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Immunophenotyping by Flow cytometry of Patient with chronic myeloid leukemia CML. Single center

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Abstract
This retrospective study aimed to highlight chronic myeloid leukemia (CML), which is characterized by an acquired mutation affecting the hematopoietic stem cell (HSCs), which leads to excessive production of immature granular cells and is correlated with mutual mutations of chromosome 9 and chromosome 22 Which leads to the formation of BCR-ABL gene (Philadelphia chromosome). It is believed that this gene association plays a key role in the formation of chronic myeloid leukemia through the activity of tyrosine kinase enzyme. The current study included 70 patients with chronic myeloblastic leukemia (41 patients treated with Tyrosinase kinase inhibitors (TKIs) as the first and second generation (imatinib & Nilotinib) and 30 new pre-treatment patients) at the Center for Oncology and Hematology at Al Hussein Medical City Karbala province, during the period from April 2017 to March 2018. With 30 individuals used as a control group (apparently healthy). Complete venous blood samples were taken for the purpose of studying the complete blood count, microscopic blood sample, and for the study of immunohistochemical patterns using surface markers CD7 and CD34 with flow cytometry. Conclusions treatment with Tyrosinase kinase inhibitors (TKIs) is effective in reversing the observed deviation in values of blood parameters measured to normal values and very close to healthy individuals. The immunophenotypic using surface markers (CD34 & CD7) with flow cytometry is a good predictive value for patients with chronic myeloid leukemia (CML).

INTRODUCTION:

Flow cytometry is measurement of cellular antigens as they are flowing in a fluid stream. It permits fast, quantitative and many parameter diagnosis of heterogenous cell population on a cell-by-cell basis (1). Cell markers usually presented on more than one cell type; so, flow cytometry panals strategies may lead to procedures for immunophenotying cells with more than one antibody at the same time by measuring the unique map of cell markers using several antibodies together, each has different fluorochrome, a given cell population can be distinguished, and quantified (2). Immunophenotyping is established by labeled antibodies that defines specific epitopes of cellular antigen. The technique employed for immunophenotyping is usually by flow cytometry (3). Chronic Myeloid Leukemia (CML) is a hematopoietic stem cell disease, characterized by a reciprocal translocation between chromosomes 9 and 22, resulting in the formation of the Philadelphia chromosome (Ph). This translocation t (9;22) results in the head -to-tail fusion of the breakpoint cluster region (*BCR*) gene on chromosome 22 at band q11 and the Abelson murine leukemia (*ABL*) gene located on chromosome 9 at band q34. The product of the fusion gene (*BCR-ABL*) is believed to play a central role in the initial development of CML (4). It is clear that CML occurs all over the world, from childhood to aging, which is most common in adults. Over the past two decades, cases of infection in Iraq have clearly increased, as reported by the Iraqi Cancer Council (5).

MATERIALS AND METHODS

Blood samples from 71 male and female CML patients (with either newly diagnosed or already treated patients) referred to the Center of Oncology for Hematology of Al-Hussein Medical City in Karbala, Iraq, for evaluation and treatment during the period Apr. 2017 to Feb. 2018 were used in this study. The CML subjects included 30 newly diagnosed (untreated) patients (15 men and 15 women) with a mean age of 41.96 ±14.29 yr. and an age range of 19 – 65 yr. Also, another 41 already diagnosed as having CML and currently receiving treatment included 21 men and 20 women with a mean age of 42.36±16.07 yr. and an age range of 11 – 70 yr. The range of duration of diseases between several months to 12 yr. Thirty control subjects were also used in this study. They included 15 men and 15 women with a mean age of 39.8±15.78 yr. with an age range of 16 – 65 yr.

Blood sampling A total venous blood sample of 2.5 ml were obtained from each patient included in this study by venipuncture and the samples were collected in EDTA tubes. Blood sample from suspected patient was examined for complete blood indices in the teaching laboratory department of the Medical City, a blood film was made by taking a drop of blood sample spreads it on a clean dry slide, and staining it by Leishmen,

Immunophenotyping flowcytometry determination of CD7 & CD34

In this study immunophenotyping CD7 &CD34 expression were investigated by using four-colour Cyflow® Cube 8 flow cytometry device (Mandery Cyflow® China), which is a fully equipped desktop Flow Cytometer (FCM)

