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AXIN2 VARIATIONS MAY CONTRIBUTE TO INCREASED RISK OF PEDIATRIC T-ALL

AXIN2 VARYASYONLARI ARTMIŞ PEDİATRİK T-ALL RİSKİNE KATKIDA BULUNABİLİR

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ABSTRACT

Objective: Deregulated WNT signaling was reported in T-ALL and other cancers. AXIN2 is a negative regulator of the active WNT signaling and *AXIN2* gene variants were associated with increased cancer risk. In this study, we aimed to determine *AXIN2* variations and compare with clinic features in T-ALL.

Methods: Thirty-two diagnostic T-ALL patients were retrospectively enrolled in the study. Coding sites of the *AXIN2* were amplified by PCR and then screened by denaturing high-performance liquid chromatography (dHPLC). Patients with differential chromatograms were evaluated by Sanger sequencing.

Results: None of the patients had pathogenic *AXIN2* variants. Besides that, *AXIN2* polymorphisms, rs2240308/ rs1133683/ rs9915936 were detected in 14 (43.7%) T-ALL patients. Genotype distributions of the rs2240308 and rs1133683 variants in T-ALL group were significantly different from controls (rs2240308, GG/GA p=0.029; rs1133683, GG/GA p<0.0001) and G allele increased the overall risk of T-ALL compared to A allele in both polymorphisms. We did not observe any clinical differences between *AXIN2* variant carriers or non-carriers.

Conclusion: *AXIN2* rs2240308 and rs1133683 variants revealed significant positive associations between susceptibility to T-ALL.

Keywords: Acute lymphoblastic leukemia, AXIN2, variation, WNT

ÖZ

Amaç: Bozulmuş WNT sinyal iletisi, T-ALL ve birçok diğer kanserde gösterilmiştir. AXIN2, aktif WNT sinyalinin negatif yönde düzenleyicisidir ve *AXIN2* gen varyasyonları artmış kanser riski ile ilişkilendirilmiştir. Bu çalışmada, T-ALL hastalarında *AXIN2* varyasyonlarının belirlenmesi ve klinik belirteçlerle birlikte değerlendirilmesi hedeflenmiştir.

Yöntem: Yeni tanı, 32 T-ALL hastası retrospektif olarak çalışmaya dahil edilmiştir. *AXIN2* geninin kodlayan bölgeleri PZR ile çoğaltılmış ve sonrasında denatüre edici yüksek performanslı likit kromatografi yöntemi (dHPLC) ile taranmıştır. dHPLC'de farklı kromatogram bulgusuna sahip örnekler Sanger dizileme yöntemi ile değerlendirilmiştir.

Bulgular: Hastaların hiçbirinde patojenik varyant belirlenmemiştir. Bunun yanında, 14 (%43,7) T-ALL hastasında *AXIN2* polimorfizmleri, rs2240308/ rs1133683/ rs9915936 belirlenmiştir. T-ALL hastalarında kontrol örneklerine göre rs2240308 ve rs1133683 varyasyonlarının genotip dağılımları anlamlı farklılık göstermiştir (rs2240308, GG/GA p=0,029; rs1133683, GG/GA p<0,0001). G aleli, her iki polimorfizm için A aleline göre artmış genel T-ALL riski ile ilişkilendirilmiştir. *AXIN2* varyasyonu taşıyan ve taşımayan hastalar arasında, klinik belirteçler açısından bir fark gözlenmemiştir.

Sonuç: *AXIN2* rs2240308 ve rs1133683 varyasyonlarının varlığı, T-ALL'ye yatkınlık açısından anlamlı düzeyde ilişkili bulunmuştur.

Anahtar Kelimeler: Akut lenfoblastik lösemi, AXIN2, varyasyon, WNT

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Introduction

Acute lymphoblastic leukemia (ALL) is the most common hematopoietic malignancy in childhood cancers that characterized by expansion of immature B- cell (B-ALL) or T- cell (T-ALL).[1,2] Improved chemotherapy protocols, molecular based risk stratification and follow up of the patients led to increase overall survival (OS) rates up to 90% for pediatric ALL in high-income countries. On the other hand, relapse of ALL remains a leading cause of cancer related death.[3]

T-ALL is a rare hematopoietic malignancy (approximately 15% of pediatric ALL) with a relatively inferior prognosis compared with B-ALL. T-ALL is associated with genetic alterations and epigenetic abnormalities in T-cell development which lead to loss of cell cycle control, increased self-renewal capacity and blastic cell proliferation. T-ALL cells frequently have overexpression of T-cell specific transcription factors such as TAL1, TAL2, LMO1, MYC and HOXA.[4] Aberrant activation of NOTCH1 pathway by NOTCH1/ FBXW7 gene mutations were commonly observed in T-ALL patients.[2,5]

