# $CD4^{+}CD25^{+high}$ FoxP3 regulatory T-Cells in typical and atypical Philadelphia +ve CML as regard response to Imatinib therapy

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**Abstract**: Regulatory T cells $CD4^{+}CD25^{+high}$ FoxP3 (Tregs) play an important role in the control of tumor immunity as well as autoimmunity. So we investigated the percentage level of Tregs in newly diagnosed CML patients and correlated it with the patients' clinical, laboratory, cytogenetic variance and response to Imatinib (TKI) therapy. **Methods:** Tregs percentage were quantified by flowcytometry and iFISH (extra signal dual-color probe) was used to detect both typical and atypical BCR/ABL gene rearrangements in 120 newly diagnosed CML patients. The response to Imatinib therapy was evaluated after 12 months .Results: Tregs% at diagnosis was significantly higher in patients with advanced Sokal score and accelerated phase and positively correlated with BM blast cells %and BCR-ABL/G6PDH% but no significant difference as regards cytogenetic variants and response to Imatinib in each group. There was significant decreased in Tregs % after therapy compared to the baseline in the responder groups while in non-responder groups it was significantly decreased only in patients who had atypical Philadelphia. During follow up those patients who had disease progression showed increase in the Tregs% compared to its level during remission but these increase was significantly only in those had typical Philadelphia. **Conclusion**: inhibition of Tregs is an immune mechanism important in the control of CML in patients with typical Philadelphia only but not in patients with atypical Philadelphia. Recommendation: to incorporate detection of such atypical Philadelphia into the routine evaluation of all CML cases and consider deletion status when weighing the risks associated with different treatment modalities.

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## 1. Introduction

Chronic Myeloid Leukemia (CML) is a malignant myeloproliferative disorder originating from a pluripotent stem cell that expresses the BCR/ABL oncogene and is characterized by abnormal release of the expanded, malignant stem cell clone from the bone marrow into the circulation. The discovery of the Philadelphia chromosome followed by identification of its BCR/ABL fusion gene product and the resultant constitutively active P210 MBCR/ABL tyrosine kinase promptedthe understanding of the molecular pathogenesis of CML[1,2].

Certain atypical forms of Philadelphia were described like minor mBCR/ABL, duplication on both chromosomes and deletions adjacent to the BCR/ABL translocation breakpoint on the derivative chromosome 9 which reported in 10–15% of CML patients with a standard t(9;22)[3].These deletions are large and so likely include unrecognized tumor suppressor genes. More important, the deletions are proved to be a powerful and independent prognostic factor more potent than the scoring systems of Sokal et al.[4] or Hasford et al.[5].

Most authors have considered that deletions on der(9) are associated with a shorter duration of chronic phase and shorter survival in patients treated with interferon therapy [6], although it has been suggested that an effective treatment with a selective tyrosine kinase inhibitor such as imatinib mesylate against the activity of BCR/ABL in CML might counter adverse prognostic factors such as der(9) deletions but the failure rate between those patients still high which explained by loss of some tumor suppressor genes or disturbance in the antitumor immune response [7].

One of the key players of immune regulation is the natural $CD4^{+}CD25^{+high}$regulatory T cells (Tregs) that express high level of the transcription factor Foxp3, which is essential for their development and suppressive function. They show a potent Immunosuppressive function and contribute to immunologic self-tolerance by suppressing potentially auto-reactive T cells. There is now substantial evidence that Tregs play an important role in the control of tumor immunity as well as autoimmunity [8,9].

Tregs were identified 3 decades ago in tumor models and provided an explanation for why highly immunogenic tumors continue to grow in spite of an antitumor immune response [10].

Very little is known about the role of Tregs in CML. Increased Tregs numbers was observed in CML

patients who relapse after allogeneic stem cell transplantation, suggesting that these cells may be detrimental to the graft-vs.-leukemia effect of allogeneic stem cell transplantation. These data indicate a possible role for Tregs in the immune control of CML[11-12].

