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Cd44 Gene Single Nucleotide Polymorphism: Impact On Efficacy Of Cd34^{+} Cell Mobilization In Autologous Haemopoietic Stem Cell Transplantation.

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Abstract

In the last few years , mobilized Peripheral blood stem cells became an important source for autologous hamopoietic stem cell transplantation in stead of bone marrow stem cells . Mobilization of stem cells into peripheral blood can be affected my many factors such as: underlying diseases, prior treatment, age and genetic polymorphisms such as G-CSFR, adhesion molecules,VCAM-1,CD 44 and chemokines SDF -1. The CD44 is a cell adhesion molecule which shows high structural heterogeneity .CD44 is multifunctional glycoprotein receptor that binds various ligands like hyalouronic acid and ostopontin as these adhesion molecules play a critical role in HSCs homing in bone marrow, any abnormalities in these adhesion molecules can impair HSC lodgment in their bone marrow niche and mediate their mobilization to peripheral blood. The aim of the present study was to assess the impact of CD44 single nucleotide polymorphism on the efficacy of mobilization of CD34^{+} hematopoietic progenitor cells in patients scheduled for autologous transplantation. The study was conducted on 46 patients were scheduled for autologous HSC mobilization and transplantation. The median age of patients was43.0 with range of(21-60) years . The group of patients with multiple myeloma (n=23), Hodgkin lymphoma (n =18), non - Hodgkin lymphoma (n = 5). □ The major criteria of poor mobilizers which are proposed by the Italian GITMO working group include: (1) After adequate mobilization (G-CSF 10 μg/kg if used alone or ≥5 μg/kg after chemotherapy) circulating CD34(+) cell peak is <20/μL up to 6 days after mobilization with G-CSF or up to 20 days after chemotherapy and G-CSF or(2) they yielded $<2.0 \times 10^{(6)}$ CD34(+) cells per kg in ≤3 apheresis. In the present study four patients fulfilled the criteria for poor mobilizer Patients homozygous for T allele had a lower total yield of CD34 cells/kg body weight with median (2.28)versus the group of allele C carriers (CC+CT)with median (2.9) with P value=0.26 and also patients homozygous or T allele had a lower number of CD34 cells collected during the first apheresis session with median of(0.9) versus the C allele carriers(CC+CT) with median of (2.23) with P value =0.15. The study revealed that 55.0% of carriers of C allele (CC+CT) reached the target of minimum of 2x 10^{6} cells /kg during the first apheresis session,12.5% of allele C carriers reached the target CD34 count during the second apheresis ,27.5% of C allele carriers reached the target CD34 count during the third apheresis session and 5% of C allele carrier group were from the poor mobilizer group. Where,16.7% of homozygous T patients reached the target of minimum of 2x 10^{6} cells /kg during the first apheresis session , 50% of homozygous T patients reached the target CD34 count during the second apheresis,0.0% of homozygous T patients reached the target CD34 count during the third apheresis session ,and 33.3% of homozygous T

patients were from the poor mobilizer group .with P value=0.006 which is statistically significant.

In conclusion, CD44 rs13347 can be used as a predictive factor for the mobilization response of HSCs in patients with hematological malignancies. has an impact on the efficacy of HSCs mobilization in patients with hematological malignancies.

Introduction:

The vast majority of autologous stem cell transplantation is performed using mobilized peripheral blood stem cells which have replaced the bone marrow as a source of stem cells for autologous HSCT .[34]

The HSC niche is a specialized microenviroment on which HSC reside, and divided into 2 anatomical parts: vascular and endosteal niche(4)

HSCs homing and lodgment in the bone marrow niche is mediated through some adhesive and chemotactic interactions via adhesion molecules such as VCAM-1 that binds its receptor integrin alpha 4 beta1expressed by HSCs, transmembrane SCF (kit ligand) that binds its receptor c-Kit (CD117) on HSCs., Stromal cell–derived factor-1 (SDF-1/CXCL12) secreted by niche cells and its receptor CXCR4 on HSCs.,CD44 a cell surface molecule which interacts with osteopontin and hyaluronic acid.(4-5)

Mobilization of stem cells can be induced by pharmacological mechanism such as G-CSF, Plerixafor(CXCR4 antagonist),cyclophosphamide and stem cell factors.(6) G-CSF induces HSCs mobilization through marked down regulation of adhesion and chemokine processes such as VCAM-1,SDF-1 and SCF mediated adhesion interactions. [53-57] and also through mediating release of large amounts of proteases such as neutrophil elastase, cathepsin G and matrix metalloproteinase-9 from the granulocytes and their progenitors, so it disrupts the HSC adhesion to their niche in the bone marrow.

