *MYCN*:*β-actin* ratio. Only samples in which the *MYCN*:*β-actin* ratio was >2.0 were considered amplified. In each assay, positive controls and non-amplified negative controls were used.

cDNA synthesis and quantitative PCR (qPCR) for CYPs. cDNA synthesis was performed by reverse transcription of extracted tumor RNA using a RevertAid First Strand cDNA Synthesis

Table I. Chemotherapeutic agents used in the treatment of Lebanese patients with neuroblastoma and their corresponding drug-metabolizing enzymes.

Anti-cancer agent	Phase I enzyme	Phase II enzyme
Cisplatin	*GSTM1*, *GSTP1*, *GSTT1*	-
Cyclophosphamide	*CYP2A6*, *CYP2B6*, *CYP2C8/9/19*, *CYP3A4*, *CYP3A5*	*ALDH*, *GSTA1*
Doxorubicin	-	*GSTP1*
Etoposide	*CYP2C9*, *CYP3A4/5*	*UGT1A1*, *GSTP1*
Ifosfamide	*CYP2A6*, *CYP2B6*, *CYP2C8/9/19*, *CYP3A4*, *CYP3A5*	*GST*
Topotecan	-	-
Vincristine	*CYP3A4*, *CYP3A5*	*GST*

Table II. Primers and probes used in genotyping, mRNA expression and amplification assays.

Gene and assay	Sequence
CYP3A4 gene	
Genotyping forward primer	5'-GGACAGCCATAGAGACAA CTGCA-3'
Genotyping reverse primer	5'-CTTTCCTGCCCTGCACAG-3'
mRNA expression forward primer	5'-CACAGATCCCCCTGAAATTAAGCTTA-3'
mRNA expression reverse primer	5'-AAAATTCAGGCTCCACTTACGGTG-3'
TaqMan probe	5'-(6FAM)-AGGACTTCTTCAACCAGAAAAACCCGTTGTTCT-(TAMRA)-3'
CYP3A5 gene	
Genotyping forward primer	5'-CATGCTTAGTAGACAGATGAC-3'
Genotyping reverse primer	5'-GGTCCAAACAGGGAAGAAATA-3'
mRNA expression forward primer	5'-ACAGATCCCCTTGAAATTAGACACG-3'
mRNA expression reverse primer	5'-CTTAGGGTTCCATCTCTTGAATCCA-3'
TaqMan probe	5'-(6FAM)-AAGGACTTCTTCAACCAGAAAAACCCATTGTTCTA-(TAMRA)-3'
MYCN amplification	
Forward primer	5'-CCCCTGGGTCTGCCCCGTTT-3'
Reverse primer	5'-GCCGAAGTAGAAGTCATCTT-3'
Fluorogenic probe	5'-CCCACCCTCTCCGGTGTGTCTGTCGGTT-3'
β-actin control gene	
mRNA expression forward primer	5'-TCACCCACACTGTGCCCATCTACGA-3'
mRNA expression reverse primer	5'-CAGCGGAACCGCTCATTGCCAATGG-3'
TaqMan probe	5'-(6FAM)-ATGCCCTCCCCCATGCCATCCTGCGT-(TAMRA)-3'
Amplification forward primer	5'-TCACCCACACTGTGCCCATCTACGA-3'
Amplification reverse primer	5'-CAGCGGAACCGCTCATTGCCAATGG-3'
Fluorogenic probe	5'-ATGCCCTCCCCCATGCCATCCTGCGT-3'

kit (Thermo Scientific). Briefly, the reaction consisted of RNA, hexamer primers (provided in the RevertAid kit), reaction buffer, RNase inhibitor, dNTP mix and reverse transcriptase which were incubated for 5 min at 25˚C and then the reaction was terminated by heating at 70˚C for 5 min. qPCR analysis was performed using a TaqMan system (Applied Biosystems) according to the manufacturer's instructions. *β-actin* was used as the reference gene. The 50-µl PCR mixture consisted of 20 ng synthesized cDNA, primers for *CYP3A4*, *CYP3A5* and *β-actin* (600 nmol/l each), 250 nmol/l TaqMan probes and PCR Master mix (Applied Biosystems) (Table II). Amplification and detection were performed with the MxPro qPCR Agilent system (Agilent Technologies, Santa Clara, CA, USA) using the following PCR reaction profile: 95˚C for 10 min followed by 40 cycles of 95˚C for 20 sec and 62˚C for 1 min. In every assay, liver DNA extracted from paraffin-embedded healthy liver tissue, archived at St George Hospital University Medical Center (Beirut, Paris) and a non-DNA template were used as positive and negative controls, respectively.

