RESEARCH ARTICLE

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Correlation of Genetic Polymorphism of CYP3A5 to Cyclophosphamide Efficacy and Toxicity in Rhabdomyosarcoma Pediatric Egyptian Cancer Patients

Cherine E. ElShereef[1], Hala F. Zaki[2], Osama A. Badary[3], Sherif Kamal[4], Mohamed Nagy[5], Dalia Makhlouf[5], Mohamed Kamal[6], Inas Elnady[7], Sameh A.Abdelshafi[6], Sherif Abou El Naga[8], Mona M. Saber[2]*

Abstract

Objectives: Rhabdomyosarcoma (RMS) accounts for 50% of soft tissue sarcomas and 7% of pediatric malignancies. Cyclophosphamide (CPA) is the cornerstone of therapy and is a prodrug that is activated by the highly polymorphic drug-metabolizing enzyme CYP3A5. We aim to examine the possible CYP3A5 polymorphism association with CPA efficacy, survival outcomes, and toxicity in Egyptian pediatric RMS patients. **Methods:** The three non-functional SNPs, CYP3A5*3 rs776746 (C_26201809_30), CYP3A5*6 rs10264272 (C_30203950_10), and CYP3A5*7 rs41303343 (C_32287188_10) were genotyped by real-time PCR. We conducted a cohort retrospective study of 150 pediatric RMS patients treated with CPA-based first-line treatment to analyze the association between these genotypes and CPA efficacy/toxicities in RMS patients. **Key findings:** The frequency of having normal, intermediate, and poor metabolizers was 4.7%, 34%, and 61.3%, respectively. There was an association between these different phenotypes, genotypes, and CPA efficacy/toxicity. Hemorrhagic cystitis and pancytopenia were present in all patients, while nephrotoxicity incidence was 87.3%. There was a notable difference in the occurrence of hemorrhagic cystitis among CYP3A5 intermediate metabolizers *1/*3, *1/*6, and poor metabolizers *3/*3, *3/*6 with a significance level of $p<0.05$. Neither CYP3A5*7 polymorphism nor *6/*6 genotype was identified in our study. **Conclusion:** Our results demonstrate that CYP3A5*3 (rs776746) and CYP3A5*6 (rs10264272) have a great association with CPA efficacy and toxicity in RMS patients.

Keywords: Rhabdomyosarcoma- cyclophosphamide- CYP3A5- Pharmacogenetics- Pharmacogenomics

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Introduction

Rhabdomyosarcoma (RMS) is a malignancy that primarily affects skeletal muscle tissue but can also occasionally affect organs like the bladder and uterus. RMS can affect anyone at any age, but children are more prone to developing it. It is a little spherical blue cell tumor that makes up 7% of all pediatric malignancies [1].

Rhabdomyosarcoma (RMS) cases are estimated to increase by 400–500 each year in the USA. More than half of rhabdomyosarcoma diagnoses are made in children and adolescents under the age of 10, who also account for the majority of cases. These tumors, known as embryonal rhabdomyosarcomas (ERMS), typically develop in the head and neck region, as well as the vaginal and urinary systems. All age groups are susceptible to alveolar rhabdomyosarcoma (ARMS), which is more frequently detected in the arms, legs, or trunk. The risk rating approach that guides RMS treatment takes into account both pre-treatment clinical Tumor , Node and Metastasis (TNM) staging and surgical grouping. Recovery rates increased from 25% in the early 1970s to 70% in the subsequent 40 years, thanks in part to risk stratification and multimodal care [2].

Vincristine, actinomycin, and cyclophosphamide (VAC) have been used as the standard chemotherapy regimen for treating RMS for many years, according to the Intergroup Rhabdomyosarcoma Study Group. Approximately 35% of RMS patients benefit from chemotherapy in achieving complete remission (CR) [3].

*[1]Clinical Pharmacy Department, Faculty of Pharmacy, Heliopolis University, Cairo, Egypt. [2]Pharmacology and Toxicology Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt. [3]Clinical Pharmacy Department, Faculty of Pharmacy, Misr University for Science and Technology, Cairo, Egypt. [4]Egypt Healthcare Authority, Cairo, Egypt. [5]Pharmaceutical Services Department and Personalized Medication Management Unit, Children's Cancer Hospital Egypt (57357), Cairo, Egypt. [6]Research Department, Children's Cancer Hospital Egypt (57357), Cairo, Egypt. [7]Pediatric Medical Oncology, Beni Suef University and Children's Cancer Hospital Egypt (57357), Cairo, Egypt. [8]Pediatric Oncology Department, National Cancer Institute, Cairo University and Children's Cancer Hospital Egypt 57357, Cairo, Egypt. *For Correspondence: mona.magdy@pharma.cu.edu.eg*

Cyclophosphamide (CPA), an oxazaphosphorine alkylating chemotherapeutic medication that is primarily used in combination with other chemotherapeutic medications to treat lymphomas, some forms of brain cancer, neuroblastoma, leukemia, and other solid tumors. [4]. It has a relatively narrow therapeutic index and a number of undesirable side effects, including cardiotoxicity, nephrotoxicity, neurotoxicity, infertility, hemorrhagic cystitis, myelosuppression/pancytopenia, and leukemogenesis [5,6]. Due to the fact that it is a prodrug, only 70–80% of the dosage is converted to its active metabolite; 4-hydroxycyclophosphamide. It is swiftly absorbed and then processed into active metabolites by the cytochrome P450 system (CYP3A5) in the liver [7]. The main active metabolite is 4-hydroxycyclophosphamide, which balances aldophosphamide that easily diffuses into cells, where it is broken down into phosphoramide mustard and acrolein [8].