Stemness gene expression was correlated with disease progression and treatment resistance in several cancers. Wingless Type (WNT) signaling pathway is an evolutionarily conserved pathway which is involved in cell fate determination, self-renewal capacity and expansion of hematopoietic progenitor cells.[6] Deregulated WNT pathway has been reported in ALL and several solid cancers.[7,11]

The WNT pathway is activated by canonical (β-catenin dependent) and non-canonical signaling. WNT ligands such as WNT3A, WNT1 start the activation cascade of the canonical WNT signal by binding to FZD receptors and LRP co-receptors.[7] These signal transduction leads to activation of DVL that inactivates disruptive complex (APC, AXIN, GSK3β) and subsequently prevents the degradation of β-catenin. Unphosphorylated β-catenin accumulates in the cytoplasm and enters the nucleus where β-catenin interacts with TCF and LEF proteins.[12] By this interaction, histone acetylases are recruited that lead transcriptional activation of various growth promoting genes such as matrilysin/matrix metalloproteinase 7, cycling D1. In the absence of WNT ligands, cytoplasmic β-catenin is phosphorylated by a disruptive complex and degraded by ubiquitin proteasome pathway. Therefore, β-catenin cannot enter the nucleus and transcriptional repression is maintained by interaction of TCF/LEF complex with transcriptional inhibitors of the gaucho family.[7,12]

AXIN1 and AXIN2 are universal targets of WNT pathway and act as scaffold proteins.[13] Upregulation of CTNNB1 and elevated AXIN2 accumulation were reported in 40% of T-ALL patients and ALL cell lines.[11] Moreover, variants of *AXIN1* and *AXIN2* genes might influence the gene activity. The aim of this study is to determine *AXIN2* variations and compare with clinic features in T-ALL.

Methods

T-ALL Patients

A total of n=32 T-ALL who were diagnosed at pediatric hematology divisions of Istanbul University, Istanbul Medical Faculty, Cerrahpasa University, Medical Faculty and Istanbul Sisli Hamidiye Research and Training Hospital were enrolled retrospectively in the study. Bone marrow (n=28) and peripheral blood (n=4) samples were obtained before treatment.

The patients aged 1 to 16 years (mean 7.9 years with standard deviation 3.64 years) were treated and followed according to modified- Berlin-Frankfurt-Münster (BFM) 90 or 2000 protocols.[14] Female to male ratio was 1:2.2, median initial white blood cell counts (WBC) was 87.7x109/L (range, 1-603.0), median initial hemoglobin counts (Hb) was 9.5 g/dL (range, 5.1-15.6), median platelet counts (Plt) was 35x109/L (range, 5.2-263.6). Seven patients had central nervous involvement (CNS). Seven patients developed relapse and six of them were death related to cancer progression. Median follow up of the patients was 20.5 month (range, 1-180).

The patients were routinely screened for SIL::TAL, BCR::ABL, E2A::PBX, MLL::AF4, TEL::AML1 translocations. Three T-ALL patients had SIL::TAL translocation. Patients' *NOTCH1/FBXW7* mutation profile and *AXIN2* expression levels were also available.[5,11] 34.4% of T-ALL patients (n=11) carried *NOTCH1/FBXW7* variations. *AXIN2* expression data were available for 29 patients and 11 of 29 patients had increased *AXIN2* expression.[11]

RNA Extraction and PCR

RNA was extracted using Qiagen RNeasy Kit (Qiagen, GmbH, Germany). RNA quantity was checked with a spectrophotometer (Nanodrop 1000, Thermo Fisher Scientific, Germany). cDNA was synthesized by random hexamers and Moloney murine leukemia virus (MMLV) reverse transcriptase according to the manufacturer's procedures (MBI Fermentas, Vilnius, Lithuania). All coding exons of *AXIN2* were amplified by specific primers and PCR products were evaluated by 2% agarose gel. Primers were designed to obtain overlapped fragments; average amplicon length was 350 base pairs (248 bp to 404bp) and primer sequences can be shared upon request.

