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CDKN2A and CDKN2B Gene Variants in Acute Lymphoblastic Leukemia in Tunisian Population

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Introduction

Acute lymphoblastic leukemia (ALL) is an aggressive lymphoid cancer, frequent in younger than in adult population with 25 new children diagnosed with ALL every year in Tunisia [1,2]. ALL constitutes 25% of cancer that affect people before their twenties; with 2 at 5years peak age. Current therapy allows the cure of approximately 60%-80% nowadays of (young) ALL patients and the five-year survival is estimated at 60% [2-5]. ALL is characterized by infiltration of monoclonal immature cells medullar and extra medullar areas [6-8]. The physiopathology of ALL is multifactorial, and includes interaction between modifiable (environmental), and non-modifiable factors, in particular genetic factors [9,10]. However, the genetic etiology dominates in younger subject, because children are less exposed to the environmental factors compared to adults [10,11]. In these regards, acquired somatic mutations contributing to ALL were reported [12]. These act by increasing the proliferation and survival of progenitor cells and impaired further cellular differentiation [12]. Though these and related alterations participate in the diagnosis and prognosis they remain insufficient to physio-pathological processes [12]. The introduction of novel diagnostic tools, such as high resolution karyo typing or CGH Array and genome wide molecular analysis may reveal additional somatic mutations not captured by standard cytogenetic analysis [7,13].

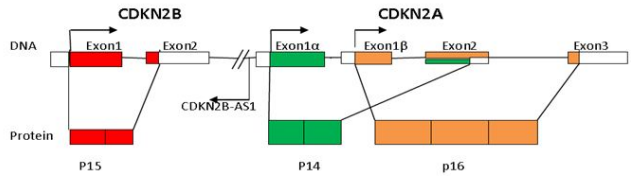

Figure 1: CDKN2A/CDKN2B gene localised in chr 9; CDKN2A encode two suppressor proteins p14ARF (exon 1α and exon 2) and p16INK4A (exon 1β, exon2 and exon 3), CDKN2B encode one protein p 15.

Earlier studied identified 18 single nucleotide polymorphisms (SNP) to be associated with ALL of which only 12 SNP had moderate odds ratio (OR) of 1.43-3.6, which included zinc finger protein subfamily 1A gene [11]. Subsequently, genome-wide associated scans (GWAS) confirmed an association between ALL and polymorphic variants in cyclin-dependent kinases-inhibitor (CDKN) 2A and CDKN2B genes in some, but not in other populations [7,14]. CDKN2A/B is important in control of cell cycle [15]. Protein CDKN2A and CDKN2B are encoded by CDKN2A and CDKN2B adjacent genes, localized on chromosome 9p21 (Figure 1) are frequently deleted, mutated and promoter methylated in some cases of cancer [16-18]. In response to these alterations, cancer cells become unable to control proliferation, and thus cell cycle regulation [19,20]. CDKN2A and CDKN2B genes were reported to be inactivated in hematologic malignancies, such as B-ALL (21%) and T-ALL (50%) patients [21]. Furthermore, heterozygous and homozygous deletions are more frequent than hyper methylation and mutations in exons 1 and 2 of CDKN2A in childhood ALL [21].

The aim of the current study is to evaluate the association between the risk of ALL in Tunisian children and CDKN2A and rs3731217 (intron1) gene variant which creates two overlapping cis-acting intronic splice enhancer motifs (CCCAGG and CAGTAC) which may regulate alternative splicing, and CDKN2A rs3734249 (exon2) missense SNP associated with alanine-to-threonine replacement [19,20,22,23]. In addition, the association of CDKN2B rs662463 in ANRIL, regulates CDKN2B expression by disrupting a transcription factor binding site for CEBPB with ALL will also be investigated [24,25].

Subjects and Methods

Subjects

The retrospective study group includes 122 children with ALL, recruited in the period January 2013 to May 217, with mean age of 7.6 ± 4.5 yr. ALL was diagnosed and classified according to morphological and immunophenotypic characterization of blast cells in the bone marrow (OMS 2016). Of these patients, 106 were diagnostic with B-ALL and 16 with T-ALL. In addition, 91 children with mean age 7.9 ± 5.0 yr served as controls, and were recruited from pediatric general service for routine checkup, and were matched to cases according to self-declared ethnic origin. Blood samples were taken from all participants in EDTA-containing tube for total genomic DNA extraction. The guardians of patients and controls were required to sign a consent form before inclusion in the study, which was approved by the Ethic committee of CHU Farhat Hached (Sousse, Tunisia).