CYP mRNA expression calculations. Gene expression was calculated by absolute quantification as previously described (26). Standard curves for the target genes *CYP3A4* and *CYP3A5*, as well as the reference gene *β-actin*, were constructed by serial dilutions of a synthesized cDNA obtained from archival liver tissue of a healthy donor. The threshold

cycle values obtained for each gene from the tumor samples were extrapolated on corresponding constructed standard curves to obtain the number of copies per gene per sample. *CYP3A4* and *CYP3A5* expression levels were then calculated as a ratio of the number of copies of the target gene to the number of copies of the reference gene obtained for each sample.

Statistical analysis. Variant genotypes were grouped for statistical analyses based on previous studies of enzymatic function or phenotypic consequence (27-30). Homozygote carriers of the *CYP3A5**3/*3 mutant genotype were compared with *CYP3A5**1/*3 heterozygotes or wild-type *CYP3A5**1/*1 homozygotes. Similarly, homozygote and heterozygote carriers of *CYP3A4**1B mutant allele (*CYP3A4**1B/1B or *CYP3A4**1A/1B) were compared with homozygote wild-type *CYP3A4**1A/1A carriers. *CYP3A4* and *CYP3A5* gene expression levels were analyzed as dichotomous variables (above or below the median expression level). Overall Survival (OS) is a standard measurement of survival defined as the duration from the time of diagnosis of disease to either death or date of last contact (31). A Kaplan-Meier survival analysis was used to estimate the OS in the total group of patients, and the *MYCN* amplification subgroups. The difference in survival between the MYCN amplified and the MYCN non-amplified groups was compared using the Log rank test (25). A Cox proportional hazard regression model was used to assess associations between mortality and *CYP3A4* and *CYP3A5* polymorphisms and mRNA expression after adjusting for *MYCN* amplification. HRs and 95% confidence intervals (CIs) were calculated. Statistical analyses were performed using the Statistical Package for Social Sciences, version 18.0.0 (SPSS, Inc., Chicago, IL, USA).

Results

Demographic and clinical characteristics. The mean age at NB diagnosis was 2.5 years (±1.1). The median OS was 3.7 years (range: birth to 11 years). Cases were more likely to arise in males than in females (gender ratio 5:4). The median time to mortality was 3.7 years (range: 9 months to 10 years) and 55.6% of the cases had stage IV disease at diagnosis. *MYCN* amplification was present in 59% of the cases. All patients underwent surgery while 59% underwent radiotherapy. Of all the patients, 22% showed tumor relapse, and 44% had succumbed to the disease by the end of follow-up (Table III).

Prevalence of CYP3A4 and CYP3A5 genotypes. The majority of the studied patients were carriers of the homozygote wild-type genotype *CYP3A4**1A/1A (85.2%), and the homozygote mutant genotype *CYP3A5**3/*3 (81.5%) (Table III and Fig 1).

Survival analysis. Kaplan-Meier survival analysis revealed that NB patients with *MYCN* amplification had a significantly lower median OS compared with those with no *MYCN* amplification (3.1 vs. 5.8 years, P=0.03) (Fig. 2). The associations between the risk of mortality and a number of genetic factors, calculated using Cox proportional hazard regression analysis, are shown in Table IV. A 4-fold increased risk of mortality was associated with *MYCN* amplification (HR 4.11, 95% CI 1.14-14.80 P<0.02). After adjusting for *MYCN* amplification, patients with *CYP3A5* expression levels above the median had a 39% lower mortality risk (HR 0.61, 95% CI 0.21-1.74 P=0.353) while patients with *CYP3A4* expression levels above median had a 2-fold higher mortality risk (HR 2.00, 95% CI 0.67-5.90 P=0.214) compared with that of patients with expression levels below the median; however, these associations were not statistically significant.

On the other hand, carriers of the *CYP3A5**3/*3 homozygote mutant genotype had a 4-fold increased risk of mortality compared with that of the homozygote wild-type or heterozygote mutant carriers (HR 4.30, 95% CI 0.56 33.3 P=0.511). Homozygote and heterozygote carriers of the *CYP3A4**1B mutant allele had a 52% lower risk of mortality compared with that of the non-carriers (HR 0.48, 95% CI 0.06-3.76, P=0.368).