Mobilization of stem cells into peripheral blood can be affected my many factors such as: underlyiong diseses,prior treatement, age and genetic polymorphism such as G-CSFR,adhesion molecules,VCAM-1,CD 44 and chemokines SDF -1.(7-8)

The CD44 is a cell adhesion molecule that is encoded by one gene located on chromosome 2 in humans, which shows high structural heterogeneity that results from a wide variety of protein polymorphisms and post-translational modifications.(9-10)

CD44 standard (CD44s) is the most abundant isoform, expressed by most mammalian cells (2) and acts as a common receptor for hyalourinic acid and also CD44 in humans perform a unique role by a specialized isoform (HCELL)(Haemopoietic cell E-/L-selectin ligand which plays an important role in cell-cell adhesive interactions so it plays a critical role in HSC homing in bone marrow.(11)

The aim of the present study is to asses the impact of CD44 gene single nucleotide polymorphism rs 13347 on the efficacy of HSC mobilization in patients with hematologica malignancies

Patients and methods:

The present study is conducted on 46 patients who were scheduled for autologous HSC mobilization and transplantation. Patients were recruited from the stem cell transplantation unit and the stem cell transplant clinic at El- Mowasah Hospital.the median age of patients is 43 years with range (21-60) years. The group of patients with multiple myeloma (n=23), Hodgkin lymphoma (n =18), non - Hodgkin lymphoma (n = 5), Table (1).The major criteria of poor mobilizers which are proposed by the Italian GITMO working group include: (1) After adequate mobilization (G-CSF 10 μg/kg if used alone or ≥5 μg/kg after chemotherapy) circulating CD34(+) cell peak is <20/μL up to 6 days after mobilization with G-CSF or up to 20 days after chemotherapy and G-CSF or(2) they yielded $<2.0 \times 10^{(6)}$ CD34(+) cells per kg in ≤3 apheresis. In the present study we have classified the patients according to number of apheresis sessions required to reach target count of CD34 cells of minimum 2 $x10^6$ cell/kg into groups (A,B,C) and group D who didn't reach the target count throughout the three apheresis sessions (poor mobilizer group) according to GITMO criteria table(2). In another approach , we have classified the patients according to their CD34 cell yield during the first apheresis session in to three groups (> or equal $4x10^6$ cells/kg),(> or equal 1 and <4)and(<$1x10^6$ cells/kg) as shown in table (3). In the present study Polymorphisms of CD44 rs13347,were evaluated. genotyping was done using standarad PCR based assays.

Table (1) Characteristics of patients

Age, median (range), yr	43 (21-60)
Sex (male/female)	30/16
Multiple myeloma	23
Hodgkin lymphoma	18
Non Hodgkin lymphoma	5
Mobilizing regimen	
G-CSF alone	45
G-CSF + mozobil	1
Remission state	
Complete remission (CR)	42
Very good partial remission (VGPR)	4

Table (2) classification of the patients according to the number of apheresis sessions required to gather the minimum count of CD34 cells of $2x10^6$ cells/kg

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Patient groups	No.	%
Group A (Reach (2 x 10^6) at pheresis session 1)	23	50.0
Group B (Reach (2 x 10^6) at pheresis session 2)	8	17.4
Group C (Reach (2 x 10^6) at pheresis session 3)	11	23.9
Group D (didn't reach the target throughout the 3 sessions)	4	8.7

Table (3) classification of the patients according to CD34 cell yield during the first apheresis

Patient groups according to CD34 (x10^6) yield in the first apheresis session	No.	%
< 1 (x10^6)	13	28.3
≥ 1 – < 4 (x10^6)	24	52.2
≥ 4 (x10^6)	9	19.6

Diagnosis of multiple myeloma based on:

Routine investigations:(complete blood count, ESR, serum LDH, Liver function tests, Bone marrow trephine biopsy with aspiration, Protein electrophoresis, Quantitative assay of immunoglobulins, Immunofixation in serum and urine, Renal function tests, Skeletal survey, ECHO cardiography, MRI in selected cases.[12-19]

Diagnosis of lymphoma based on: Routine investigations:(complete blood count, ESR, serum LDH, Liver function tests, Excisional lymph node biopsy and immunohistochemistry, Bone marrow trephine biopsy, CT scan (neck, chest, abdomen, pelvis, ECHO cardiography [20-25].