I. Reagents and Assay Procedure CyLyse® stands for an erythrocyte lysing reagent kit with a complete preservation of the surface proteins and particularly no loss of cells**.**
   1. **Antibody labeling**
      Antibody labeling was done by mixing 100 microlitre of blood with conjugated antibody (10 microlitre) in a test tube, mixed thoroughly, then incubated in the dim light at room temperature for fifteen minutes.
   2. **. Leukocyte fixation**
      For leukocyte fixation, 100 microlitre of reagent A has been added and mixed well and incubated at room temperature for at least ten minutes in the dim light.
   3. **Erythrocyte lysis**
      For erythrocyte lysis 2.5 ml of reagent B was added, vibrate gently and then incubated in the dim light for twenty minutes. After that the sample has been analyzed by flow cytometry.

II. Determination of the Aberrant Phenotype Identification of blast cells was performed using forward scatter (FSC) versus side scatter (SSC) parameters. Antigen expression was considered to be positive when the percentage of positive blast cells was equal or greater than 20% forCD7 and equal or greater than 10% foe CD34. Similarly, aberrant phenotypes were defined when at least 20% of the blast cells expressed that particular phenotype (6).

RESULTS:

This study recruited 71 patients; with a Mean ± SD (42.44± 15.91) yr., Age range was 11–70; median age was 43 years, thirty control subjects were also used in this study. They included 15 men and 15 women with a mean age of 39.8±15.78 yr. with an age range of 16 – 65 yr. Patient characteristics (treated and newly diagnosis) showed in Table 1.

The role of FC in the chronic phase of chronic myeloid leukemia (CP-CML) is unknown. Immunephenotyping alone have a limited role in the diagnosis of CML but are often used in advanced stages, especially in the Blast crisis (BC-CML), so we hypothesized that the finding of abnormal (immature) cells using FC in CP-CML might predict an early shift to the BC phase. This study was planned to evaluate the results of the distribution of some markers surface in the peripheral blood with FC for a number of Patients with chronic CML and registered with one health institution Comparing them with a number of healthy individuals for the purpose of follow-up and assessing the predictive value of the markers (CD34 & CD7) in pathological prediction of disease.

I. Surface markers CD34 & CD7 in CML patients and control:

Table (2) shows the distribution of percentage of expression of surface markers (CD34 & CD7) in CML patients and control group. All samples were positive for CD7+ expression in control groups and new patients (100%) 30/30 compared to treated patients (92.7%) 38/41 positive (7.3%) 3/41 with statistically significant differences P ≤0.05. All samples had a negative expression (aberrant CD34 expression) of the CD34 surface area in the control groups and the new patients (100%) 30/30, compared to the treated group where the positive samples (7.3%) were 3/41, while the negative was (92.7%) 38/41. were observed significant differences P ≤0.05.

Table 1: Baseline characteristics of seventy-one participants

Patient's characteristics	**Mean ± SD**	**Range**
Age / years	42.44± 15.91	11-70
Gender		
Male Female	36 (50.7%) 35 (49.3%)	M/F 1.03:1
HB (g/dL)	(11.63± 2.16)	(6.90-15.80)
WBC count (10^9/L)	(68.02± 100.75)	(3.30-440)
Eosinophils%	(3.0±3.3)	(0 -15)
Basophils %	(1.52±1.73)	(0-7)
Myeloblast%	(1.65± 4.55)	(0- 35)
PLT (10^9/L)	(298.68± 157.58)	(39-990)
Spleen size	(4.5-6.34)	(0-22.3)

Duration of Treatment (months)	**Treatment n= 41**	**Newly diagnosis n=30**	**Total (n=71)**
Mean ± SD Range	62.84±35.41 (144-12)	6.00± (6-0)	68.84±35.41 (144-0)
Type of Treatment			
Imatinib	27 (%65.9)	21(% 70)	48 (%67.6)
Nilotinib	14 (%34.1)	9 (% 30)	23 (%32.4)
Phase of disease			
Chronic phase	38 (%92.7)	30 (%100)	68 (%95.8)
Advanced phase	3 (%7.3)	0 (%0.00)	3 (%4.2)

Table (2) Distribution of the percentage of expression of surface markers (CD34 & CD7) in CML patients and control group