In the present study, we attempted to investigate the percentage levels of T regulatory cell (CD4+CD25+$^{high}$FoxP3+) in newly diagnosed typical and atypical Philadelphia +ve CML patients and their implication in the extent of the disease activity. Then to assess the effect of TKI (Imatinib) treatment on Tregs% levels.

**2. Subjects and Methods:**

This study was done between August 2009 to March 2011 at Clinical Pathology and Medical Oncology Departments, Zagazig University hospitals. It was carried out on 120 CML patients. Their ages ranged from 37 to 57 years. They were 64 males and 56 females. 80 healthy, age and sex-matched volunteers were recruited as a control group. The study was approved by the local ethics committee. Formal consent was obtained from all studied subjects.

Patients with hereditary blood disease, chromosomal anomalies other than Philadelphia chromosome and patients with congenital abnormalities were excluded. Patients were subjected to: full history taking, proper clinical examination, complete blood count and neutrophil alkaline phosphate (NAP) score.

Fluorescence in situ hybridization (FISH) using locus-specific extra-signal (LSI BCR/ABL ES) dual-color translocation probe (VysisInc., Downers Grove, IL, USA), labeled with spectrum green and spectrum red, was applied on bone marrow (BM) samples to locate the BCR/ABL fusion gene, detection of (9q34) deletion and to distinguish between the major (M) and minor (m) breakpoint. At least 20 metaphases or interphase per patient were analyzed (cutoff level was established at 2%).

Interpretation of the results illustrated in table (1) and Photo(1).

Pattern A: typical iFISH patterns of BCR/ ABL gene rearrangements involving the MBCR rejoin consisted of one yellow fusion signal der (22), one green non-rearranged 22and two red for der(9)plus the non-rearranged chromosome 9 -signals.

Pattern B: one fusion, one red and one green signals, extensive deletions of 9q sequences proximal to the breakpoint occurred explaining the loss of one red signal – as compared to typical MBCR/ABL iFISH.

Pattern C: two fusion, one green and two red signals correspond to supernumerary Philadelphia chromosome with MBCR/ABL.

Pattern D: mBCR breakpoints in which the red signal corresponding to der(9)is replaced by a second fusion signal .

Flow cytometeric assay of CD4$^{+}$CD25$^{high}$ (Tregs) and FoxP3 expression.

a. Sample preparation and flow cytometry analysis.

Lymphocytes were purified from peripheral blood by Ficoll-Hypaque (Biochrom, Berlin, Germany). Staining was performed using mouse anti-human monoclonal antibodies (mAbs) (anti-CD25) Phycoerythrin (PE) and (anti-CD4) Peridinine chlorophyll protein (Per-CP) conjugate (Dako). Measurement of CD4/CD25 expression using a FACS Calibur flowcytometry Becton Dickinson, San Jose, CA).

b. FoxP3 staining:

FoxP3 staining was performed according to the manufacturer's protocol {FITC antihuman Foxp3 Staining Set (eBioscience)}. Cells were first stained with surface mAb of interest (anti CD4/antiCD25) followed by FoxP3 intracellular staining using permealizing solution. To avoid nonspecific Fc receptor staining, we used appropriate isotype controls of mouse anti-human mAbs.

With multigated analysis, The percentage of FoxP3 expression were determined on CD4$^{+}$ T-cells that are very high in CD25$^{+}$ to avoid contamination with other CD4$^{+}$CD25 low/intermediate effector T-cells. FACS-acquisition and analysis were performed immediately with FACS Cell Quest software (BD Biosciences) (Fig 1).

Real-time RT-PCR quantitative detection of BCR/ABL.

RNA was extracted from bone marrow aspirate using (QIAamp RNA blood kit from Qiagen for total RNA purification from human whole blood). Real-time RT-PCR of BCR/ABL were done from the extracted RNA using the Light Cycler. t(9, 22) Quantification kit (RQ-PCR kit, Roche). Relative expression levels of different samples were calculated by standardizing the amount of BCR-ABL transcripts in a sample to the amount of an endogenous-expressed housekeeping gene, G6PDH. The values for BCR-ABL and G6PDH for each sample are calculated by the Light Cycler software by comparing the crossing points to the standard curve. In our study, we expressed the BCR-ABL/G6PDH ratio as a percentage by multiplying ratio x 100.

