ABSTRACT: Despite the efficacy of imatinib mesylate (IM) in treating chronic myeloid leukemia (CML), there is a high degree of resistance. Alpha-1-acid glycoprotein may reduce drug efficacy through its ability to interact with IM and blocks it from reaching its target, while protein glycoprotein (PGP) may reduce the intracellular concentration of the drug via an active pump mechanism. We thus investigated the correlation between AGP and PGP levels and the resistance/response to treatment. A total of 26 CML patients were investigated for AGP and PGP levels at diagnosis and during treatment. There was no significant difference or correlation between AGP levels and the different groups of patients. There was also no significant difference in the fluorescence intensities of PGP levels among the different patient groups. The resistance observed in our CML patient population could not be correlated with AGP and PGP levels. There was no significant pattern of AGP and PGP expression, irrespective of the response or resistance to treatment.

KEYWORDS: CML, drug resistance, binding protein, AGP, PGP, Qatar

Introduction

Although imatinib mesylate (IM) was shown to be efficacious in treating chronic myeloid leukemia (CML) patients, an increasing rate of resistance has been reported not only for IM but also subsequently for other generations of tyrosine kinase inhibitors (TKIs). We previously reported a high rate of resistance to IM that reached up to 54% in CML patients in Qatar. We investigated the possible mechanism of this high rate of resistance through a series of experiments and reported the impact of mutations/single-nucleotide polymorphisms (SNPs) and additional chromosomal abnormalities (ACAs) on CML resistance to IM. While mutations/SNPs explained only 8% of resistant cases, ACAs were the most common cause of treatment failure in 23% of our CML patient cohort; intolerance, on the other hand, was further noticed in 8% of patients, and the mechanisms of resistance remained unknown in 15% of patients. Here, we report on our investigation of alpha-1-acid glycoprotein (AGP) and protein glycoprotein (PGP) as the possible causes of CML resistance to IM treatment.

Alpha-1-acid glycoprotein. The human serum AGP is predominantly synthesized in the liver as a single-chain glycoprotein that belongs to the family of acute-phase reactants and is elevated in various physiological and pathological conditions. The native protein with a molecular weight of 40,000 has a low isoelectric point, resulting in a negatively charged molecule at physiological pH.

AGP is mainly known to bind basic and neutral drugs such as IM to prevent them from binding their targets. AGP is known to reduce drug efficacy through its ability to interact with IM and blocks it from reaching its target. AGP may reduce the intracellular concentration of the drug via an active pump mechanism. We thus investigated the correlation between AGP and PGP levels and the resistance/response to treatment. A total of 26 CML patients were investigated for AGP and PGP levels at diagnosis and during treatment. There was no significant difference or correlation between AGP levels and the different groups of patients. There was also no significant difference in the fluorescence intensities of PGP levels among the different patient groups. The resistance observed in our CML patient population could not be correlated with AGP and PGP levels. There was no significant pattern of AGP and PGP expression, irrespective of the response or resistance to treatment.
resistance via reducing the intracellular concentration of IM, and its overexpression has been frequently implicated in tumor resistance to different chemotherapeutic drugs.

This protein carries out an adenosine triphosphate (ATP)-dependent efflux of structurally diverse lipophilic compounds and expels anticancer chemotherapy agents out of cells.

Various authors demonstrated that IM is a substrate of PGP and that some aspects of resistance to IM treatment may therefore be caused by an increased PGP activity.

In vitro generated models of resistant CML (LAMA84-R) cell lines showed overexpression of PGP and BCR-ABL1.

Therefore, we also investigated the correlation between PGP levels and the high resistance to IM treatment in our CML patients.

**Design and Methods**

**Patient recruitment.** Patients aged 16–65 years with Philadelphia chromosome positive (Ph+) CML for at least 12 months and receiving only IM treatment during November 2006 to December 2011 were included in the study. Patients gave their written, informed consent to participate, and blood samples were collected. The study was conducted in accordance with the principles of the Declaration of Helsinki, and approved by Hamad Medical Corporation Research committee (HMC research proposal no. 400/06).

**Treatment regimen.** Patients in the chronic phase (CP) received 400 mg of IM orally once daily, while patients in the accelerated phase (AP) received 600 mg once daily as per the European LeukemiaNet (ELN). Patients’ response to IM was assessed according to the ELN 2006, 2009, and 2013 guidelines as described previously.

**Assessment of patients’ response.** Patients’ responses based on the absolute quantification of BCR-ABL1 were assessed as reported earlier. The BCR-ABL1 kinase domain mutations and ACA results are reported elsewhere.

**AGP and PGP studies.** The levels of AGP and PGP were measured in patients treated with IM and compared with 10 healthy individuals as a control group.