Immunological markers		Experimental group			P. Value
		Newly diagnosis	Treated patients	Control (healthy)	
		N: 30	N: 41	N: 30	
Positive	**CD7**	30/30(%100)	41/38(%92.7)	30/30(%100)	
Negative	**(≥20%) positive**	30/0 (%0.00)	41/3 (%7.3)	30/0 (%0.00)	
Mean ± S.D		65.36±8.05	63.27±16.44	59.69±11.58	**0.00**
Minimum		50.5	1.79	43.9	
Maximum		78.57	80	83.06	
Positive	**CD34**	30/0 (%0.00)	41/3 (%7.3)	30/0 (%0.00)	
Negative	**(≥10%) positive**	30/30(%100)	41/38(%92.7)	30/30(%100)	
Mean ± S.D		2.46±2.14	4.03±10.32	4.18±1.89	**0.00**
Minimum		0.78	0.3	2.11	
Maximum		12.7	66.6	9.21	

Table (3) Distribution of surface markers (CD7 & CD34) in CML patients compared to control group depending on the gender Mean ± S. D.

Gender	Experimental group	N	CD34	CD7
			Mean ± S. D	Mean ± S. D
Male	Treated patients	21	2.29 ±0.74	66.33 ±11.76
	Newly diagnosis	15	4.02 ±1.21	57.30 ±5.51
	Control (healthy	15	2.59 ±0.92	65.02 ±8.10
	Total	51	2.71 ±1.09	64.25 ±10.09
Female	Treated patients	20	2.68 ±1.21	64.74 ±11.11
	Newly diagnosis	15	4.38 ±2.51	62.04 ±8.12
	Control (healthy	15	2.47 ±0.75	65.66 ±6.72
	Total	50	2.89 ±1.50	64.62 ±9.16
Total	Treated patients	41	2.48 ±1.01	65.56 ±11.33
	Newly diagnosis	30	4.19 ±1.86	59.51 ±7.03
	Control (healthy	30	2.53 ±0.83	65.34 ±7.32
	Total	101	2.80 ±1.30	64.43 ±9.59
Gender P. Value			0.444	0.575
Experimental group P. Value			0.000	0.043
Experimental group LSD			0.72	5