SNP Genotyping

Total genomic DNA was isolated from peripheral blood leukocytes by the salting-out method. Genotyping of rs3731217, rs3731249 (CDKN2A) and rs662463 (CDKN2B) was done using TaqMan® SNP Genotyping Assay, and specific primer pairs for each SNP. A standard 10 µl PCR reaction consisted of 1X TaqMan® Genotyping Master Mix (Applied Biosystems), 1X SNP Genotyping Assay Mix and 20 ng DNA.

Statistical analysis

Statistical analysis was performed on SPSS v. 23.0 (SPSS Inc., Chicago, IL). Data were expressed as percentages of total (categorical variables), or mean ± SD (continuous variables). Student's t-test was used to determine differences in means, and Pearson χ^2 test was used to assess inter–group significance. Genotypes were tested for departures from Hardy–Weinberg equilibrium (HWE) in the control population using Haploview 4.2 (www.broadinstitute.org/haploview). All analyses were conducted under additive genetic model. Pairwise linkage disequilibrium (LD) values were calculated with Haploview 4.2 and haplotype reconstruction was performed by the expectation maximization method. Logistic regression analysis was performed in order to determine the odds ratios (OR) and 95% confidence intervals (95%CI) associated ALL after controlling for age and gender as covariates, taking control subjects as the reference group. Statistical significance was set at P<0.05.

Results

Study subjects

The characteristics of ALL patients and control subjects are shown in Table 1.

Parameter	Cases (122)	Controls (142)	P1
Age[2]	7.6 ± 4.5	7.9 ± 5.0	0.687
≤ 10 years of age[3]	38 (30.9)	43 (30.3)	1.000
Gender (M:F)	73:49	79:63	0.533
WBC[2]	532,855 ± 4,883,861	13582 ± 18,724	0.206
WBC: 20,000 – 100,000[3]	23 (18.7)	11 (7.7)	<0.001
WBC >100,000[3]	33 (26.8)	0 (0.0)	
RBC[2]	10.9 ± 2.0	8.1 ± 2.9	<0.001
Platelets[2]	92,881 ± 105,428	352,316 ± 155,183	<0.001
Positive family history (%)	93.9	NA	NA
Immunophenotype: LAL-B[3]	107 (87.0)	NA	NA
LAL-T[3]	16 (13.0)	NA	NA

[1]Student t-test for continuous variables, chi-square analysis for categorical variables.

[2]Mean ± SD

[3]Number (percent total)

Table 1: Characteristics of Study Participants.

No significant inter-group differences were recorded for age (P=0.687), or gender (P=0.533). All hematological indices were significantly different between ALL patients and control subjects.

Association studies

Summarizes the association between CDKN2Ars3731249, rs3731217, and CDKN2B rs662463SNPs and ALL in case-control subjects Table 2.

Locus	SNP	Position1	Alleles	Cases2	Controls2	HWE	χ	P 3	OR (95% CI)	Power
CDKN2A	rs3731249	21970917	C:T	7 (0.03)	10 (0.04)	0.16	0.01	0.92	0.95 (0.35 – 2.53)	0.24
	rs3731217	21984662	G:T	24 (0.14)	28 (0.13)	0.21	0.01	0.92	1.03 (0.57 – 1.85)	0.28
CDKN2B	rs662463	22030439	C:T	41 (0.20)	38 (0.18)	0.51	0.32	0.57	1.15 (0.71 – 1.88)	0.36

MAF, Minor allele frequency; HWE, Hardy-Weinberg Equilibrium.
1. Location on chromosome based on dbSNP build 125.
2. Minor allele (frequency).

3. Adjusted P value, adjusted for age and gender.

Table 2: Distribution ofCDKN2A and CDKN2B alleles in cancer cases and control subjects.