Phosphoramide mustard creates DNA crosslinks between and within DNA strands causing cell death [9]. Acrolein however, can induce hemorrhagic cystitis, which is characterized by microscopic or significant hematuria and, in rare cases, dysuria [10]. Hemorrhagic cystitis can be prevented by drinking enough fluids, avoiding nighttime doses, and using MESNA (sodium 2-mercaptoethane sulfonate), a sulfhydryl donor that binds and detoxifies acrolein [11]. When cyclophosphamide is administered less frequently, the total medicine dose is decreased, and the amount of acrolein that enters the bladder is decreased [12]. Many hepatic cytochrome P450 (CYP450) enzymes, including CYP3A4/5, CYP2B6, CYP2C9, and CYP2C19, have been connected to CPA activation. According to earlier investigations, the main CYP450s involved in CPA activation include CYP3A4 and CYP3A5 [13,14]. The polymorphic enzyme CYP3A5 has a large number of recognized variations [15,16]. There are now nine recognized CYP3A5 single nucleotide polymorphisms (SNPs) in humans with various ethnic frequencies [17]. In comparison to the CYP3A5*1 wild type , polymorphic CYP3A5 variants have been shown to have less favorable metabolic features than the CYP3A5*1 [18,19].

The three allelic variations of CYP3A5 that are most prevalent in Caucasians are CYP3A5*3, CYP3A5*6, and CYP3A5*7. A single nucleotide polymorphism in intron 3 of the CYP3A5*3 causes an early termination codon, whereas studies have indicated that the allelic variants CYP3A5*6 and CYP3A5*7 produce little to no active CYP3A5 enzyme [20,21]. The remaining genotypes indicated are essentially devoid of active CYP3A5 enzyme since people without the CYP3A5*1 produce such a minimal amount of this enzyme. Cyclophosphamide-induced toxicity and efficacy were shown to be higher in non-expressers (poor metabolizers) than in expressers (normal metabolizers) [22,23]. Researchers have been examining the role of common non-functional CYP3A5 SNPs in predicting response to various treatments and assisting in the individualization of dose for each patient to improve chemotherapeutic effectiveness while reducing toxicity in response to the growing importance of personalized medicine [24].

Understanding the factors that affect how CPA is metabolized could aid in explaining why different people have varying reactions to CPA thus leading to the development of more effective CPA dosing regimens. This emphasizes the importance of dose individualization, which can enhance therapeutic efficacy and toxicity. To the best of our knowledge, there is no information regarding the impact of the CYP3A5 polymorphism on CPA efficacy and toxicity in pediatric cancer patients in Egypt. Therefore we aim to ascertain the prevalence of three CYP3A5 common non-functional SNPs in a cohort of Egyptian pediatric RMS patients CYP3A5*3 (rs776746), CYP3A5*6 (rs10264272), and CYP3A5*7 (rs41303343) and to examine the possible association of CYP3A5 polymorphism and cyclophosphamide efficacy, survival outcomes, and toxicity in Egyptian pediatric RMS cancer patients to increase treatment success with low side effects.

Materials and Methods

Patient eligibility and treatment

This pharmacogenetic study is a cohort observational retrospective analysis on pediatric Egyptian cancer patients at Egypt's 57357 children's cancer hospital. A total of 150 RMS patients under the age of 18 were included in this retrospective analysis. They were recruited in January 2013 till November 2016 at the Children's Cancer Hospital of Egypt (CCHE) and treated with a combination of vincristine, actinomycin, and cyclophosphamide (VAC) as the first-line standard clinical treatment for RMS, according to Intergroup Rhabdomyosarcoma study group (IRSG-IV) protocol. All patients took their treatment protocol and were followed-up in the past and their samples and data were collected from the hospital's biobank and files in the hospital system. The regimen included Vincristine (1.5 mg/m^2) given as an IV push (max. 2 mg), actinomycin (0.045 mg/kg) given as a 5 min intravenous infusion, and cyclophosphamide (1.2 gm/m^2) infused over 30–60 min, with hydration maintained at 3000 ml/m^2/day and vincristine given once a week for two weeks.

For all types of RMS, cyclophosphamide was administered every three weeks throughout the ten-month RMS protocol treatment period. According to the treatment regimen, radiotherapy or surgery was performed. Patients were followed-up on till their treatment was completed.

Clinical evaluation of cyclophosphamide-related efficacy (survival rate and response) and toxicity (grade and occurrence) were based on occurrences noted in each patient's medical record. For the individuals in this study, one whole blood sample (5ml) had been taken from the biobank for DNA extraction and genotyping. Taqman Real-Time polymerase chain reaction assay was used to genotype the CYP3A5 (3, 6 & 7) SNPs rs776746 (C_26201809_30 CYP3A5*3), rs10264272 (C_30203950_10 CYP3A5*6), and rs41303343 (C_32287188_10 CYP3A5*7), respectively.

RMS protocol doses according to age or weight: Cyclophosphamide dose: ≥3 years were given 1200 mg/m^2 IV as a 30- to 60-minute infusion with IV fluids and MESNA. Patients ≥1 year < 3 years were given 40 mg/

kg/dose IV as a 30- to 60-minute infusion with IV fluids and MESNA. Patients < 1 year were given 40 mg/kg IV as a 30- to 60-minute infusion with IV fluids and MESNA, as shown in Table 1.

Cyclophosphamide was combined with MESNA and fluids to avoid and indicate the incidence of hemorrhagic cystitis. The recommended total daily MESNA dose was 100% of the daily cyclophosphamide dose given at 0, 3, 6, and 9 hours after cyclophosphamide was started. Hydration with 200 mL/m^2/hour fluids was done before cyclophosphamide administration and after CPA with 3L/m^2 for 24 hours.

Ethics statement

This study was reviewed and approved by the Faculty of Pharmacy's Ethical Committee (Approval number: PT 2612). The Children's Cancer Hospital Egypt 57357 Biobank Committee has approved the sample release. Under the Biobank Research Consent, all participants' parents or legal guardians had to sign a Biobank research consent form.

Blood sampling and DNA extraction

Participants' peripheral blood (5 mL) was taken and collected in EDTA vacutainers. We make DNA extraction from Qiagen kits according to the manufacturing instructions. DNA was measured by the The Nanoquant TM spectrophotometer which was used to determine the purity and concentration of DNA. Until pharmacogenetic analysis, DNA was stored at –20°C.

Eligibility Criteria

Subjects between the ages of one and eighteen years who had a confirmed early diagnosis of RMS and were using cyclophosphamide as part of their treatment protocol from 2013 to 2016 were eligible for this study. Patients with a history of chronic renal disease, hepatic disease, or any other type of cancer other than RMS were excluded from the trial. Patients who were pregnant were also excluded.