Denaturing High-Performance Liquid Chromatography (dHPLC) and Sanger Sequencing

PCR products were screened by denaturing high-performance liquid chromatography (dHPLC) (WAVE DNA Fragment Analysis System 3500, Transgenomic Inc. Omaha, Nebraska, USA). Each amplicon was run once in the dHPLC with two different melting temperatures. Patients' chromatograms were compared with the wild-type profile (two wild-type control samples were run for each amplicon and run), and samples with different characters (eluted before normal homoduplexes) were selected for Sanger sequencing. dHPLC primers were used in a combination to obtain longer amplicons length (~700 bp) such as, *AXIN2* exon2-4 region were amplified

by three different primer pairs in dHPLC analysis, first amplicons' forward primer and second amplicons' reverse primer were combined for Sanger analysis. Sanger sequences results were analyzed using CLC combined Workbench software (V.3.6.1, Denmark).

Statistical Analysis

The correlation between variation status and clinical parameters (sex, age, WBC count at diagnosis etc.) was examined by the Fisher's exact test. The Kaplan Meier method was used to estimate the survival rates while the OS was measured based on the time of diagnosis until the patients' death or their last follow-up. Relapse free survival (RFS) calculations were based on the present therapy response time to the date of patients' relapse or death. Log rank test was used for survival analyses. The data distribution was tested with Shapiro-Wilk test. The Hardy-Weinberg equilibrium test was used for the analysis of genotype distribution in the patient group. Hardy-Weinberg equilibrium and allele frequency of each polymorphism between the cases and controls were evaluated by a chi-squared test. *CTNNB1*, *CYCLOPHILIN* and *ABL* were used as reference genes.[11] Expression data were evaluated according to the delta Ct method.[15] Genotype and expression analysis were done by Welch's t test. P value of <0.05 (two-sided) was considered significant. The statistical analyses were done by IBM SPSS Statistics 28.0.0.0 software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism software 8 (Dotmatics, San Diego, CA., USA).

Results

A total of 32 T-ALL patients were screened for *AXIN2* variations by dHPLC and Sanger sequencing. We did not detect any pathogenic variant in the ALL cohort. Besides that, AXIN2 polymorphic variants were detected in 14 (43.7%) T-ALL samples; 11 patients carried a missense variant rs2240308 (c.148C>T, p.Pro50Ser), one patient had synonym rs1133683 (c.1386C>T), one patient had synonym rs9915936 (c.1365A>G) variant and one patient carried both rs1133683 and rs9915936 variations. The variants rs2240308 and rs1133683 were classified as benign, the rs9915936 variant was classified as likely benign according to the ACMG criteria.

Pinarbasi et al. screened 100 healthy Turkish men for the variants rs2240308, rs1133683, and rs9915936.[16] Although, gender and age distribution were not matched with our patient cohort, we used genotype frequencies according to the published control data since it was the only proper population data for Turkey.[16] The genotype distribution for the variants was consistent with the Hardy-Weinberg equilibrium both in control and patients.

Allele frequencies of the variants were listed in Table 1. Genotype distribution of the rs2240308 variant in the T-ALL group was significantly different from controls and the G allele increased the overall risk of T-ALL compared to the A allele. The frequency of heterozygous genotype (GA) and homozygous genotype[17] of rs2240308 were significantly different among the patients compared to controls (GA, p= 0.029, OR= 2.58, 95% CI= 0.13- 5.90; AA, p= 0.008, OR= 10.59, 95% CI= 1.73-115.8). Non-Finnish European population had 47.5%, T-ALL patients showed 79.7% of G allele frequency for rs2240308 variant.[18] The G allele frequency was significantly higher in T-ALL group compared to the controls (p= 0.0017, OR= 2.84, 95% CI= 1.447-5.68).

Non-Finnish European population minor allele frequency for rs9915936 (T) was 10.4%, T-ALL patients had 3.1% frequency for the same allele.[18] Homozygous genotype of rs9915936 (TT) was not detected in T-ALL and control groups. Genotype frequency and allele frequencies of rs9915936 were not different between the T-ALL and controls (p= 0.744, OR= 1.3, 95% CI= 0.29-6.37). The *AXIN2* rs1133683 allele A had higher representation in non-Finnish European population (66.4%) than the African (27.2%) and East Asian (35.2%).[18] The *AXIN2* rs1133683 allele G was the dominant allele both in Turkish control cohort (68%) and T-ALL (96.8%). The frequency of rs1133683 GG was significantly higher in T-ALL (93.75%) compared to controls (44%) (p<0.0001, OR= 16.36, 95%CI=3.99-71.65). rs1133683 AA genotype was not presented to any of T-ALL patients.