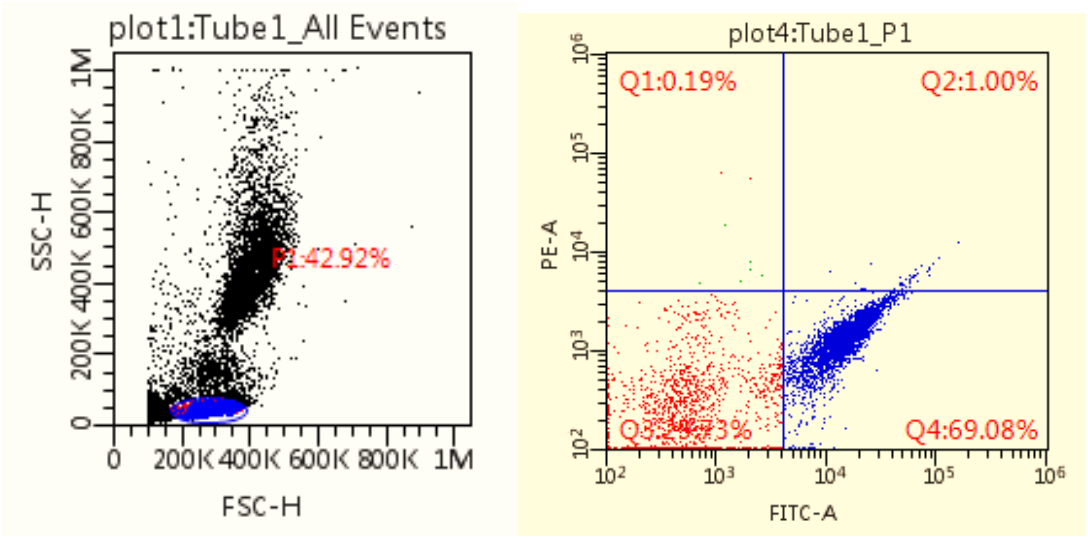

Image (1) Patient showed common expression of CD7 & CD34

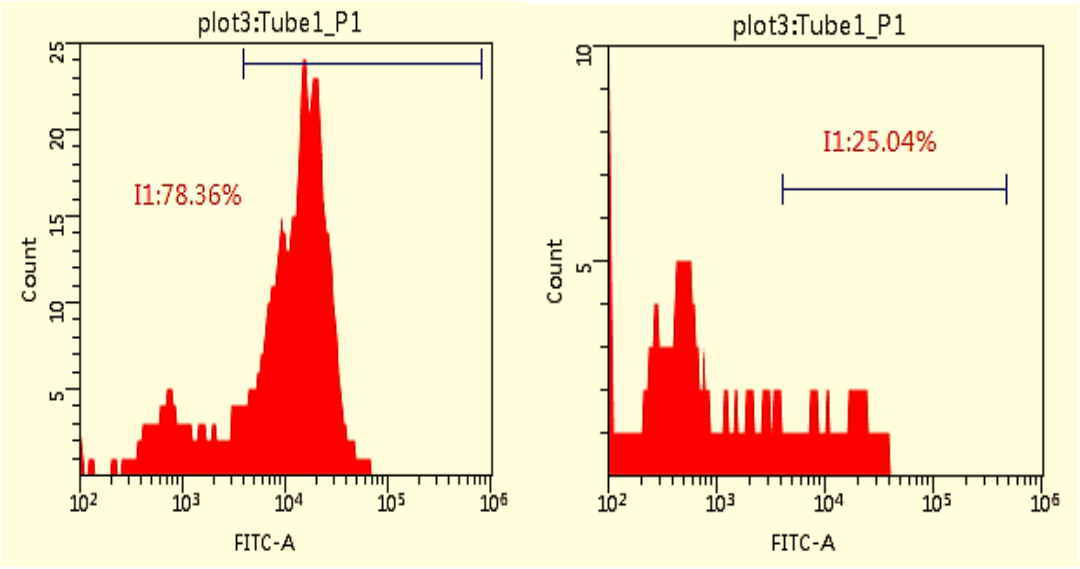

Image (2) CD7 Expression of Fluid Flow Technology (FC)

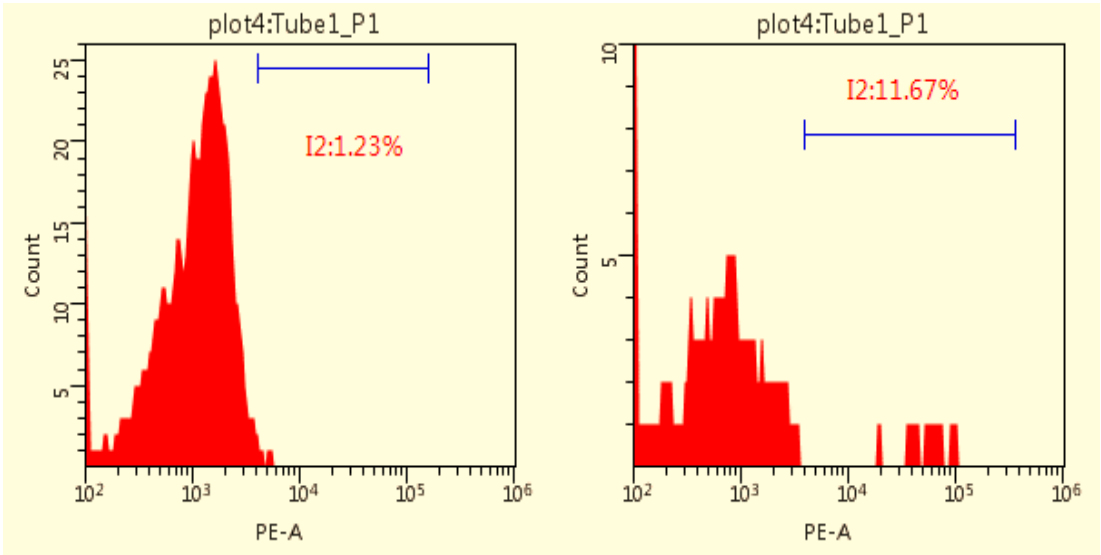

Image (3) CD34 positive and negative expression by flow cytometry (FC)

The FSC vs SSC plot was used to gate on the blast cell population. This is the older technique to gate on blast cells. The newer technique is by using CD45 vs SSC. However, using the older technique of FSC vs SSC is still useful especially in patients with high blast cell. The FITC dye is associated with CD7 and PE dye coupled with CD34.

Table (4) Effect of duration of treatment and age groups in surface markers (CD7 & CD34) compared to control group Mean ± S. D.