The genotypes of CDKN2A rs3731249 (P=0.16), rs3731217 (P=0.21), and CDKN2B rs662463 (P=0.51) were in HWE among control population. Minor allele frequency (MAF) of CDKN2A rs3731249 (P=0.92), rs3731217 (P=0.92), and CDKN2B rs662463 (P=0.57) were not significantly different between ALL cases and control subjects, even before applying the Bonferroni correction method for multiple testing. Setting homozygous major allele as reference (OR=1.00), results from Table 3 demonstrated lack of association of CDKN2A rs3731249 (P=0.54), rs3731217 (P= 0.06), and CDKN2B rs662463 (P=0.47) genotypes with ALL under the additive model, as well as dominant or recessive models (data not shown).

Gene	SNP	Genotype	Cases	Controls	P	aOR1 (95% CI)
CDKN2A	rs3731249	C / C	94 (0.93) 2	128 (0.93)	0.54	1.00 (Reference)
		C / T	7 (0.07)	8 (0.06)		1.19 (0.42 3.40)
		T / T	0 (0.00)	1 (0.01)		0.00 (0.00 – NA)
	rs3731217	G / G	66 (0.76)	76 (0.73)	0.06	1.00 (Reference)
		G / A	18 (0.21)	28 (0.27)		0.74 (0.38 1.46)
		A / A	3 (0.03)	0 (0.00)		NA (0.00 – NA)
CDKN2B	rs662463	C / C	65(0.64)	69(0.66)	0.47	1.00 (Reference)
		C / T	31(0.31)	34 (0.32)		0.97 (0.53 1.75)
		T / T	5 (0.05)	2 (0.02)		2.65 (0.5014.16)

Table 3: Genotype frequencies of CDKN2A and CDKN2B variants in cases and controls.

Correlation studies

We next examined the correlation between CDKN2A and CDKN2B and ALL-associated hematological and biochemical indices. Spearman correlation calculation demonstrated correlation between CDKN2A rs3731249and LAL-B/LAL-T phenotype (r=0.244, P=0.014), and between CDKN2B rs662463 and the liver function tests ALAT (r=-0.372, P=0.036), ASAT (r=-0.415, P=0.018), and LDH (r=-0.379, P=0.043) (Table 4). Lack of correlation was noted between CDKN2Ars3731217and the biochemical and hematological examined in ALL patients Table 4.

	rs3731249		rs3731217		rs662463	
Parameter	r	P	r	P	r	P
Age	0.09	0.372	-0.049	0.649	0.013	0.898
BMI	-0.207	0.478	0.108	0.724	0.369	0.194
Blasts	0.055	0.713	-0.268	0.09	-0.05	0.738
LALB/ LALT	0.244	0.014	0.094	0.389	0.096	0.342
WBC	0.126	0.208	-0.028	0.799	0.137	0.17
RBC	-0.067	0.506	-0.026	0.811	0.085	0.398
Platelets	0.04	0.69	0.123	0.257	0.138	0.168
ALAT	-0.227	0.196	0.266	0.163	-0.372	0.036
ASAT	-0.222	0.207	-0.044	0.823	-0.415	0.018
LDH	0.222	0.257	303	0.141	-0.379	0.043
Urea	-0.17	0.369	0.191	0.371	-0.331	0.092

Table 4: Correlation of CDKN2A and CDKN2B variants with clinical features.

Haplotype analysis

Three-locus [rs3731249, rs3731217 (CDKN2A) and rs662463 (CDKN2B)] haplotype analysis demonstrated that the majority of haplotype diversity was captured by 5 haplotypes in controls (96.1%) and cases (100.0%), which comprised CGC, CGT, CTC, TGC, and TGT haplotypes. Taking the common CGC haplotype as reference (OR=1.00), univariate and multivariate analysis demonstrated lack of association of any of the indentified haplotypes with ALL, even before correcting for multiple testing Table 5.