CYP3A5 genotyping

Blood samples from 150 unrelated patients were used to obtain genomic DNA. For the detection of CYP polymorphisms, a real-time PCR was utilized with the TaqMan SNP Genotyping Assay (Applied Biosystems) for CYP3A5*3, CYP3A5*6, and CYP3A5*7 from thermo fisher company. All PCR amplifications were carried out according to manufacturing instructions (Table 2). The TaqMan 5'-nuclease test chemistry from Applied Biosystems makes SNP genotyping findings quick and easy. The thermal cycling conditions of both SNP and DME is presented in Table 3.

Clinical evaluation & toxicity criteria

All patients' tumor responses were classified as complete response (CR): Disappearance of all target lesions for a period at least one month, partial response (PR): ≥30% decrease in the sum of the longest diameters of the target lesions,taking as reference the baseline sum of the longest diameter, stable/stationary disease (StD): Neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD, taking as reference the smallest sum of the longest diameter since the treatment

Table 1. RMS Protocol Doses According to Age or Weight.

	Drug	Age	Dose
V	Vincristine	< 1 year	0.025 mg/kg IV x 1
		≥1 year and < 3 years	0.05 mg/kg IV x 1 (maximum dose 2mg)
		≥3 years	1.5 mg/m^2 IV x 1 (maximum dose 2mg)
A	Dactinomycin	< 1 year	0.025 mg/kg IV x 1
		≥1 year	0.045 mg/kg (maximum dose 2.5mg) IV x 1
C	Cyclophosphamide	< 3 years	40 mg/kg/dose IV x 1
		≥3 years	1200 mg/m^2 IV x 1

*Mesna and fluids will be used with cyclophosphamide.

Table 2. Primers Used for the Analysis of CYP3A5 Polymorphisms

Allele	SNP	Polymorphism	Primers Sequence
CYP3A5*3	Rs776746	T/C, Transition, Substitution	ATGTGGTCCAAACAGGGAAGAGATA[T/C]TGAAAGACAAAAGAGCTCTTTAAAG
CYP3A5*6	Rs10264272	C/T, Transition, Substitution	CTAAGAAACCAAATTTTAGGAACTT[C/T]TTAGTGCTCTCCACAAAGGGGTCTT
CYP3A5*7	Rs41303343	A/-, Insertion/Deletion	CCATCTGTACCACGGCATCATAGGT[A/]AGGTGGTGCCTGGAAGGAAAGAAAC

Table 3. Thermal Cycling Conditions

Steps	Predesigned SNP and Custom			DME		
	Temp.	Duration	Cycles	Temp.	Duration	Cycles
AmpliTaq Gold®, UP, EnzymeActivation	95°C	10 minutes	HOLD	95°C	10 minutes	HOLD
Denaturation	95°C	15 seconds	40	95°C	15 seconds	50
Annealing/Extension	60°C	1 minute		60°C	90 seconds	

Table 4. Patient Demographics and Characteristics

Variables		Total number of patients = 150	
Age (years)	Mean ± SD	5.72 ± 4.04	
Weight (Kg)	Mean ± SD	21.5 ± 15.6	
Height (cm)	Mean ± SD	109.7 ± 25.7	
BSA(m^2)	Mean ± SD	0.96 ± 0.44	
		Number of patients	Percentage
Gender	Male	87	58%
	Female	63	42%
Primary site	Abdominal Wall	1	0.70%
	Biliary Tract Liver	3	2.00%
	Chest Wall	3	2.00%
	Extremities	16	10.70%
	Genitourinary (Non-Bladder/Non-Prostate)	8	5.30%
	Head & Neck	70	46.70%
	Pelvic	25	16.70%
	Perineal Region	1	0.70%
	Retroperitoneal Pelvic	1	0.70%
	Urinary bladder	21	14.00%
	Missing data	1	0.70%
Stage	Stage I	23	15.30%
	Stage II	11	7.30%
	Stage III	80	53.30%
	Stage IV	35	23.30%
	Missing data	1	0.70%
Type of RMS	Alveolar	18	12.00%
(Histopathology)	Embryonal	132	88.00%
Tumor Site	Favorable	132	88.00%
	Unfavorable	18	12.00%
Initial risk	High Risk	35	23.30%
	Intermediate Risk	106	70.70%
	Low Risk	8	5.30%
	Missing data	1	0.70%
Metastasis	No	114	76.00%
	Yes	35	23.30%
	Missing data	1	0.70%
Response	Complete Remission	67	44.70%
	Partial Remission	20	13.30%
	Progressive Disease	51	34.00%
	Stationary Disease	6	4.00%
	Missing data	6	4.00%
Nephrotoxicity	No	19	12.70%
	Yes	131	87.30%
Hemorrhagic cystitis	Yes	150	100%
Pancytopenia	Yes	150	100%

started, or progressive disease (PD): ≥ 20% increase in the sum of the longest diameter of the target lesions, using the response evaluation criteria [25] in solid tumors (Response evaluation criteria in solid tumor-RECIST) guidelines version 1.0, and we graded each toxicity using the updated Common Terminology Criteria for Adverse Events (CTCAE) guidelines.

Table 5. Genotyping and Allele Frequency

Variables		Number of patients	Percentage
CYP3A5 Interpretation	CYP3A5 1*1	7	4.70%
	CYP3A5 1*3	33	22.00%
	CYP3A5 1*6	2	1.30%
	CYP3A5 3*3	92	61.30%
	CYP3A5 3*6	16	10.70%
Metabolizing Status	Intermediate Metabolizer	51	34.00%
	Normal Metabolizer	7	4.70%
	Poor Metabolizer	92	61.30%

Measurements & Parameters

Radiological assessment by MRI and CT scan was done to mark the end of treatment, and was obtained from the patient's records. Cumulative incidence of toxic mortality "overall survival" and event free survival "relapse-free survival" were recorded. Hemorrhagic cystitis was documented upon occurrence of and hematuria by urine analysis. When RBC above 50 /HPF and in case of double the standard dose of MESNA was used. Complete blood count for measuring pancytopenia/ neutropenia after CPA dose administration was performed every 3 weeks along with liver function tests (ALT, AST and total bilirubin) and kidney function tests (serum creatinine and BUN). Toxicities grading were according to CTCAE guidelines. Modifications in CPA dosage was done if the patient had substantial hematuria (>50 RBCs/ HPF). Cyclophosphamide was stopped and restarted when hematuria has cleared for at least 1 week at a 50% dose and escalated to 100% if tolerated. MESNA was given as a continuous infusion with its dose equivalent to double the daily CPA dose. The MESNA continuous infusion was begun at the same time as the cyclophosphamide infusion and ceased no sooner than 8 hours after the CPA infusion has finished.