Experimental group	Age categories	number	**CD34**	**CD7**
			Mean ± S.D	Mean ± S.D
Treated patients	< 20	4	2.88 ±1.08	63.73 ±9.28
	21 - 30	5	1.69 ±0.28	64.32 ±12.98
	31 - 40	10	2.83 ±1.51	67.10 ±11.49
	41 - 50	7	2.55 ±0.66	66.26 ±13.04
	51 - 60	9	2.42 ±0.77	69.93 ±8.61
	> 60	6	2.29 ±0.83	57.87 ±12.89
	Total	41	2.48 ±1.01	65.56 ±11.33
Newly diagnosis	< 20	1	5.33 ±	54.22 ±
	21 - 30	8	3.47 ±1.22	63.74 ±7.40
	31 - 40	6	5.58 ±3.46	55.83 ±8.55
	41 - 50	5	3.92 ±1.99	58.61 ±6.21
	51 - 60	6	4.73 ±1.81	56.53 ± 8.22
	> 60	4	3.89 ±1.21	58.77 ±6.48
	Total	30	4.48 ±1.93	57.89 ±7.37
Control (healthy)	< 20	2	3.35 ±0.00	54.00 ±0.00
	21 - 30	8	2.62 ±0.64	70.39 ±4.32
	31 - 40	7	2.47 ±1.18	66.50 ±6.65
	41 - 50	4	1.76 ±0.41	66.44 ±7.57
	51 - 60	5	2.80 ±0.39	64.30 ±7.42
	> 60	4	2.47 ±1.08	59.11 ±6.55
	Total	30	2.53 ±0.83	63.45 ±6.50
Total	< 20	7	3.85 ±1.08	9.28± 57.31
	21 - 30	22	2.60 ±1.00	66.15 ±8.24
	31 - 40	22	3.12 ±1.98	63.14 ±8.89
	41 - 50	17	2.52 ±1.05	63.77 ±8.94
	51 - 60	19	2.74 ±0.96	63.53 ±8.08
	> 60	14	2.80 ±1.18	58.58 ±8.64
	Total	101	2.93 ±1.20	62.08 ± 8.67
Experimental group P. Value			0.000	0.050
Age categories P. Value			0.071	0.205
Experimental group LSD			0.71	4.52

Table (5) Effect of treatment type and by period of months in surface markers CD7 & CD34 for Mean ± S. D. CML patients

(TKIs)	Treated time	Number	CD34	CD7
			Mean ± S.D	**Mean ± S.D**
Imatinib	6-0	21	3.04± 2.78	65.56±6.12
	48 -12	14	2.55 ±0.97	66.44 ±7.72
	84 - 60	10	2.49 ±0.55	64.65 ±14.32
	144 - 96	3	2.56 ±1.60	66.06 ±13.96
	Total	48	2.66 ±1.47	65.67 ±10.53
Nilotinib	6	9	2.84± 2.73	63.48±14.69
	48 -12	6	2.94 ±1.75	58.73 ±12.14
	84 - 60	4	1.91 ±0.64	75.41 ±5.03
	144 - 96	4	2.20 ±0.23	61.65 ±12.25
	Total	23	2.40 ±1.20	64.18 ±11.03
Total	6	30	2.92±2.75	64.52±10.40
	48 -12	20	2.66 ±1.22	64.13 ±9.63
	84 - 60	14	2.30 ±0.62	68.24 ±12.91
	144 - 96	7	2.35 ±0.96	63.54 ±12.06
	Total	71	2.55 ±1.38	65.10 ±11.25
P. Value treatment			0.613	0.907
P. Value Time			0.343	0.176

The FSC vs SSC plot was used to gate on the blast cell population. This is the older technique to gate on blast cells. The newer technique is by using CD45 vs SSC. However, using the older technique of FSC vs SSC is still useful especially in patients with high blast cell. The FITC dye is associated with CD7 and PE dye coupled with CD34.

The results of the current study showed that the levels of expression of CD7 and CD34 surface markers in the study groups showed significant differences between P≤0.05 and the three experimental groups of the study, whereas there was no gender effect (male and female). On the gender of the study subjects on the values of the surface markers as shown in Table 3.

As for the results shown in Table (4), there is also no statistically significant difference at P ≤0.05 when comparing the distribution of the expression levels of the CD7 and CD34 surface markers by age groups. However, there was a significant difference at P ≤0.05 when comparing the three experimental groups among them for surface markers.