Pre- transplantation investigations: Complete blood count, serum LDH, ESR. Assessment of remission status with CT scan, PET scan, bone marrow biobsy, Liver function

tests, Left ventricular ejection fraction (echocardiograh, Electrocardiogram, Serological survey: HCV, HBV, HIV, CMV, EBV, Toxoplasmosis, Pulmonary function tests, Chest x-ray, Repeat of protein electrophoresis and immunofixation,CD34 stem cells counting by flowcytometry in an aliquot of each apheresis product throughout the leukapheresis sessions is routinely done.[26-31]

Genotyping for CD44 gene SNPs rs(13347):

Genomic DNA was extracted from the patients peripheral blood samples{N=46)using PureLink® Genomic DNA Mini Kit[33]. Then CD44 gene polymorphisms detection by allelic discrimination using the 5` nuclease assay. Real time PCR was performed using rotagene.rs(13347) CD44 were analyzed using1ug genomic DNA, Taqman Genotyping Master mix 2X and 0.5Um specific primers. The thermal profile was: holding at 95□C for 10 minutes, followed by 45 cycles of:

- Denaturation: 95□C for 15 seconds.
- Annealing/Extension: 60□for 1 minute.

Statistical Analysis

The results are presented as medians and ranges for continuous variables. SNPs were analyzed for deviation from the Hardy-Weinberg equilibrium, using the chi-square test. The Wilcoxon matched-pair test was used to compare groups of dependent continuous variables. The chi-square test with Yates correction and the exact Fischer test were used to investigate the dependence between variables. Correlations between variables were assessed by the Spearman rank correlation coefficient (r). Multivariate logistic regression was performed. Comparisons and correlations were considered significant if P <.05.

Results:

Mobilization efficacy:

The present study revealed that 55.0% of carriers of C allele (CC+CT) reached the target of minimum of 2x 10^6 cells /kg during the first apheresis session versus 16.7% of homozygous T group,12.5% of allele C carriers reached the target CD34 count during the second apheresis versus 50% of homozygous T group,27.5% of C allele carriers reached the target CD34 count during the third apheresis session versus 16.7% of homozygous T group and 5% of C allele carrier group were from the poor mobilizer group versus 16.7% of homozygous T group with P value=0.006 which is statistically significant.table(4) In the present study four patients fulfilled the criteria for poor mobilizer.

The present study results revealed that Patients homozygous for T allele had a lower total yield of CD34 cells/kg body weight with median (2.28)versus the group of allele C carriers (CC+CT)with median (2.9) with P value=0.26 and also patients homozygous or T allele had a lower number of CD34 cells collected during the first apheresis session with median of(0.9) versus the C allele carriers(CC+CT) with median of (2.23) with P value =0.15

Based on classification of the cases into 3 groups according to CD 34 cell yield during the first session: (> or equal 4 x10 ^{6} cells/kg› or equal 1 to < 4 and, < 1) there was no statistical difference between these 3 groups according to the allele frequency. Table(6)

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Allele Frequencies in CD44:

according to CD44 SNP rs13347 (n = 46) ,the result divided our studied cases into 26 cases with CC genotype ,6 cases with TT genotype and 14 cases with CT genotype .

CD44 SNP rs13347	No.	%
(CC)	26	56.5
(CT)	14	30.4
(TT)	6	13.0
CC + CT (R)	**40**	**86.9**
Allele frequency		
C	66	71.7
T	26	28.3