Complete molecular response (CMR) was defined as a point when BCR-ABL mRNA was undetectable by RT-PCR Real-time quantitative.

Patients had treated with stander dose of Imatinib and evaluation after 12 months, those had CMR were followed up for two years [13].

Statistical Analysis:

Data were entered, checked and analyzed using SPSS program package version 16 for windows. Results were expressed as mean± standard deviation (SD), Chi-square, student-t test and Mann–Whitney analysis was used for statistical comparisons between two groups of patients' parametric data. Correlation analysis was performed with Pearson correlation test. P-values below 0.05 were considered significant.

## 3. Results

120 Ph +ve CML patients were recruited into this study, 86 in chronic phase and 34 in accelerated phase diagnosed on the basis of clinical, hematological and cytogenetic criteria.

Cytogenetic findings of the patients are given in table (1). A sole typical MBCR t(9;22)(q34;q11) translocation was found in 84 (70%) patients, 26 (21.7%) with MBCR, del-der(9) and 10 (8.3%) with supernumerary Philadelphia (MBCR ,+ Ph).

The patients were classified into two main groups; group A: patients with Atypical Philadelphia (positive for del-der(9)and MBCR ,+ Ph) and group B: with sole typical MBCR t(9;22).No statistical significant difference as regard the clinical and hematological data (except for basophil; p = <0.014) between the two groups (A and B). While there was statistical difference in all parameters when compared to control group. Therewas statistical significant different as regard clinical stages (p<0.001) Sokal score (p=0.008) andresponse to Imatinib (p=0.033) between group A and B table (2).

Analysis of the percentages of CD4+CD25+$^{high}$FoxP3 (Tregs%) in the study groups at diagnosis before starting therapy (fig 1) revealed a significant increase of these cells in patients in high and intermediate Sokal score compared to those in low stage (p=0.024) and in accelerated phase compared to those in chronic stage (p<0.038) but no significant difference as regards cytogenetic variants and response to Imatinib in each group table (3).

Correlation between the percentage of CD4+CD25+$^{high}$FoxP3 Tregs cells showed a significant positive correlation with number of blast cells in BM and with BCR-ABL/G6PDH% ratio (p<0.001 and p=0.025 respectively) (Fig 2 & 3).

After 12 months of treatment with Imatinib the Tregs % numbers in responder (achieved complete molecular remission) and non-responder to therapy before and after therapy as regards cytogenetic variants, there was significant decrease in the percentage of Tregs after therapy compare to the baseline (before therapy) in all responder groups while in non responders there was a significant decrease in Tregs % also after therapy in patients with atypical Philadelphia only (Table 4).

Patients had remission were followed up for two years some of them had a progression to accelerated or blastic phases and showed re-elevations of the number of Tregs which was significant only (p<0.003)in those with typical sole MBCR (Table 5).

Table (1): FISH patterns with the ES probes in BCR/ABL +ve CML at diagnosis.

| iFISH pattern with BCR/ABL ES probe | Chromosomes signals Fusion Red Green | Cases(120) N % | Interpretation |
|---|---|---|---|
| Pattern A: 1F 2R 1G | 1F (Ph) 2R (9,9q+) 1G (22) | 84(70) | t(9;22), MBCR |
| Pattern B: 1F 1R 1G | 1F (Ph) 1R (9) 1G (22) | 26 (21.7) | t(9;22), MBCR del(9q+) |
| Pattern C: 2F 2R 1G | 2F (Ph, Ph) 2R (9,9q+) 1G(22) | 10 (8.3) | t(9;22), MBCR ,+ Ph |
| Pattern D: 2F 1R 1G | 2F (Ph; 9q+) 1R (9) 1G (22) | 0 (0) | t(9;22)mBCR |