II. Surface markers CD34 & CD7 in CML patients:

The results in Table (4), which show the type of treatment and treatment time for patients with CML (new and treated), showed no statistically significant differences at P ≤0.05 indicating that the expression levels of CD7 and CD34 were not affected by treatment type for patients Who are treated with IMT as a first line of treatment or other targeted treatment (Nilotinib) for patients as first or second line. As well as the length of time to take treatment by patients did not notice significant differences statistically at the value of P ≤0.05.

DISCUSSION

The prognosis for patients with CML depends on the stage of the disease at presentation, but, even for patients diagnosed in CP, there is significant variability in survival. CML consists of myeloid cells at all stages of maturity in patients with chronic phase (CP). The flow cytometry (FC) technique allows for the detection of abnormal cell surface markers that, unlike acute myeloid leukemia (AML), FC is a simple diagnostic tool, (CP-CML) due to loss of braking of cells in maturity and are reserved to determine the proportions of immature cells (Blast) in the advanced stage of the disease (7). We hypothesized that such an outcome would help identify a small number of patients who progress to the BP-CML shortly after the initiation of tyrosine kinase inhibitors (TKIs).

The results of analysis of surface markers CD7 and CD34 (immunephenotypes by flow cytometry) of samples of CML patients and a group of individuals (apparently healthy) who were considered a control group are shown in tables 2 and 3. CD7 was positively expressed at the fraction value greater than or equal to 20% of the mono-nuclear cells in the peripheral blood of most study subjects. In a slightly different manner, 3/101 subjects were found to have aberrant CD7 expression of CD7 (<20%), CD7 was initially identified as a marker of early differentiation of T cells and natural killer, but is now known to be expressed on immature cells capable of producing T, B and myeloid cells. Thus, the CD7 study may be important for studying the cellular origin of neural development. CML, in addition, the cells in the epithelial gland, the majority of mature T cells and natural killer cells, and some common lymphatic joint cells express CD7. These data suggest that the $CD7^+$ expression in the peripheral blood cells of patients in the chronic phase CP-CML may reflect the expansion of spinal cord strains in this disease, and that the transformation of the cells into the BC-CML stage where transiently expressed CD7 may be represented Good arrest of maturation of hematopoietic predecessors at an early stage of the nucleus of the blood. Marti'n-Henao et al. (8) noted no correlation between increased CD7 expression in chronic phase and length of alpha-interferon therapy as well as our current results. There were no significant differences between CD7 expression and TKIs treatment. Hirose et al. (9) suggested in their study a lower response to chemotherapy in $CD7^+$ BC-CML patients than those with immature CD7 cells. Patients with CML may have cells with a positive expression of CD34 more than in healthy patients, and increase as the disease progresses from the chronic to advanced stage (10). Can be used as an indicator of hematopoietic population changes associated with CML progression. During the blood formation process, the CD34 surface marker is distinguished between immature HSCs and $CD34^+$ promising precursors and $CD34^+$ is characteristic of the progression of CML (11). The increase in immature cells (increased expression of $CD34^+$) results in the formation of $CD34^+$ (more mature) cell clusters with a CD34-negative expression. Thus, the ratio between $CD34^+$ and $CD34^-$ increases as the disease develops. Calculation of the most mature cells ($CD34^-$), especially when discontinuation of treatment or a sudden relapse of the disease as a result of mutation or other. (12). In addition, CD34 is predicted to be shorter than BC-CML, which may be related to treatment resistance due to over-expression of P-glycoprotein (13). Pietarinen et al. (14) noted in the results obtained by studying two groups of patients that all CP-CML patients under study responded to TKIs *in vivo* treatment. When CP-CML cells were sorted based on CD34 expression, positive CD34 cells showed positive a good sensitivity to TKIs, whereas the more mature $CD34^-$negative cells were significantly less sensitive. Thus, in CP-CML, TKIs are predominantly targeted mostly at the progenitor cell group while the different leukemia cells (mostly cells of the granular chain) are not affected by BCR-ABL1 inhibitor. These results have implications for drug discovery in CP-CML and suggest a fundamental biological difference between CP-CML and advanced forms of chronic myeloid leukemia. The expression of CD34 has a good predictive value, its absence was associated with a higher rate of complete calm, and it turned out to be an independent predictive factor for better survival (15). All immature cells in the terrestrial stage are CD34, All immature cells in the terrestrial phase are CD34, and most studies confirm this (16, 17). CD34 may sometimes be variable, this change in CD34 expression may be related to the sensitivity of monoclonal antibodies used and technical factors (eg flow cytometry sensitivity) and the criteria used to determine a positive outcome

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