Table (4)Distribution of the studied cases according to CD44 SNP rs13347

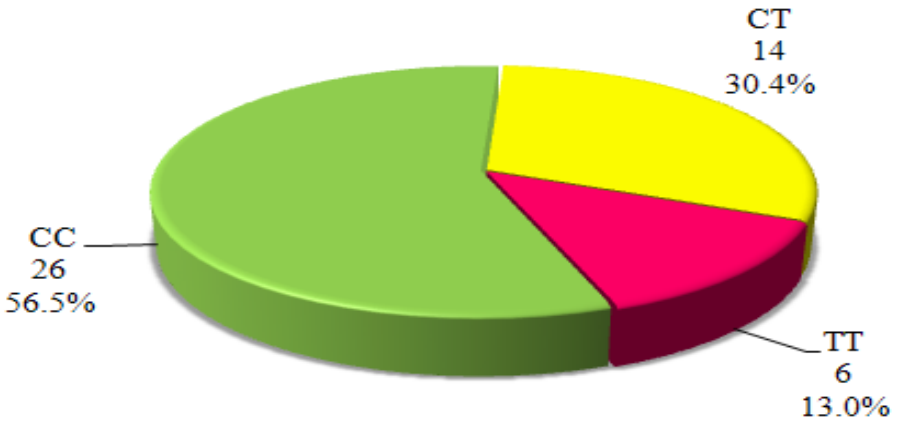

Figure (1) Distribution of the studied cases according to CD44 SNP rs13347

Table (5): Relation between CD44 SNP rs13347 alleles and number of apheresis sessions required to reach the minimum count of CD34 cells ($x10^6$) (n = 46)

	CD44 SNP rs13347				χ^2	^{MC}p
	CC + CT (n =40)		TT (n =6)			
	No.	%	No.	%		

CD34 (x10^6)						
Reach (2 x 10^6) at session 1	22	55.0	1	16.7		
Reach (2 x 10^6) at session 2	5	12.5	3	50.0		
Reach (2 x 10^6) at session 3	11	27.5	0	0.0		
Did not reach the target count of CD34 cell(2x10^6) throughout the three apheresis sessions (poor mobilizer)	2	5.0	2	33.3	10.046	0.006

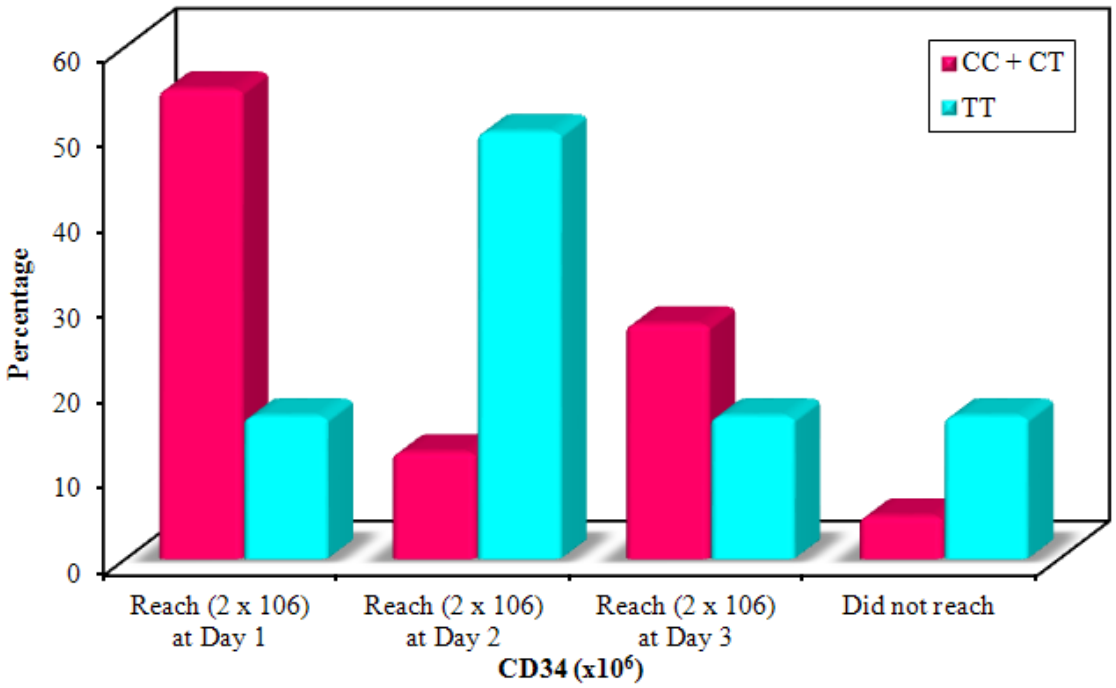

Figure (2): Relation between CD44 SNP rs13347 alleles and number of apheresis sessions required to reach the minimum count of CD34 cells (x10^6) (n = 46).

Table(6): Relation between CD44 SNP rs13347 alleles and CD34 (x10^6) yield during the first apheresis session and throughout the three sessions.