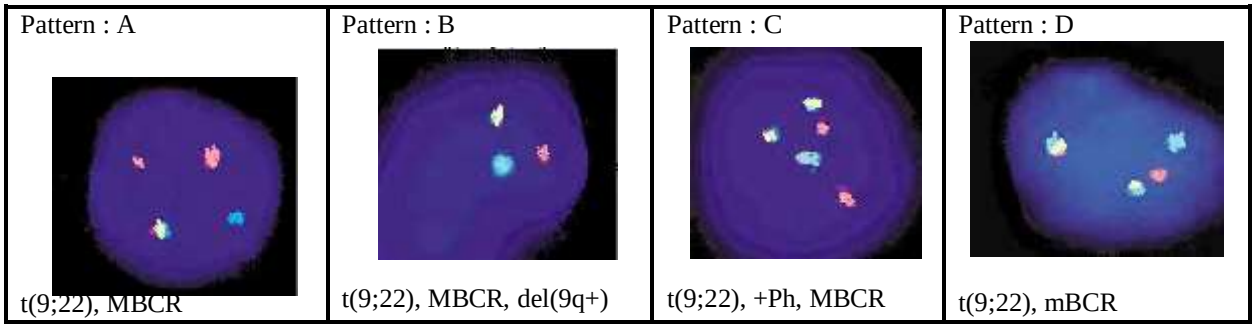
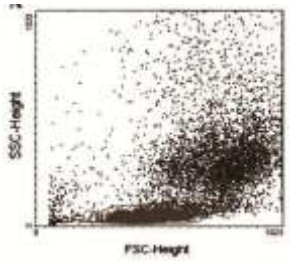



Photo (1): Interpretation of the different FISH patterns

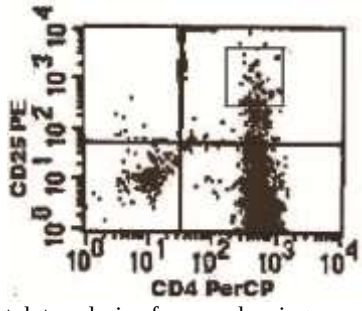
Table (2): Selected clinical and laboratory characteristics of CML patients with typical and atypical Philadelphia(at diagnosis) compared to control group and response to Imatinib.

| Parameters | Group A Atypical Philadelphia (n = 36) | | Group B Sole typical Philadelphia (n= 84) | | | Control (n=100) |
|---|---|---|---|---|---|---|
| | mean±SD | P* | mean±SD | P* | P** | |
| Spleen size (cm) | 15.4± 3.5 | <0.001 | 16.2± 3.1 | <0.001 | 0.215 | 6.8±1.2 |
| HB g/dL | 10.1± 1.8 | 0.001 | 10.7 ±2.1 | <0.003 | 0.137 | 12.2±1.9 |
| TLC x10^9/ul | 93.4±35.6 | <0.001 | 89.2±31.2 | <0.001 | 0.581 | 7.4±2.9 |
| Basophil % | 6.8±2.1 | <0.001 | 5.9±1.7 | <0.001 | <0.014 | 0.3±0.21 |
| Platelets x10^9 /ul | 506.6 ± 97.9 | <0.001 | 488.4 ± 79.7 | <0.001 | 0.287 | 235±57.4 |
| PB Blast % | 7.4±2.3 | --- | 6.8±2.1 | --- | 0.166 | - |
| BM Blast % | 9.5±2.7 | <0.001 | 8.8±2.4 | <0.001 | 0.161 | 0.7±0.3 |
| NAP score | 4.8±1.2 | <0.001 | 5.2± 1.4 | <0.001 | 0.137 | 68±21 |
| CD4+25+$^{high}$foxP3+% | 1.97±0.4 | <0.001 | 1.82±0.4 | <0.001 | 0.080 | 0.82±0.27 |
| BCR-ABL/G6PDH % | 32.9 □10.7 | --- | 35.5 □11.2 | --- | 0.240 | - |
| | N (%) | | N (%) | | | P |
| Phase of disease<br>Chronic phase (86)<br>Accelerated phase(34) | 16 (44.4 %)<br>20 (55.6%) | | 70(83.3%)<br>14(16.7%) | | | <0.001 |
| Sokalscore<br>Low (39)<br>Intermediate(35)<br>High (46) | 5(7.7%)<br>11(30.8%)<br>20 (61.5%) | | 34(40.5%)<br>24(28.6%)<br>26(30.9%) | | | 0.008 |
| Response to Imatinib(after one year)<br>CMR (75)<br>NCMR(45) | 15 (41.7.5%)<br>21 (58.3%) | | 60(71.4%)<br>24(28.6%) | | | 0.033 |