Statistical Analysis

Power analysis has been conducted prior to the study, revealing that with a sample size of 150 patients and alpha targeted as 0.05, we achieve a 97% power to detect odds ratio of 3.75 [26,27]. Descriptive statistics for categorical variables included frequencies and percentages, while continuous variables were summarized using means with standard deviations or medians with interquartile ranges based on data distribution. For univariate analysis, continuous outcomes were compared using Student's t-test / Wilcoxon test (according to distribution), while categorical outcomes were analyzed using Chi-square or Fisher's exact test. Endpoints for overall survival (OS) are measured from the date of diagnosis to the date of death or last contact, and event-free survival (EFS) is measured from the date of diagnosis to the date of failure or last contact for failure-free patients. Multivariate logistic regression and Cox Proportional Hazard Models were used to adjust for covariates, including patient demographics, clinical characteristics, CYP3A5 mutation, and toxicities. Model selection employed backward elimination and was evaluated using the Akaike Information Criterion (AIC). A two-sided probability of $p<0.05$ is considered

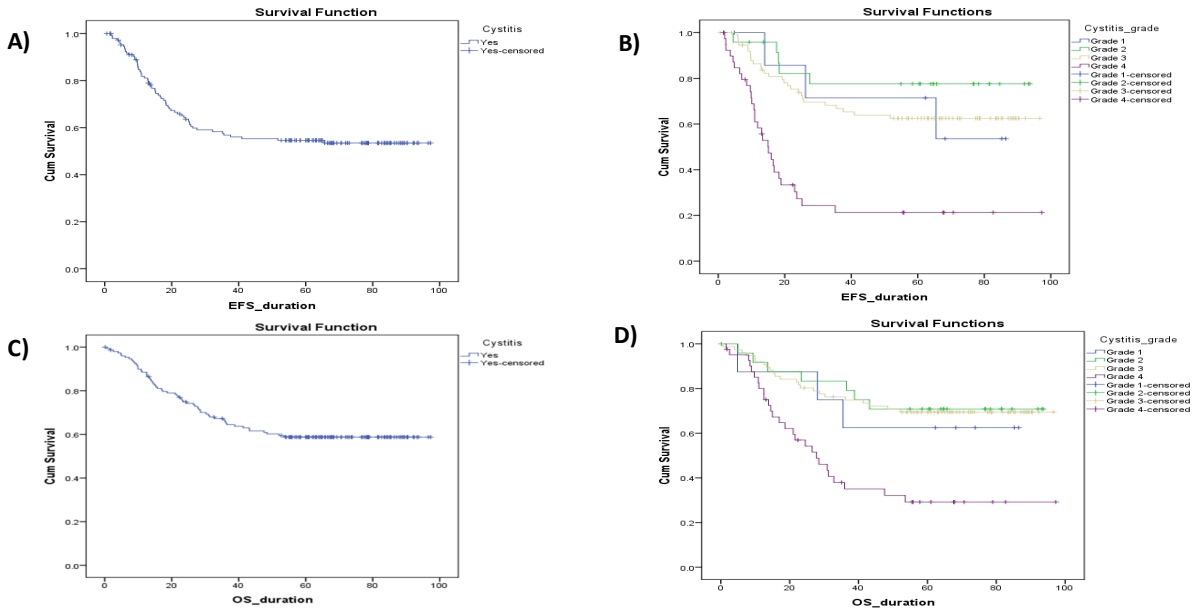

Figure 1. Kaplan-Meier Illustrating OS and EFS with the Hemorrhagic Cystitis & Its Grading Toxicity of the Study Cohort

statistically significant. The analyses are conducted using SPSS version 20 and R v4.2.1.

Results

Patient demographics and characteristics

Table 4 illustrates patients' characteristics. A total of 150 pediatric RMS patients were enrolled in receiving CPA as part of their clinical treatment protocol for RMS. The study population had a mean age of 5.72 ± 4.04 years and included 87 male and 63 female patients. The mean body weight, height, and surface area were 21.5 ± 15.6 kg, 109.7 ± 25.7 cm, and 0.96 ± 0.44 m^2, respectively. The gender distribution in that cohort study was 58% male and 42% female. Frequencies of pancytopenia and hemorrhagic cystitis were 100 % while the incidence of nephrotoxicity was 87.3 %. The incidence of RMS primary sites at the abdominal wall, biliary tract, liver, chest wall, extremities, genitourinary (non-bladder or non-prostate), head &neck, pelvic, perineal region, retroperitoneal pelvic, and urinary bladder was 0.7%, 2%, 2%, 10.7%, 5.3%, 46.7%, 16.7%, 0.7%, 0.7%, and 14%, respectively. RMS stage frequencies from stages I, II, III, and IV were 15.3%, 7.3%, 53.3%, and 23.3%, respectively. The types of RMS distribution in the study were 12% alveolar and 88% embryonal, while tumor site