CD34 ($x10^6$)	CD44 SNP rs13347		U	P
	CC + CT	TT		
Day 1	**(n= 40)**	**(n= 6)**	75.00	0.150
Min. – Max.	0.16 – 9.20	0.80 – 4.06		
Mean ± SD.	2.74 ± 2.19	1.45 ± 1.28		
Median	2.23	0.98		
Total days	**(n= 40)**	**(n= 6)**	86.00	0.267
Min. – Max.	0.66 – 9.20	1.30 – 5.70		
Mean ± SD.	3.52 ± 1.82	3.19 ± 1.93		
Median	2.99	2.28		

Table(7): Relation between CD44 SNP rs13347 alleles and CD34 cell count ($x10^6$) gathered during the first apheresis session(n = 46)

	CD44 SNP rs13347				χ^2	^{MC}p
	CC + CT (n =40)		TT (n =6)			
	No.	%	No.	%		
CD34 ($x10^6$) at first apheresis session						
< 1 ($x10^6$)	10	25.0	3	50.0		
≥ 1 – < 4 ($x10^6$)	22	55.0	2	33.3	1.724	0.441
≥ 4 ($x10^6$)	8	20.0	1	16.7		

Discussion:

There are multiple factors that can affect the efficacy of stem cell mobilization to the peripheral blood such as age, gender, previous lines of administrated treatment and the type of the primary disease[34-38], in our study we couldn't judge the relation between these factors and the mobilization failure as the poor mobilizer group consisted of only 3 patients.

The group of poor mobilizer had an equal result of the genotype, where the result was one case with CC genotype, one case with CT genotype, and one case with TT genotype.so we can't asses the relation between the genotype and the poor mobilization.

We have classified our subjects according to number of apheresis sessions required to reach target count of CD34 cells of minimum 2 $x10^6$ cell/kg into groups (A,B,Cand D) group(A) included patients who reached the target count of CD34 Cell (> or equal 2 x 10^6 cell /kg)at the first apheresis session ,group(B) included patients who reached the target count of CD34 cells at the second apheresis session, group(C) included patients who reached the target CD34 cell count at the third apheresis session and group(D) included patients who didn't reach the target count throughout the three apheresis sessions (poor mobilizer group) according to GITMO criteria . Based on this classification, the present study revealed that 55.0% of carriers of C allele (CC+CT) were from group(A),12.5% of C allele carriers were from group(B),27.5% of C allele carriers were from group (C) and 5%of C allele carriers were from group (D).Where ,16.7% of homozygous T patients were from group (A), 50% of homozygous T patients were from group (B),0.0% of homozygous T patients were from group(C) ,and 33.3 % of homozygous T patients were from group (D)the poor mobilizer group .with P value=0.006 which is statistically significant.

In 2014, Anna Szmigielska-Kaplon et al conducted a similar study which studied the effect of polymorphisms of CD44 gene and other adhesion molecules VCAM-1 and CXCR-4 on HSC mobilization efficacy in patients with hematological malignancies . This study was applied on 110 patients,where she studied the effect of the gene polymorphism of various adhesion molecules which included CD44 gene rs (13347),VCAM1 gene rs (1041163),and CXCR4 gene rs(2680880). She observed that only CD44 gene SNP rs (13347) has an impact on the mobilization efficacy of HSCs in to peripheral blood in patients with haematological malignancies and the polymorphism of both of VCAM1 and CXCR4 has no influence on the mobilization efficacy.

According to this study, Anna Szmigielska-Kaplon et al reported that a higher frequency of TT genotype in rs 13347(CD44) gene was found in the poor mobilizer group as the poor mobilizer group was 15 patients and 4 of the 15 patients (26%)were homozygous for T allele where as among the good mobilizers the TT genotype patients were only 7 of 95(7%) (p=0.02) , which is in concordance with the present study as the poor mobilizer group was 4 patients and 2 of the 4 patients were homozygous for T allele with p value =0.006 . Detection of gene expression was performed in this study and she reported thatTT genotype resulted in a higher CD44 mRNA expression at the time of apheresis but in carrier of C allele it was observed that CD44 gene expression is decreased at the time of apheresis.[41]

She observed also in her study that patients with TT genotype had a lower number of CD34 cells gathered during the first apheresis session with median of (1.2) p value=0.04 ,

Which is in concordance with our study where the TT genotype had the lower number of CD34 cells gathered in the first apheresis session with median of(0.98) where p value=0.15 which is statistically non significant.[34-41]