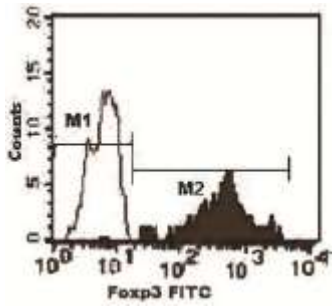
CMR complete molecular response, NCMR complete molecular response not achieved
*against controls, **between patients groups
Atypical Philadelphia mean those had t(9;22) MBCR del(9q) and t(9;22) MBCR ,+ Ph.




Forwardand side scatter histogram was used to define thelymphocytes.




Dotplot analysis of a case showing co-expression of CD4+CD25$^{high}$.




Histogram analysis of FoxP3 expression

Fig. (1): Analysis of the percentages of CD4+CD25$^{high}$ that express FoxP3 .

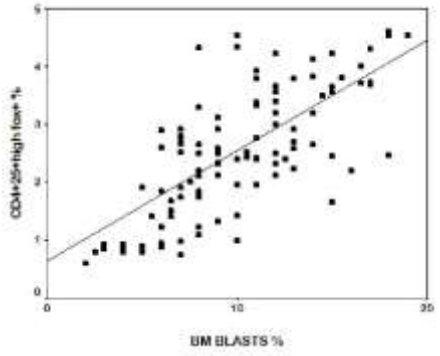



Fig. (2): A significant positive correlation between BCR- ABL/G6PDH% ratio and Tregs%. (p=0.015).

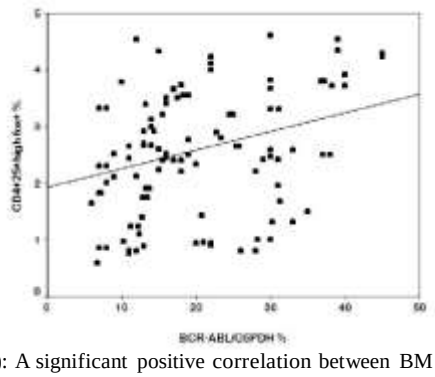



Fig. (3): A significant positive correlation between BM Blast cells and Tregs%. (p <0.001).

**Table (3): Comparison of the Tregs percentage in patients as regard staging, phase of disease, cytogenetics and responder to Imatinib.**

| Variable | N | Regulatory T cells percentage (At diagnosis) mean±SD | P |
|---|---|---|---|
| Sokal score (n=120) | | | |
| Low | 39 | 1.23± 0.36 | |
| Intermediate | 35 | 2.16±0.49 a | 0.024 |
| High | 46 | 2.37± 0.61a | |
| Phase of disease (n=120) | | | |
| Chronic | 86 | 0.92±0.31 | 0.038 |
| Accelerated | 34 | 2.31±0.71 | |
| Cytogenetic (n=120) | | | |
| t(9;22), MBCR | 84 | 1.80± 0.42 | |
| t(9;22), MBCR del(9q) | 26 | 1.97 ± 0.47 | 0.592 |
| t(9;22), MBCR ,+ Ph | 10 | 2.01 ±0.59 | |
| t(9;22), MBCR (n=84) | | | |
| Responder to Imatinib | 60 | 1.78± 0.43 | 0.710 |
| Non responder Imatinib | 24 | 1.82± 0.48 | |
| (9;22), MBCR del(9q) (n=26) | | | |
| Responder to Imatinib | 10 | 1.91± 0.39 | 0.886 |
| Non responder Imatinib | 16 | 2.03± 0.61 | |
| t(9;22), MBCR,+ Ph(n=10) | | | |
| Responder to Imatinib | 5 | 1.98± 0.50 | 0.935 |
| Non responder Imatinib | 5 | 2.04± 0.52 | |