AXIN2 expression levels were available for 29 T-ALL patients and compared between genotypes of the variants.[11] *AXIN2* mRNA expression levels were similar between different genotypes; rs2240308 GG vs GA/AA genotypes (p=0.841), rs9915936 CC vs TC genotypes (p=0.571), rs1133683 GG vs GA genotypes (p=0.708) (Figure 1).

The patients with *AXIN2* variants were stratified according to the clinical and genetic characteristics. No significant difference was observed for any features between *AXIN2* variant carriers and non *AXIN2* variant carriers (Table 2). Kaplan-Meier survival analysis did not indicate any significant differences of T-ALL patients with *AXIN2* variants for OS and RFS (OS, p=0.08; RFS, p= 0.227).

Discussion

WNT signaling pathway involves in embryonic development, cell proliferation, immune control and stemness.[9,19] Aberrant WNT pathway activation, and mutations or epigenetic silencing of the pathway components were detected in many cancers.[9] AXIN2 is a negative regulator of activated WNT signaling pathway and has been related to tumor progression and metastasis.[13] In our previous work, we detected deregulated WNT pathway in a group of T-ALL with increased *AXIN2* expression.[11] In this study, we screened exons of *AXIN2* gene for variants in 32 pediatric T-ALL patients by dHPLC that is a reliable method for candidate gene scanning with a sensitivity of 1-5% in variants.[20] Then the samples with different chromatograms were sequenced by Sanger sequencing.

AXIN2 variants were reported mostly in solid tumors such as hepatocellular carcinoma, prostate cancer, ovarium or

lung cancer.[16,21,23] On the other hand, *AXIN2* mutation is a rare event in pediatric leukemia, only 3.1% of T-ALL patients and 2.1% of B-ALL patients showed *AXIN2* mutations in St. Jude pediatric cancer cohort.[24] In line with St. Jude pediatric cancer cohort data, we did not detect any pathogenic variants in our T-ALL cohort. However, 14 of 32 T-ALL (43.7%) patients had benign/likely benign polymorphic variants; rs2240308, rs1133683 and rs9915936. *AXIN2* variants might alter the protein's function and were reported in various cancers, nevertheless, the association between *AXIN2* polymorphisms and cancer risk are still controversial. Herein, we identified a potential association between T-ALL risk and rs2240308 and rs1133683. Our results suggested the allele G of rs2240308 and the allele G of rs1133683 may increase T-ALL susceptibility. In case-control population-based studies, rs2240308 variant was detected in colorectal cancer, prostate cancer, head and neck cancer, astrocytoma, oral cancer, ovarian cancer, hepatocellular carcinoma, gallbladder cancer and breast cancer.[21,23,25] Kanzaki et al. reported an association between rs2240308 polymorphism and lung cancer in Japanese population.[26] Furthermore, serum AXIN2 expression levels in prostate cancer patients with GG/AG genotypes were found higher than patients with AA genotypes in Chinese population.[25] On the other hand, this polymorphism was not correlated with prostate cancer susceptibility in Turkish population.[16]

Rs1133683 polymorphism had association with overall cancer risk and was detected in lung cancer, prostate cancer, astrocytoma, ovarian and colorectal cancers.[16,27,28] In T-ALL cohort, G allele was the dominant allele and the frequency significantly higher compared to controls (p=<0.001). rs9915936 polymorphism was observed in prostate cancer, astrocytoma and significantly correlated with lung cancer.[21,25] In our study, no obvious association was demonstrated between the rs9915936 C/T polymorphism in T-ALL risk.

Furthermore, we evaluated *AXIN2* mRNA expression levels according to the genotypes. We did not observe any differential expression between different genotypes of rs2240308, rs1133683 and rs9915936 for *AXIN2*. Although *AXIN2* mRNA expression levels were low, AXIN2 protein expressions were found high in a group of T-ALL.[11] According to our results, *AXIN2* mRNA levels were not very informative and protein levels are needed to understand possible genotype-phenotype correlation. Unfortunately, protein samples of T-ALL patients were not available for any further experiment. In addition to the limited number of participants, another limitation of the study was the lack of the pediatric Turkish control group.

In conclusion, our study suggests that *AXIN2* rs2240308, and rs1133683 polymorphisms are associated with T-ALL cancer risk. AXIN2 polymorphisms were detected in many cancers but we did not find any study about polymorphic variants of *AXIN2* and risk of ALL. Future large scale and well-designed research are required to validate these effects in more detail.