Table III. Descriptive data on the studied population.

Characteristic	Patients (n=27)
Age at diagnosis (years), mean (SD)	2.5 (±1.1)
OS (years), median (range)	3.7 (0-11)
Gender, n (%)	
Male	15 (55.6)
Female	12 (44.4)
Tumor stage, n (%)	
III	12 (44.4)
IV	15 (55.6)
CYP3A4 genotype, n (%)	
*CYP3A4**1A/*1A	23 (85.2)
*CYP3A4**1A/*1B	3 (11.1)
*CYP3A4**1B/*1B	1 (3.7)
CYP3A5 genotype	
*CYP3A5**1/*1	2 (7.4)
*CYP3A5**1/*3	3 (11.1)
*CYP3A5**3/*3	22 (81.5)
MYCN amplification, n (%)	
Yes	16 (59.3)
No	11 (40.7)
Surgery, n (%)	
Yes	27 (100.0)
No	0 (0)
Radiotherapy, n (%)	
Yes	16 (59.3)
No	11 (40.7)
Relapse, n (%)	
Yes	6 (22.2)
No	21 (77.8)
Mortality, n (%)	
Yes	12 (44.4)
No	15 (55.6)

SD, standard deviation; OS, overall survival.

Table IV. Association of cytochrome P450 expression, genotypes, and *MYCN* amplification with the risk of mortality in patients with neuroblastoma.

Variable (reference)	HR (95% CI; P-value)
MYCN amplification (No amplification)	4.11 (1.14-14.80; 0.020)
CYP3A4 genotypes *CYP3A4**1B/1B or *CYP3A4**1A/1B (*CYP3A4**1A/1A)	0.48 (0.06-3.76; 0.368)
CYP3A5 genotype *CYP3A5**3/*3 (*CYP3A5**1/*3 or *CYP3A5**1/*1)	4.30 (0.56-33.30; 0.511)
CYP3A4 expression>median (<median)	2.00 (0.67-5.90; 0.214)
CYP3A5 expression>median (<median)	0.61 (0.21-1.74; 0.353)

All *CYP3A4/5* genotypes and expression associations with mortality risk were adjusted for *MYCN* amplification.

These associations were also adjusted for *MYCN* amplification and they did not achieve statistical significance.

Discussion

In the present study, the associations between survival time and the expression levels or genetic polymorphisms of *CYP3A4* and *CYP3A5,* as well as *MYCN* amplification, were analyzed in Lebanese children diagnosed with NB. *CYP3A4**1B and *CYP3A5**3 polymorphisms were specifically targeted as being the most common among multiple populations, with some insight into their effect on enzymatic activity and expression (27). In concordance with previous studies, *MYCN* amplification was observed to be the strongest predictor of an unfavorable clinical outcomes in children with NB in the present study (10). In addition, after adjusting for *MYCN* amplification, the results of the present study demonstrated that individuals with the *CYP3A5**3/*3 mutant genotype and lower tumor *CYP3A5* expression levels demonstrated a higher mortality risk, while those with the *CYP3A4**1B mutant allele and lower tumor *CYP3A4* expression levels demonstrated a lower mortality risk; however, these associations did not achieve statistical significance, likely owing to the small sample size.

CYP3A4 and *CYP3A5* are considered to code for important CYP isoenzymes pertaining to drug metabolism, due to the diversity of their anti-cancer substrates and their relative abundance in humans (32). While no previous studies have examined the association between these enzymes and clinical outcome in NB, both enzymes have been shown to be important prognostic modifiers in several common types of malignancy. High protein expression levels of the *CYP3A4/5* genes have previously been reported to predict metastasis and poor prognosis in osteosarcoma (33). On the other hand, patients with breast cancer carrying the *CYP3A4**1B and *CYP3A5**1 alleles were found to have a significantly lower mean survival time compared with that of carriers of the wild-type alleles (17).