Responder = those showed (CMR) complete molecular response later onafter 1 year of therapy..
a= significant with the first group.

**Table (4): Comparison of the Tregs percentage in patients groups before and after one year of therapy.**

| Variable | N | Percentage of Tregs Cells mean±SD | | P |
|---|---|---|---|---|
| | | Before therapy | After therapy | |
| (9;22), MBCR Responder | 60 | 1.78 ± 0.43 | 0.46±0.18 | <0.001 |
| t(9;22), MBCR Non responder | 24 | 1.82 ± 0.48 | 1.76±0.32 | 0.761 |
| (9;22), MBCR del(9q) Responder | 10 | 1.91± 0.49 | 0.64±0.21 | 0.011 |
| t(9;22), MBCRdel(9q) Non responder | 16 | 2.03± 0.61 | 0.68±0.22 | 0.046 |
| (9;22), MBCR, + Ph Responder | 5 | 1.98± 0.50 | 0.34±0.11 | 0.012 |
| t(9;22), MBCR,+ Ph Non responder | 5 | 2.04 ± 0.52 | 0.31±0.12 | 0.011 |

Responder = those showed (CMR) complete molecular response later on after 1 year of therapy.

**Table (5): Comparison of Tregs percentage in(9;22), MBCR patients groups with and without del(9q) at remission (responders) and after progression**

| Variable | Tregs % | | | | P |
|---|---|---|---|---|---|
| | At remission n | mean±SD | After progression* n | mean±SD | |
| t(9;22), MBCR | 60 | 0.46±0.12 | 12 | 1.45±0.42 | 0.003 |
| t(9;22), MBCR , del(9q) | 10 | 0.64±0.21 | 5 | 1.02±0.33 | 0.331 |
| t(9;22), MBCR,+ Ph | 5 | 0.34±0.11 | 3 | 0.98±0.36 | 0.077 |

progression* mean patients had accelerated or crisis phase after remission during 2 years follow up.

**4. Discussion**

Patients with CML display considerable clinical heterogeneity during the chronic phase of the disease. The molecular basis for this variability remains obscure. The pathogenic consequences of an apparently "simple" translocation may frequently be more complex than previously realized.In our study, we found del-der(9) in 21.7% of the patients these group showed a significant higher Sokal score and advanced stage with more resistance to Imatinib therapy compared to those who had sole typical MBCR while, supernumerary Philadelphia chromosome was found in 8.3% these results near to that reported in other studies[14-16].

There are multiple mechanisms involved in resistance to therapy, which could impact on disease progression. These include expression of the MDR-1 gene[17], reduplication of BCR-ABL[18], decreased apoptosis and possibly defective drug transport[19].

The researchers suggest that regulatory T cells (Tregs) are defined by expression of the fork head transcription factor family FoxP3. Expression of FoxP3 is required for regulatory T cell development and suppressive function [20].

In this study, FoxP3 has been shown to be expressed on $CD4^{+}$ $CD25^{high}$ only. Fontenot et al. [21] reported that FoxP3 is a unique marker of $CD4^{+}CD25^{+}$ regulatory T cells, distinguishing them from activated $CD4^{+}CD25^{+}$T cells .