Table 1. Distribution, odds ratio and allele frequencies of rs2240308, rs9915936 and rs1133683 variants in pediatric T-ALL and control

	Genotypes	Patient* (n=32)	Control* (n=100)	p value	OR (95% CI)
rs2240308	GG	20 (62.5)	34 (34)	1	Reference
	GA	11 (34.3)	48 (48)	**0.029**	2.58 (1.13-5.90)
	AA	1 (3.2)	18 (18)	**0.008**	10.59 (1.73-115.8)
	Total	32 (100)	100 (100)		
Allele Frequency	G	51 (79.7)	116 (58)	1	Reference
	A	13 (20.3)	84 (42)	**0.0017**	2.84 (1.447-5.68)
	Total	64 (100)	200 (100)		
rs9915936	CC	30 (93.7)	92 (92)	1	Reference
	TC	2 (6.3)	8 (8)	0.744	1.3 (0.29-6.37)
	Total	32 (100)	100 (100)		
Allele Frequency	C	62 (96.9)	192 (96)	1	Reference
	T	2 (3.1)	8 (4)	0.749	1.29 (0.3-6.17)
	Total	64 (100)	200 (100)		
rs1133683	GG	30 (93.75)	44 (44)	1	Reference
	GA	2 (6.25)	48 (48)	**<0.0001**	16.36 (3.99-71.65)
	AA	0	8 (8)	*0.023*	
	Total	32 (100)	100 (100)		
Allele Frequency	G	62 (96.8)	136 (68)	1	Reference
	A	2 (3.2)	64 (32)	**<0.0001**	14.59 (3.8-62.11)
	Total	64 (100)	200 (100)		

*Values expressed as n (%), OR: Odds Ratio

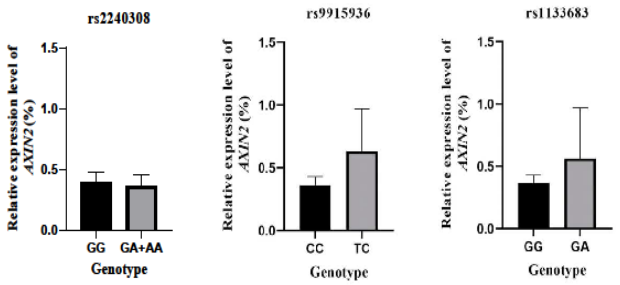

Figure 1. Expression levels of *AXIN2* in rs2240308 G/A, rs9915936 C/T, rs1133683 G/A genotypes of T-ALL patients

Table 2. Clinical and molecular features with respect to AXIN2 variants presentation

	Patients with *AXIN2* Variant* (n=14)	Patients without *AXIN2* Variant* (n=18)	p value
Age Groups			
1-10 years	3 (21.3)	10 (55.6)	0.266
>10 years	11 (78.6)	8 (44.4)	
Gender			
Male	8 (57.1)	14 (77.8)	0.267
Female	6 (42.9)	4 (22.2)	
Risk Category at Diagnosis			
Standard	0 (0)	2 (12.5)	0.342
Medium	3 (27.3)	2 (12.5)	
High	8 (72.7)	12 (75)	
WBC Count/L			
<50 000	6 (50)	6 (35.3)	0.471
>50 000	6 (50)	11 (64.7)	
CNS Involvement			
No	11 (78.6)	13 (76.5)	1
Yes	3 (21.4)	4 (23.5)	
Translocation			
No translocation	8 (80)	8 (88.9)	1
Ph or MLL	2 (20)	1 (11.1)	
Response to Induction Therapy (day 33)			
Response	11 (91.7)	12 (85.7)	1
Absence of response	1 (8.3)	2 (14.3)	
***NOTCH1/FBXW7* Mutation**			
Wild type	9 (64.3)	12 (66.7)	1
Mutant	5 (35.7)	6 (33.3)	
***AXIN2* Expression**			
Up	6 (50)	5 (29.4)	0.438
Down	6 (50)	12 (70.6)	
Relapse			
Yes	3 (21.4)	4 (25)	1
No	11 (78.6)	12 (75)	
Outcome			
Dead	3 (21.4)	7 (38.9)	0.446
Alive	11 (78.6)	11 (61.1)	

*Values expressed as n (%)

Compliance with Ethical Standards

The ethical committee of Istanbul Medical Faculty (reference number and date: 2008/305 and 20.02.2008) approved this study and written informed parental consents were obtained.

Conflict of Interest

The authors declare no conflicts of interest.

Author Contribution

Authors contributed equally to this work.

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