Haplotype1	Cases	Controls	P	OR 2 (95% CI)	aP	aOR2 (95% CI)
CGC	0.6523	0.695		1.00 (Reference)		1.00 (Reference)
CG T	0.173	0.141	0.26	1.42 (0.78 -2.61)	0.23	1.45 (0.79 -2.67)
CTC	0.141	0.097	0.22	1.55 (0.77 -3.13)	0.20	1.58 (0.783-21)
TGC	0.007	0.025	0.32	0.36 (0.05 -2.65)	0.33	0.36 (0.05-2.79)
TGT	0.028	0.003	0.48	18.38 (0.01 -625.02)	0.56	22.87 (0.00 815.71)

1. Haplotype containing .rs3731249, rs3731217 (CDKN2A) and rs662463 (CDKN2B); haplotype frequency determined by the maximum likelihood method.

2. aOR = adjusted odds ratio; covariates that were controlled for were age and gender.

3. Haplotype frequency.

Table 5: CDKN2Aand CDKN2B haplotypedistribution in cases and controls.

Discussion

This is the first study to analyze the association of CDKN2Ars3731217 and rs3731249and CDKN2B rs662463 variants with ALL in North-African Arab-speaking Tunisians. ALL is a common childhood cancer. The genetic alterations increase cells proliferation, prolonged their survival, and/or impair the differentiation of the lymphoid hematopoietic progenitors [26,27]. Genetic alteration of CDKN2A/2B was reported as a risk factor in many type of cancer including ALL [9]. Migliorini G [7] and Lopez-Lopez E [14] have shown that the alterations of the CDKN2A–CDKN2B locus was associated with increased leukemia risk in some population but not in other [14,28]. In this study, we replicated the results from previous studies which evaluated the association of the risk ALL with rs3731217 and rs3731249 (CDKN2A) and rs662463 (CDKN2B). Our data showed lack of association of the tested CDKN2Aand CDKN2B variants with ALL, irrespective of the genetic model used (codominant, dominant or recessive). The five common haplotypes identified were also not associated with ALL.

The rs3731217 could affect gene expression by different mechanisms. It creates two overlapping cis-acting intronic splice enhancer motifs, CCCAGG and CAGTAC that may regulate alternative splicing of CDKN2A [24]. Its association with ALL is controversial, highlighted by the independent association of rs3731217 with ALL in Polish, Hispanic, and Thai populations [29-31]. The results of two GWAS of the scale study, and Latvian population reported comparable frequency of rs3731217 in ALL patients and controls comparable to our results, and with a negative association with childhood ALL [32,33]. On the other hand, studies on German, French and Canadian populations reported strong association of rs3731217 and ALL [32,34]. Collectively, this suggests that rs3731217 constitutes a European ALL-susceptibility locus [23,30]. In European population the risk remains statistically significant after adjustment for multiple testing. This SNP is associated with Pediatric B-cell precursor acute lymphoblastic leukemia by alteration of mRNA stability of the two tumor suppressors proteins p16 and p14 ARF [24].

GWAS identified rs3731249 as risk factor [24]. This SNP is localized in exon 2 of CDKN2A gene and encoded two proteins: p16INK4A, a negative regulator cyclin-dependant kinase, and p14ARF, a p53 activator Figure 2 [31].

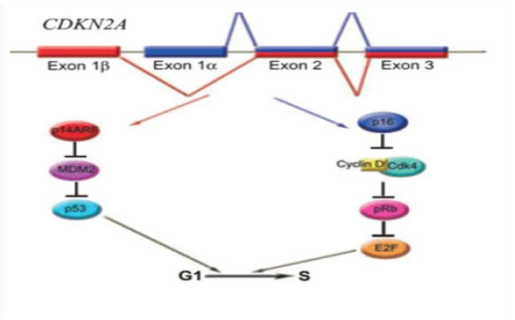

Figure 2: The CDKN2A gene is made up of four exons (exon 1β, exon 1α, exon 2, and exon 3) encode two suppressor protein p14ARF and p16INK4A which are transcribed from the same second and third exons but alternative first exon: p16 from exon 1α and p14 ARF from exon 1β; p16 is a specifically inhibit cyclin/CDK-4/6 and p14ARF an activator of p53. Both p16 and p14ARF block cell division during the G1/S phase of the cell cycle and Loss it its proliferation control and cycle regulation.