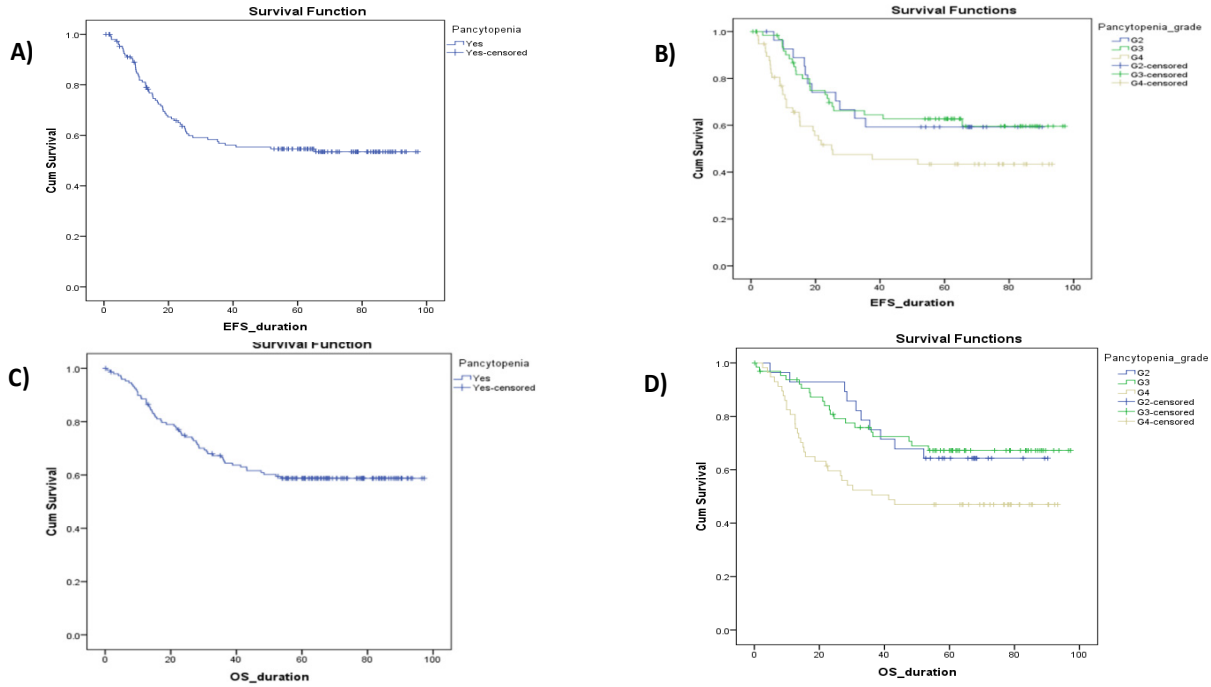

Figure 2. Kaplan-Meier Illustrating OS and EFS with the Pancytopenia & Its Grading Toxicity of the Study Cohort.

Table 6. Factors Influencing the 5 Years Overall Survival

Factors influencing the OS		Case Processing Summary		OS (5 years)	95% CI Mean survival duration		P value
		Event number	Censored Number	Probability %	Lower	Upper	
Gender	Female	32	31	49.3	48.078	67.384	< 0.05*
	Male	30	57	65.7	62.669	78.301	
Primary site	Abdominal Wall	0	1	100	-	-	0.079
	Biliary Tract Liver	2	1	33.3	-	-	
	Chest Wall	1	2	66.7	-	-	
	Extremities	8	8	50	-	-	
	Genitourinary	1	7	87.5	-	-	
	Head & Neck	27	43	62.9	-	-	
	Pelvic	16	9	35.2	-	-	
	Perineal Region	1	0	100	-	-	
	Retroperitoneal	1	0	100	-	-	
	Urinary bladder	5	16	57.6	-	-	
Stage	Stage I	7	16	73.2	62.176	85.644	< 0.05*
	Stage II	3	8	70.7	61.622	97.396	
	Stage III	26	54	68.4	64.587	80.205	
	Stage IV	26	9	21.6	22.5	42.007	
Type of RMS	Alveolar	8	10	55.6	38.315	73.251	0.06
(Histopathology)	Embryonal	54	78	59.1	59.468	72.48	
Tumor site	Favorable	54	78	59.1	59.468	72.48	0.06
	Unfavorable	8	10	55.6	38.315	73.251	
Initial risk	High Risk	26	9	21.6	22.5	42.007	< 0.05*
	Intermediate Risk	35	71	68.3	66.429	79.814	
	Low Risk	1	7	87.5	68.156	99.098	
CYP3A5 Interpretation	CYP3A5 1*1	5	2	42.9	-	-	0.28
	CYP3A5 1*3	14	19	57.6	-	-	
	CYP3A5 1*6	0	2	100	-	-	
	CYP3A5 3*3	39	53	57.6	-	-	
	CYP3A5 3*6	4	12	71.8	-	-	
	Intermediate Metabolizer	18	33	63.3	61.47	80.689	0.15
Metabolizing Status	Normal Metabolizer	5	2	0	18.279	75.744	
	Poor Metabolizer	39	53	57.6	55.5	71.762	
Metastasis	No	36	78	67.9	67.839	80.578	< 0.05*
	Yes	26	9	21.6	22.5	42.007	
Response	CR	7	60	92.4	85.902	95.355	< 0.05*
	Partial Remission	7	13	65	44.533	65.598	
	Progressive Disease	42	9	13.8	24.705	39.66	
	Stationary Disease	2	4	66.7	33.605	85.521	
	Yes	5	6	54.5	35.844	75.697	
Hemorrhagic cystitis	Yes	60	90	58.8	59.895	72.304	-
Grade	Grade 1	3	5	62.5	40.514	84.65	< 0.05*
	Grade 2	7	18	70.8	60.434	86.421	
	Grade 3	23	53	69.4	65.847	81.768	
	Grade 4	27	14	29.2	31.602	54.782	
Pancytopenia	Yes	60	90	58.8	59.895	72.304	-
Grade	G2	10	18	64.3	57.94	79.941	< 0.05*
	G3	20	45	67.2	64.531	82.172	
	G4	30	27	47	42.628	62.907	
Nephrotoxicity	No	19	6	66.9	50.643	87.663	0.63
	Yes	131	54	57.6	58.749	71.795	
Grade	G1	96	37	60.5	60.886	75.547	< 0.05*
	G2	28	12	55.1	45.724	71.749	
	G3	7	5	28.6	6.914	45.78	