Again, she stated that the TT genotype had the lower number of CD34 cells in the total days of collection with median (3.6)where p value =0.01 which gives the same impression of our study which revealed that TT genotype patients had the lower total number of CD34cells with median of(2.28) with p value=0.267 which is still statistically non significant.[41]probably, statistical significance would be achieved if we extend the study to be applied on large number of cases

Similar observations were reported by Beatriz Martin Antonio.He applied his study on two groups: group(1) consisted of 112 individuals who were given G-CSF(10ug/kg SC for 5 days) to stimulate CD34 cell mobilization for HLA identical sibling allogenic HSCT and group 2 which consisted of 107 voluntry donors., and in this study, SNP of 16 genes (CXCL12,CXCR4,VCAM-1,VLA-4,G-CSF, CD34,CD44,CXCR2, CXCL2, Kit ligand, c-kit, MMP-9, CTSG, GNAS).[42]

Beatriz Martin Antonio reported that there was no correlation between age or gender and the CD34 count in the peripheral blood before or after G-CSF administration which is consistent with our study.

In his study, two out of 28 SNP tested, one in VCAM-1 and one in CD44 were associated with CD34 cell count in peripheral blood after G-CSF administration. The genotype (CC) CD44 rs13347 was associated with higher count of CD34 cells in peripheral blood (p=0.04),with a higher number of CD34 count of donor(p=0.025)and with the number of CD34 cells after first apheresis(p=0.02) which is in concordance with our study in which CC genotype showed higher CD34 Total count with median of3.14 with P value= 0.14 and higher CD34 count after the first apheresis with median of2.52 and P value=0.16[41,43]Although ,Martin Antonio's study was applied on healthy donors but his findings were hand in hand with our study which was applied on patients with hematological malignancies.

Anna Szmigielska-Kaplon et al observed in her study that the level of mRNA expression of CD44 rs (13347) is higher in subjects with TT genotype .Where Martin Antonio observed that CD44 rs(13347) mRNA expression didn't show any significant difference between the three genotypes during the steady state before G-CSF administration but after G-CSF administration , he detected that mRNA expression of CD44 is significantly decreased in subjects with CC and CT genotypes ,This decrease in mRNA expression couldn't be detected in subjects with TT genotype.

In another study, Karin Zaatar Cecyn studied the Expression of the adhesion molecules on CD34+ cells from steady-state bone marrow before and after mobilization and their association with the yield of CD34+ cells. In this study , they determined the expression of the following adhesion molecules {CD106(VCAM-1), CD135(FLT-3), CD184(CXCR4), CD44,

CD62(L-selectin, CD49d(VLA-4), and Cd11a(LFA-1)} on CD34 cells from bone marrow in steady state for both healthy donors and patients with hematological malignancies. Also they analyzed the correlation between these CAMs and chemokine expression with good and poor CD34 yield and they found that a statisitical significant difference in CD106(P=0.007) ,CD44(P=0.027) ,CD49d(p=0.014) is present and those with poor CD34 yield have shown a higher expression of these three adhesion molecules than those with good CD34 yield. They also determined the expression of these CAMs on the CD34+ cells from the BM after HPC mobilization for both donors and patients. They observed that there was reduced expression of CD44 mRNA after HSC mobilization with a statistical difference P=0.011. As in the previous studies Martin Antonio observed that G-CSF administration caused a decrease in the expression of CD44 mRNA and this effect is significant in subjects with the(CC) genotype(P=0.02) and CT variants (P=0.009), but this decrease in the gene expression was not evident in those with the TT variant .so these results can give the same impression of our study .

Contradictory results were reported by Seiji Mishima who studied the influence of SNP of VCAM-1 rs (1041163) , CD44 SNP rs (13347) on the mobilization efficacy of HSCs from bone marrow to the peripheral blood in patients with hematological malignancies .He demonstrated that the T allele of VCAM-1 gene is associated with higher CD34 cell count and the CC genotype showed a lower CD34 cell count in the peripheral blood at the time of apheresis but there was no statistical difference between the studied groups. This study also demonstrated that there is no association between the CD44 SNP rs(13347) and the CD34 count in the peripheral blood at time of apheresis the peak PB CD34-positive as there was no statistical difference between the studied groups regarding the allele frequency of CD44 SNP rs (13347) and this contradiction may be due to , the wide range of the primary diseases that were included in the study and the different ethnic group from our patients.

In conclusion, our results indicate that SNPs, CD44 rs13347 can be used as a predictive factor for the mobilization response of HSCs in patients with hematological malignancies who are scheduled for autologous HSCT .

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