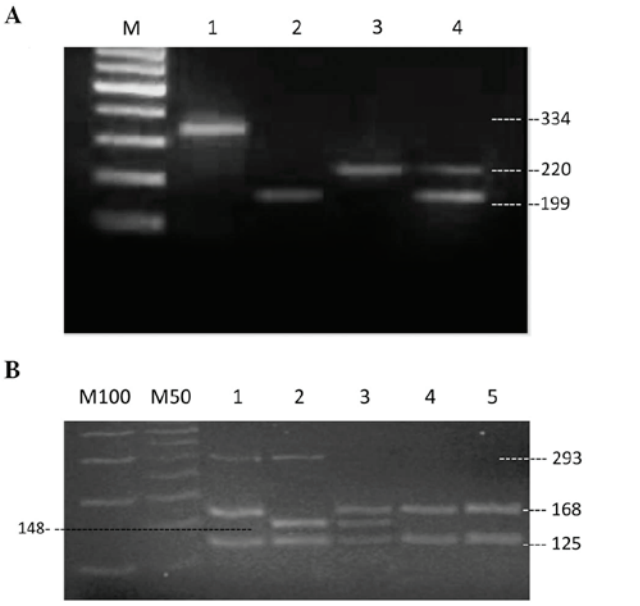

Figure 1. Genotyping gel electrophoresis for *CYP3A4* and *CYP3A5* following polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP). (A) *CYP3A4*. Lane M: 100-bp DNA marker, lane 1: 334-bp PCR product, lane 2: *CYP3A4**1B/*1B homozygote mutant, lane 3, *CYP3A4**1A/*1A homozygote wild type, lane 4: *CYP3A4**1A/*1B heterozygous genotype. (B) *CYP3A5*. Lane M100: 100-bp DNA marker, lane M50: 50-bp ladder, lanes 1, 4 and 5: *CYP3A5**3/*3 homozygote mutant, lane 2: *CYP3A5**1/*1 homozygote wild type, lane 3: *CYP3A5**1/*3 heterozygote genotype.

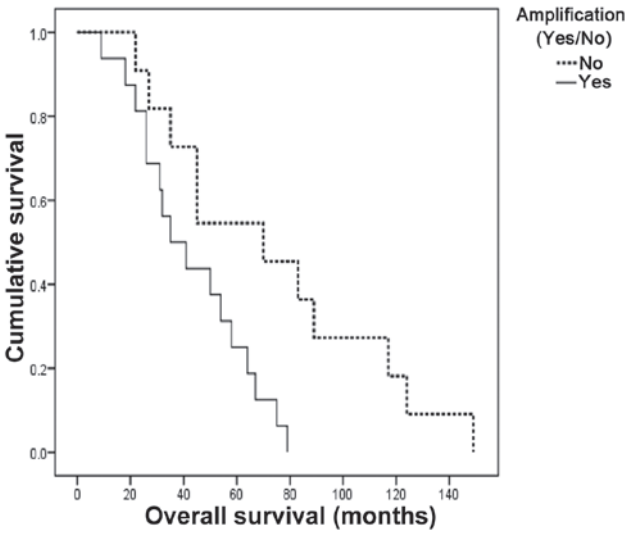

Figure 2. Kaplan-Meier estimates of the overall survival (OS) of children with neuroblastoma by *MYCN* amplification status. The median OS was higher in patients with no *MYCN* amplification (5.8 vs. 3.1 years, P=0.03).

Furthermore, *CYP3A5**1 was observed to be associated with an improved prognosis in a group of Caucasian patients with T-cell acute lymphocytic leukemia (34) and *CYP3A5**3 was found to modulate chemotherapy-induced toxicity in a group of Swedish patients with ovarian cancer (35).

*CYP3A5**3 consists of an A to G transition in intron 3, creating a cryptic splice site (27). Inappropriate splicing leads to a premature termination codon, resulting in a truncated protein with 101 amino acids (AA) compared with 502 AA in the

normal protein (28). mRNAs with premature stop codons are more unstable and rapidly degraded, which explains the lower levels of *CYP3A5* mRNA in *CYP3A5**3 homozygote carriers compared with those in wild-type carriers (36). *CYP3A5**3/*3 carriers were determined to have >50% reduction in catalytic activity for the metabolism of midazolam compared with that of carriers of the *CYP3A5**1 wild-type allele (37). The results of the present study regarding *CYP3A5* are in agreement with these findings. The *CYP3A5**3/*3 genotype and lower expression levels of *CYP3A5* mRNA were consistent in predicting poor clinical outcomes in NB patients. As such, it can be hypothesized that homozygote carriers of the *CYP3A5**3 allele may have much lower levels of enzymatic activity than carriers of other *CYP3A5* variants, and that the unfavorable outcome observed in the patients with NB in the present study may be partially due to slower bioactivation of used chemotherapeutic drugs, particularly etoposide and the nitrogen mustard drugs, based on the literature (16,19,20,38). This may be of importance clinically, particularly due to the high prevalence of the defective allele in the studied population (87%). The frequency of the allele reported in the Lebanese patients is almost identical to that reported in Caucasian patients (91%), and higher than that reported for populations of African descent (50%) (24,39). The hypothesis proposed in the present study, that the *CYP3A5* genotype and associated phenotype are part of the prognosis profile of patients with NB, was based on the reported hazard ratios and supporting molecular biological evidence from the literature. However, this hypothesis could not be accepted with confidence as the results were not statistically significant, possibly due to the small sample size of the study.