Table 7. Factors influencing the 5 years Event Free Survival

Factors influencing the EFS		Case Processing Summary		EFS (5 years)	95% CI Mean survival duration		P value
		Event number	Censored Number	Probability %	Lower	Upper	
Gender	Female	27	36	50	46.06	67.617	0.51
	Male	36	50	58.1	53.848	71.27	
Primary site	Abdominal Wall	0	1	100	-	-	< 0.05*
	Biliary Tract Liver	1	2	50	-	-	
	Chest Wall	1	2	66.7	-	-	
	Extremities	9	7	34.9	-	-	
	Genitourinary	1	7	87.5	-	-	
	Head & Neck	25	45	59.9	-	-	
	Pelvic	17	8	29.3	-	-	
	Perineal Region	1	0	0	-	-	
	Retroperitoneal	1	0	0	-	-	
	Urinary bladder	7	14	66.3	-	-	
Stage	Stage I	6	17	72.7	57.962	85.716	< 0.05*
	Stage II	3	8	68.6	50.099	95.97	
	Stage III	27	53	64.6	60.172	77.266	
	Stage IV	27	8	14.7	13.935	32.381	
Type of RMS	Alveolar	9	9	50.7	32.628	68.682	0.38
(Histopathology)	Embryonal	54	77	55.6	54.28	68.724	
Tumor site	Favorable	54	77	55.6	54.28	68.724	0.38
	Unfavorable	9	9	50.7	32.628	68.682	
Initial risk	High Risk	27	8	14.7	13.935	32.381	< 0.05*
	Intermediate Risk	35	71	65	62.153	77.068	
	Low Risk	1	7	87.5	63.035	100.605	
CYP3A5 Interpretation	CYP3A5 1*1	4	3	51.4	-	-	0.41
	CYP3A5 1*3	14	19	55.1	-	-	
	CYP3A5 1*6	0	2	100	-	-	
	CYP3A5 3*3	41	50	51.2	-	-	
	CYP3A5 3*6	4	12	72.7	-	-	
	Intermediate Metabolizer	18	33	62.2	55.785	77.772	0.28
Metabolizing Status	Normal Metabolizer	4	3	51.4	21.171	71.436	
	Poor Metabolizer	41	50	51.2	48.743	66.442	
Metastasis	No	36	78	66.8	63.785	77.996	< 0.05*
	Yes	27	8	14.7	13.935	32.381	
Response	CR	7	60	90.8	84.855	95.268	< 0.05*
	Partial Remission	6	14	64.7	43.848	66	
	Progressive Disease	49	2	0	11.718	16.49	
	Stationary Disease	1	5	80	47.174	91.317	
Hemorrhagic cystitis	Yes	64	85	54.6	53.338	66.932	-
Grade	Grade 1	3	5	53.6	42.211	85.314	< 0.05*
	Grade 2	5	19	77.6	63.318	89.839	
	Grade 3	27	49	62.5	58.831	76.439	
	Grade 4	29	12	21.2	19.38	42.443	
Pancytopenia	Yes	64	85	54.6	53.338	66.932	-
Grade	G2	11	17	59.3	48.52	74.854	< 0.05*
	G3	23	41	62.7	56.717	76.525	
	G4	30	27	43.4	37.433	59.201	
Nephrotoxicity	No	19	5	70.6	52.095	90.454	0.23
	Yes	130	59	52.3	50.965	65.319	
Grade	G1	95	44	52.7	50.119	66.668	< 0.05*
	G2	28	10	58.6	46.029	73.843	
	G3	7	5	17.9	2.609	39.338	

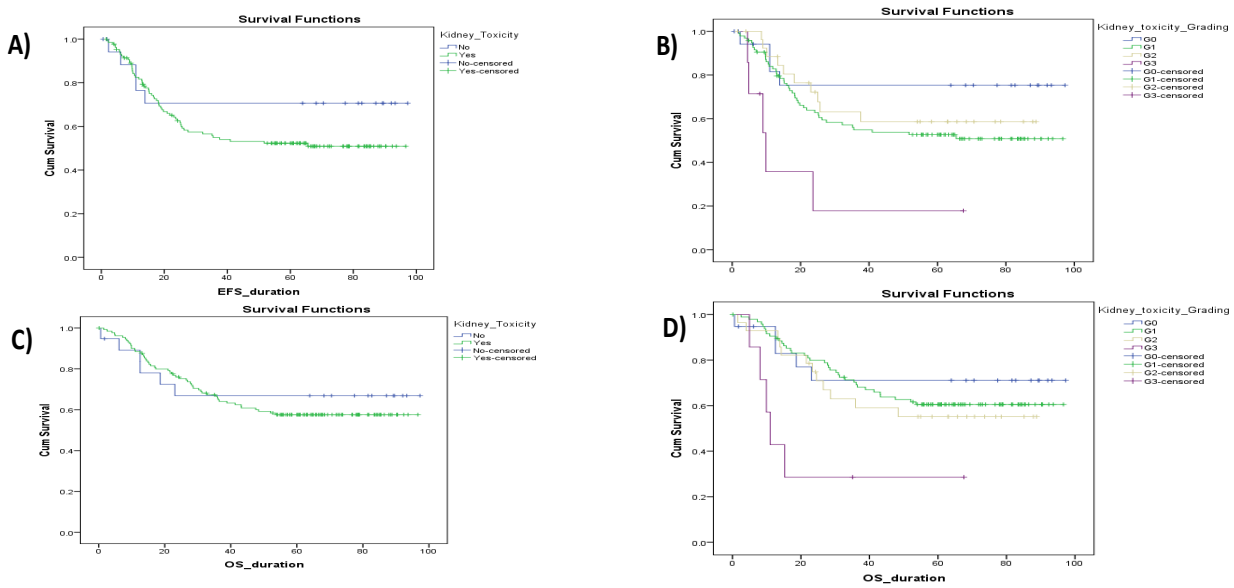

Figure 3. Kaplan-Meier Illustrating OS and EFS with the Kidney Toxicity & Its Grading Toxicity of the Study Cohort.

distribution was 12% unfavorable and 88% favorable as well. RMS risk incidence was 23.3% high risk, 70.7% intermediate risk, and 5.3% low risk, respectively. There's a 23.3% metastasis in that cohort study. RMS treatment protocol responses show 44.7% complete remission (CR), 13.3% partial remission (PR), 34% progressive disease (PD), and 4% stationary disease (StD).