We found that Tregs percentage was significantly increased in patients group compare to control ($p<0.001$) and slightly increased in patients group with atypical Philadelphia compare to those with typical sole MBCR group but hasn't touched the significant level. The possibilities of the increased proportion of Tregs in malignancy include recruitment and/or expansion from the circulating Tregs population through production of chemokine CCL22 another explanation is that the regulatory phenotype is induced from conventional T cells within the tumor microenvironment[22], Curti et al. [23] showed that AML cells induce CD25+Treg cells from CD25-cells via modulation of tryptophan catabolism.

CML cells can be targeted by cytotoxic T lymphocytes as CML cells display tumor-specific peptides on the cell surface. In spite of this, CML cells persist in patients. Tregs may play a role in limiting this existing immunity in CML in some patients. It remains unclear whether this increase in Tregs suppresses some of the immune control of CML resulting in an increased of the CML burden, or is only a consequence to the expansion of CML cells [24].

Bach and Francois, [25] reported that the mechanism by which Tregs suppress other T cell responses may involve cytokines through enhance TGF-β, TNF and suppress IL2 production also, Rojas et al. [26] found that Tregs inhibited the T-cell production of granzyme-B to a HLA-A3 restricted BCR-ABL peptide. These data suggest that Tregs can inhibit anti-CML immune responses there by enable cancer cells to evade immune surveillance, and might contribute to the progression of human tumors [27].

By analysis of the percentage on $CD4^{+}$ $CD25^{high}$ FoxP3% we found a significant increase of these cells in patients in high and intermediate Sokal score compare to those in low stage and in accelerated phase compared to those in chronic stage these results go hand in hand with that reported by Zahranetal. [28] but no significant difference as regards cytogenetics variants and response to Imatinib in each group.

We found a significant positive correlations between the percentage of Tregs with BCR-ABL/G6PDH% level which indicates that it has an immune modulating effect may be important in the progression of CML also Tregs % is positively correlated with BM blast cells % again this may indicate that higher levels of Tregs are associated with poor course of the disease or transformation into accelerated phase or blast crisis. Similar results have been described by Hus et al. [29].

Imatinib exerts its effects through two main ways and both are needed. The first by competing for ATP binding of the BCR/ABL kinase so inhibit phosphorylation of the downstream signaling proteins required for tumor-cell survival and proliferation. The second by modulating the immune response through activated CD8+ T cells and induced Tregs apoptosis[30].

We found a significant decrease in Tregs% numbers in patients who achieved CMR in both typical and atypical Philadelphia patients groups, while in non responder groups it is significantly decreased only in patients with atypical Philadelphia.

In CML patients with atypical Philadelphia, the proliferation capacity and tumor burden are increased, so the stander dose of Imatinib may be not effective, either due to supernumerary Philadelphia chromosome or due to loss of one or more of tumor suppressor genes within the deleted region on der9 like Gene producing GTPase activating protein which binds and inhibits p21racactivity [31]. Another two known genes, the ribosomal RNA processing protein 4 (RRP4) and the positive regulatory (PR) domain zinc finger PR which appear to function as negative regulators of oncogenesis [32]. So enhances the antitumor effect through suppression of Tregs seems to be not efficient in those patients with atypical Philadelphia that is why in non responder groups of those patients, Tregs% in spite of being significantly decreased they were still non responder and the patients had progression during follow up period not

showed any significant increase in Tregs% while it was significantly increased in patients with standard sole MBCR.

That this why Quintas-Cardama et al.[33] recommended for higher-dose Imatinib therapy to neutralizing effect on the poor prognosis of patients with CML who have del-der(9).

In conclusion normal hosts contain effector T cells whose activation and expansion is suppressed by the presence of Tregs which may play a role in the pathogenesis and progression of CML, so Tregs might have a clinical value in evaluating the effects of therapy specially in patients with typical Philadelphia while, in patients had atypical Philadelphia they may need different treatment modalities as, in spite of significant decrease of Tregs%, a group of them are still non responder. This conflict needs more studies with different Imatinib doses and with other treatment strategies.

It is recommended to evaluate such atypical Philadelphia into the routine evaluation of all CML cases as it will be necessary to be considered when analyzing future clinical trials of both conventional and novel therapies.

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