*CYP3A4**1B is an A to G transition within the 5'-flanking region of the gene. This polymorphism is considered to be associated with enhanced expression due to reduced binding of a repressor that may affect the transcriptional rate (29,30). However, no associations have been found between the *CYP3A4**1B allele genotype and pharmacokinetics of its substrates (40,41). Regulation of *CYP3A4* expression is known to be essentially pre-translational and its mRNA levels allow a good estimate of its enzymatic activity (42). The *CYP3A4**1B polymorphism, however, cannot explain the observed mRNA expression levels (43). In the current study, the possible association between a higher mortality risk and higher *CYP3A4* expression levels may be more significant than the reported protective effect for the *CYP3A4**1B polymorphism, particularly since it has a low frequency in the studied group (8.7%). This is similar to frequencies reported in Caucasian individuals (3.6-9.6%) and much lower than those reported in African individuals (53-67%) (44, 45). A potential association between higher *CYP3A4* expression levels and clinical outcome in NB may be hypothesized, particularly since similar associations have been previously reported in breast cancer (46), however, the results of the present study are inconclusive. Phenotypes may vary with tissue and may be dependent upon other endogenous and environmental factors. Further studies are required to elucidate the function of *CYP3A4* in the clinical outcome of NB.

There were a number of limitations in the present study that should be considered when interpreting the results. The sample size of the study was smaller than desired, due to the low incidence of NB in the Lebanese population and missing tumor samples for a number of identified patients. Another limitation of this study is the possibility that other DMEs may be modifying the response to drugs received by patients. Additional *CYP3A4/5* SNPs, GSTs, *N*-acetyltransferases, and other CYPs not investigated by the current study may influence the metabolic outcome of anticancer drugs most commonly used in the NB setting (Table I). The effects of these factors were not adjusted for and may explain why the obtained results were not significant. In addition, due to the retrospective nature of the study, serum samples from patients were not available to conduct direct measurements of drug levels. Furthermore, investigation of the mechanisms by which *CYP3A4/5* polymorphisms and expression levels may act to influence patient outcome following high-dose therapy was not possible and further studies should be conducted to address this point. Additionally, DNA from blood samples was not available to check genotype concordance between the blood and the tumor.

However, this study showed some strength. Despite the small sample size, the prognostic value of *MYCN* amplification was found to be statistically significant, in concordance with the literature. Furthermore, *CYP3A5**3/*3 genotype and lower mRNA expression levels predicted similar clinical outcome, which reflects the internal validity of the reported results.

The hypothesis that *CYP3A5**3 polymorphism and low expression levels may be associated with an inferior clinical outcome in children diagnosed with NB is the first of its kind. Although the observed effects did not achieve statistical significance, the suggested associations may apply for a substantial proportion of cases given the high prevalence of this particular polymorphism in numerous populations including Lebanese. The Lebanese population appears to have a high *CYP3A5**3 mutant genotype frequency, similar to that of the Caucasian population. The results of the current study may have an important clinical value. Since *CYP3A5* represents at least half the total hepatic CYP3A content, it may be an important genetic contributor to inter-individual and interracial differences in clearance and response to NB anticancer drugs, particularly in *CYP3A5* homozygote mutant patients (33). Characterization of associations between *CYP3A5* polymorphisms and clinical outcome could be used to genetically define subgroups of patients with NB who may be more predisposed to therapeutic failures or adverse drug reactions. However, the prognostic value of functional polymorphisms of *CYP3A5* and *CYP3A4* remains to be fully determined. We recommend building upon the observations of the current study by conducting cohort studies which incorporate larger sample sizes. Confirming these hypotheses and generating supportive mechanistic data will ultimately allow for an individualized and optimized NB therapy that may improve survival.

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