This SNP is a mis-sense variant, associated with C-to-T substitution resulting in alanine-to-threonine replacement [25]. This substitution induces a missense change for p16INK4A open reading frame, reduce the capacity of tumor suppressor CDK4 and CDK6 that favorite the B cell proliferation [23]. In p14ARF, the rs3731249 is in the 3ÂUTR region, where the T allele creates a miRNA binding site for miR-132-5p and miR-4642 [35]. These miRNAs could modify p14ARF expression, and then increased its function as cyclin inhibitor. Therefore, T allele of C/T SNP inCDKN2A caused B-ALL through its effect on the function of both p16INK4A andp14ARF.

The association ofrs3731249 with ALL remains controversy [23]. It has high frequency of risk in African-American children than other population. Gutierrez-Camino reported a positive association between rs3731249 and ALL risk in Spanish population. Also this variant is associated with B-ALL subtypes, including B-hyperdiploid ALL, and ETV6-RUNX1 ALL [25].

German (n=1155) and U.K. (n=824) GWAS of B-ALL patients demonstrated that Ala148Thr variant is a factor risk of B-ALL with calculated OR of 2.46 (95% CI=1.84-3.28), and 2.48 (95% CI=1.77-3.48) [31]. It is noteworthy that rs3731249 is implicated in other cancers, such as colorectal, lung and melanoma [36,37]. We have not established an association between CDKN2A Ala148Thr variant and ALL risk in our patient cohort children. MAF of Tunisian controls and case patients (0.04 *vs.* 0.03) were comparable to those of German (0.03 *vs* .0.08) and UK (0.03 *vs.* 0.05) populations [31].

We also examined the association between rs662463 CDKN2B variant and ALL. This variant is localized in the long non-coding RNA encoded in the chromosome 9p21 region, ANRIL [31,38]. ANRIL regulate the expression of CDKN2A/B tumor suppressors, and thus control the cell proliferation, apoptosis, senescence and aging [39,40]. The presence of rs662463 in ANRIL alters the binding site for CEBPB transcription factor, frequently mutated in BCP-ALL, increased the BALL risk by diminishing the level of p 15 tumor suppressor [24]. This variant was demonstrated to be a B-ALL risk factor African-Americans and populations of European descent [24]. The CDKN2B rs662463 variant is in almost complete LD with rs2811712 (r^2= 1.00), which is a

B-ALL risk locus in European-Americans and African-Americans [24].

While our results did not establish a correlation between rs662463 and ALL risk, there was correlation between CDKN2B rs662463 and liver function tests ALAT, ASAT, and LDH. This prompts the speculation as to possible diagnostic capacity of this variant, given that increased liver enzyme are likely due to hepatic injury from leukemic infiltrates or treatment toxicity [41]. Our data also established correlation between CDKN2A rs3731249 and LAL-B/LAL-T phenotype, with CDKN2A/2B polymorphisms being more correlated with LAL-T than LAL-B [42-47].

Conclusion

Our data don't show a correlation between the three SNPs, although, the OR is nearly of the significativity for the SNPs. The controversies of the results are probably associated to the variation of the ethnicity that may be influence the mechanism physiopathology of ALL, the still of patient live, age and patient cohort. Our study has several strengths namely, which the first analysis of three SNPs in Arabic speaking populations, and that revealed the correlation between CDKN2Brs 662463 and the liver function. However some limitations of this case – control study relate that the cohort and the power are insufficiently, because the ALL prevalence is weak in Tunisia.

Authors' Roles

Sana Mahjoub: genotyping and paper writing.

Rabbeb Ghali: Paper writing.

Verra Chaieb: DNA extraction.

Fatma Megddiche: patients recrutement

Malmek Souayed: genotyping technical

Faouzi Janhani: Project funding.

Abdelaziz Soukri: Head Moroccan of research project

Wassim Youssef Almawi: Statistic study.

Touhami Mahjoub

Highlights

This work is the first study associated three CDKN2A/2B genotype and haplotype in Arabic speakin population. It is one of few study replicated those rs with ALL in African populations Sperman correction reveled the correlation between CDKN2B rs 662463 and the liver function and between CDKN2A rs3731249 and LAL-B/LAL-T phenotype.

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Conflict of Interest

None

Disclosure Statement

The authors have nothing to disclose

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