Genotyping and allele frequency

The distribution of the CYP3A5 genotypes are shown in Table 5. In our cohort, 4.7% CYP3A5 *1/*1 (homozygous/normal metabolizer), 22% CYP3A5 *1/*3 (heterozygous/intermediate metabolizer), 1.3% CYP3A5 *1/*6 (heterozygous/intermediate metabolizer), 61.3% CYP3A5 *3/*3 (homozygous/poor metabolizer), and 10.7% CYP3A5 *3/*6 (heterozygous/ poor metabolizer). The frequency of having normal, intermediate, and poor metabolizers was 4.7%, 34%, and 61.3%, respectively.

Factors influencing the 5 years Overall Survival (OS)

Figures 1, 2, and 3 show Kaplan-Meier illustrating OS with the hemorrhagic cystitis and its toxicity grading, the pancytopenia and its toxicity, and the kidney toxicity and its toxicity grading of the study cohort, respectively. There's a significant difference between OS and gender, tumor stage, initial risk, metastasis, response, hemorrhagic cystitis grade, pancytopenia grade, and nephrotoxicity grade, with a $p<0.05$. No significant difference was observed with types of RMS, tumor site, CYP3A5 interpretations, or metabolizing status, with p values of 0.06, 0.06, 0.28, and 0.15, respectively (Table 6).

Factors influencing the 5 years Event Free Survival (EFS)

Figures 1, 2, and 3 show Kaplan-Meier illustrating OS with the hemorrhagic cystitis and its toxicity grading, the pancytopenia and its toxicity, and the kidney toxicity and its toxicity grading of the study cohort, respectively. There's a significant difference between EFS and primary site, tumor stage, initial risk, metastasis, response, hemorrhagic cystitis grade, pancytopenia grade, and nephrotoxicity grade, with $p<0.05$. There was no significant difference with gender, types of RMS, tumor site, CYP3A5 interpretations, or metabolizing status, with p values of 0.38, 0.38, 0.41, and 0.28, respectively (Table 7).

CYP3A5 Interpretation

As shown in Supplementary Table 8, there's a significant difference between hemorrhagic cystitis grade and CYP3A5 polymorphism with a p value <0.05, and the metabolizing status had a significant difference with the CYP3A5 polymorphism with a p value <0.05. There is no significant difference between nephrotoxicity and pancytopenia with a p value >0.05. Also, there's no significant difference between gender, RMS tumor primary site, tumor stage (from stage I to IV), type of RMS (alveolar or embryonal), tumor site (favorable or unfavorable), initial tumor risk (high, intermediate, or low risk), cancer metastasis, clinical treatment response, and the CYP3A5 interpretations of the following CYP3A5 *1/*1, *1/*3, *1/*6, *3/*3, and *3/*6, respectively, with a p value >0.05.

Discussion

Cyclophosphamide (CPA) is an anticancer prodrug that must be bioactivated to become the active alkylating metabolite, 4-OH-CPA, in order to exercise its antitumor activity [28]. The response to CPA treatment varies significantly. There is a significant heterogeneity in CPA treatment responses. CYP3A5 was identified as the major CPA 4-hydroxylase catalyzing the metabolism of CPA [13,29]. Decisions on treatment doses, whether to strengthen or decrease chemotherapy dosages, or the length of treatment may benefit from strategies to identify individuals at risk of treatment failure, high toxicity incidence, and those who will need further dose adjustment. Several studies looked into how CYP3A5

affected various medicines worked clinically [30].

Our study strategy was focused on specific SNPs in the CPA activation pathway that are expected to affect response and toxicity to CPA treatment. The three non-functional SNPs identified in CYP3A5 exons 3, 6, and 7 are rs776746 (C_26201809_30/CYP3A5*3), rs10264272 (C_30203950_10/CYP3A5*6), and rs41303343 (C_32287188_10/CYP3A5*7). They were carefully chosen based on evidence that they are widespread in Caucasians, they alter CYP3A5 coding, which could modify the expression or activity of the CYP3A5 enzyme, and they are linked to a change in CPA activation and metabolism. Our study states that CYP3A5 functioning may affect the pharmacological activity and toxicity of CPA, which may have an impact on how well a patient responds to treatment. Hence, the CYP3A5 polymorphic variations may change CPA metabolism, which could account for some of the interpatient heterogeneity in CPA responsiveness. In light of these considerations, the aim of this study was to study the effect of genetic polymorphisms in CYP3A5 on the efficacy and toxicity of cyclophosphamide in Egyptian cancer patients. As a predictive biomarker for response and toxicity, toxicity grades can also be used to assess if they affect treatment efficacy and survival outcomes. A total of 150 RMS-affected kids were enlisted, and genotyping results for the chosen SNPs together with all of the clinical information and results were examined.

Although CYP3A5 is known to exhibit inter-ethnic variation, there is a significant level of genetic diversity among various groups. In our cohort, the percentage of patients carrying at least one copy of CYP3A5*3 (rs776746) and CYP3A5*6 (rs10264272) was 94% and 12%, respectively, as shown in Table 4. The main finding in our study was that in RMS patients treated with VAC as the first line of treatment, there was an association between rs776746 and rs10264272 and response to CPA-based treatment. We found that patients who received cyclophosphamide-based adjuvant chemotherapy for RMS cancer and had the CYP3A5 *1/*3, *1/*6, *3/*3, *3/*6 had significantly greater toxicity and efficacy than those who were CYP3A5 *1/*1 wild-type. These findings support our hypothesis that reduced phase-I enzyme activity (via the CYP3A5 polymorphism) leads to huge toxicity with lower efficacy after cyclophosphamide-based treatment chemotherapy, presumably as a result of slower activation of cyclophosphamide to the active form 4-hydroxycyclophosphamide.

CYP3A5 polymorphism affects the survival rates as there's a significant difference between OS and gender, tumor stage, initial risk, metastasis, response, hemorrhagic cystitis grade, pancytopenia grade, and nephrotoxicity grade and a significant difference between EFS and primary site, tumor stage, initial risk, metastasis, response, hemorrhagic cystitis grade, pancytopenia grade, and nephrotoxicity grade. A previous study by Labib et al., [26] examined the association of CYP2B6 single nucleotide polymorphisms (SNPs) with the survival of RMS in a cohort of 73 pediatric RMS patients treated with CPA-based first-line treatment. They analyzed the association between those genotypes and the survival outcome of RMS. They demonstrated that CYP2B6 rs2279343 may predict EFS in RMS patients and warrant future studies to clarify the pharmacogenetics of CPA in pediatrics which enforced our results.

Gor et al., [31] evaluated the cyclophosphamide-metabolizing enzyme polymorphisms and survival outcomes after adjuvant chemotherapy for node-positive breast cancer. They performed a retrospective cohort study of 350 women enrolled in a multicenter, randomized adjuvant breast cancer chemotherapy trial. Cox regression models were computed to determine associations between genotypes (individually or in combination) and disease-free survival (DFS) or overall survival (OS), adjusting for confounding clinical variables. Pinto et al., [6] analyzed the associations between event-free survival and 394 single-nucleotide polymorphisms (SNP) in 14 drug metabolizing enzymes or transporters involved in CPA pharmacokinetics which suggested that a pharmacogenomic approach to therapy personalization of cyclophosphamide in intermediate-risk rhabdomyosarcoma.

Another study suggests that genetic polymorphisms in enzymes involved in the activation of cyclophosphamide, such as CYP3A4, and CYP3A5, can lead to decreased enzyme activity and an increased risk of developing graft-versus-host disease (GVHD). Understanding these genetic factors may assist in improving the clinical management of transplant recipients by better predicting and managing the risks associated with cyclophosphamide therapy [12].

Abuelsoud & El Khateeb, 2023 investigated the association between the CYP2B6 c.516G>T polymorphism (rs 3745274) and various parameters related to the efficacy and tolerability of cyclophosphamide (CPA) in Egyptian patients with lupus nephritis (LN). The study findings suggest that pre-treatment evaluation of CYP2B6 rs 3745274 may help account for individual differences in treatment response for LN patients receiving cyclophosphamide therapy. This highlights the potential importance of genetic factors in predicting treatment outcomes and guiding personalized treatment strategies.

Also, CYP3A5 polymorphism showed an effect on the hemorrhagic cystitis grade and CYP3A5 metabolizing status. Other researchers examined cyclophosphamide-induced hemorrhagic cystitis. A series of 100 patients with hemorrhagic cystitis induced by cyclophosphamide was studied. [33]. While several medications may induce CYP activity, potentially modifying the expected effect of genotype, one would anticipate that such effects would be relevant only if a large proportion of the study subjects were taking such medications during chemotherapy. CYP3A5 polymorphism poor and intermediate metabolizers of CYP3A5*3 and CYP3A5*6 showed higher toxicity with lower efficacy than the normal metabolizers. CYP3A5*7 wasn't present in the study participants; CYP3A5*3/rs776746 (homozygous and heterozygous) showed more association with cyclophosphamide efficacy and toxicity than CYP3A5*6/rs10264272 (homozygous and heterozygous).

In Conclusion, in summary, our findings paint a picture of the role of CYP3A5 polymorphisms in RMS. This study shows that among patients receiving cyclophosphamide

as a component of chemotherapy used to treat RMS, a polymorphism in the cyclophosphamide-metabolizing enzyme CYP3A5 independently influences the results from cyclophosphamide-based RMS chemotherapy. When considered in conjunction with the results of earlier studies, these findings support retrospective research to further examine the connection between genetic variation, metabolite levels, and outcome to see if pharmacogenetic dosing regimens can enhance the efficacy and toxicity of this therapy. Considering CYP3A5 rs776746 and rs10264272 prior to therapy may help to explain some inter-individual variations in treatment response, according to our data. The integration of genetic factors with clinical and molecular characteristics could be applied. Additionally, this might simplify clinical judgment, enhance CPA therapy, and hence enhance clinical success. The pharmacokinetic modeling of CPA activation will help stratify RMS patients according to their genetic composition and provide a clearer understanding of the inter-individual diversity in medication response. It is estimated that the non-functional CYP3A5 *1/*3, *1/*6, *3/*3, and *3/*6 were shown to have higher cyclophosphamide efficacy and toxicity than the functional CYP3A5*1/*1.

Limitation

Due to low participants of the normal CYP3A5 *1/*1 wild, we need more normal samples to make more accurate correlations. We also need to perform additional research on all the medications used in RMS protocols to obtain more accurate clinical investigations and interpretations of the RMS patients.

Author Contribution Statement

Cherine E. ElShereef; Writing-Original draft preparation, Investigation. Hala F. Zaki; Validation, Writing-Review and Editing. Osama A. Badary; Conceptulization, Methodology. Sherif kamal; Data Curation. Mohamed Nagy; Formal analysis. Dalia Makhlouf; Investigation. Mohamed Kamal; Formal analysis. Inas elnady; Resources. Sameh A.Abdelshafi; Project administration. Sherif Abou El Naga; Supervision. Mona M. Saber; Writing-Review and Editing, Formal analysis.

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Data availability

The data underlying this article are available in the article

Abbreviations

CCHE: Children's Cancer Hospital of Egypt; RMS: rhabdomyosarcoma; VAC: vincristine, actinomycin, cyclophosphamide; MESNA: sodium 2-mercaptoethane sulfonate; CYP: cytochrome P450; CPA: cyclophosphamide; DME: drug metabolizing enzymes; SNPs: Single nucleotide polymorphisms; rs: reference SNP cluster ID for every allele; ES: event-free survival; OS: overall survival; CR: complete remission; PR: partial remission; PD: progressive disease; StD: stationary disease; ERMS: embryonal rhabdomyosarcomas; ARMS: alveolar rhabdomyosarcomas; TNM staging: Tumor, Node and Metastasis staging; EDTA: Ethylenediaminetetraacetic acid; CTCAE: Common Terminology Criteria for Adverse Events; RECIST: Response evaluation criteria in solid tumors; DFS: disease-free survival; AIC: Akaike Information Criterion; IRSG-IV protocol: Intergroup Rhabdomyosarcoma Study Group Protocol; PCR: polymerase chain reaction ; ALT: alanine transaminase; AST: aspartate aminotransferase; GVHD: graft-versus-host disease; LN: lupus nephritis.

Conflict of interest

None.

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