**AGP levels.** Serum samples were obtained from patients at diagnosis and during treatment. Determination of serum AGP levels was carried out by immunoturbidimetric analysis in the COBAS INTEGRA® 400 system using the cassette COBAS INTEGRA® alpha 1 acid glycoprotein. This assay is based on the formation of an AGP precipitate with a specific antiserum that could be measured turbidimetrically at 340 nm.

Precinorm and precipath proteins were used as quality control for monitoring the accuracy and precision of the machine.

The machine was calibrated for the detection of AGP using Calibrator For Automated Systems (C.F.A.S) protein (normal range 0.5–1.20 g/L).

Pearson correlation coefficient was used to examine the linear relationship between AGP, platelets (PLTs), white blood cells (WBCs), absolute basophils (Abs. Baso), and lactate dehydrogenase (LDH) during different stages of the disease.

**PGP levels.** Peripheral blood and bone marrow samples were collected in ethylenediaminetetraacetic acid (EDTA) to study the PGP levels. Total WBCs were collected and washed in 1x phosphate-buffered saline (PBS) and adjusted to 5 x 10^6 to 2 x 10^6 cells/μL in PBS. Then, 100 μL of cell suspension was incubated for 15 minutes at room temperature with 20 μL 7-aminoactinomycin D (7AAD), 20 μL monoclonal antibody (MAb) CD243-phycocerythrin (PE) conjugated, 20 μL CD34-fluorescein isothiocyanate (FITC), and 10 μL CD45 phycocerythrin-Texas Red conjugate (energy coupled dye) ECD (Beckman Coulter). For negative control, cells were incubated with 10 μL Immunoglobulin IgG2a-phycocerythrin-cyanine5 conjugate PC5, 20 μL mouse IgG2a-PE, 20 μL IgG1-FITC, and 10 μL IgG2-ECD (Beckman Coulter).

To further purify the sample, red blood cells were lysed using VersaLyse reagent (Beckman Coulter), by adding 1 mL of red blood lysis buffer and incubating at room temperature for 10 minutes. Cells were then centrifuged twice at 300 x g for five minutes, supernatant was discarded, and the WBC pellet was resuspended in 0.5 mL PBS.

Lyophilized human lymphocytes were used to establish and adjust four-color compensation settings, and PGP expression was gated on viable WBCs.

The samples were processed in an FC 500 flow cytometer (Beckman Coulter), and data were collected using four colors, one laser protocol in List Mode data files. 7AAD was measured on FL4 detector, PGP labeling was measured on FL2 detector, CD34 was measured on FL1 detector, and CD45 was measured on FL3 detector. Forward scatter and side scatter (SSC) were collected using linear scales, and fluorescence signals were collected using logarithmic scales. Data acquisition and analysis were performed on 10,000 viable cells with the CXP analysis software package (Beckman Coulter).

The logarithmically amplified signals were converted to linear values, which refer to the dynamic range of signal intensities (1–10,000). The differences in fluorescence intensity between isotypic controls and test samples were measured. PGP results were expressed as a ratio of arithmetic mean fluorescence intensity of CD243 MABs/arithmetic mean fluorescence intensity of isotypic control. This technique was optimized and adopted from University College London protocols.

**Results**

**Patients recruited into the study.** The clinical profiles of patients were reported elsewhere.

A total of 40 patients with CML presented to the National Centre for Cancer Care and Research (NCCCR) between November 2006 and December 2011. Among them, 33 patients (27 males, 6 females, median age 40.5 years (range: 16–62 years); 28 patients in CP and 5 in AP) met the inclusion criteria and were recruited into the study.

Due to multiethnic nature of Qatar, 7 patients were lost to follow-up because they traveled back to their home countries during the first three months of diagnosis, leaving...
only 26 patients in the study. Of the 26 patients, 22 (P1–P8, P10–P22, P25, and P31) were in CP and 4 (P5, P9, P23, and P26) in AP.

Twelve patients (P1, P2, P5, P7, P9, P10, P14, P17, P20, P23, P25, and P26) responded optimally, and 14 patients (P3, P4, P6, P8, P11–P13, P15, P16, P18, P19, P21, P22, and P31) failed treatment (failure and suboptimal response), as reported previously. Of the 14 patients who failed treatment, 12 (P3, P7, P8, P12, P13, P15, P16, P18, P19, P21, P22, and P31) showed a strong correlation between AGP and LDH (P = 0.0001), WBCs (P = 0.002), and Abs. Baso (P = 0.03; Table 1).

Molecular monitoring and patients’ response. Patients were followed up every 3 months until the end of the study, and all the 12 responders achieved a major molecular response (MMR) at 12 months onward.

On the other hand, of the 14 patients who failed treatment, 8 did not achieve MMR and 6 had primary resistance to IM and showed neither hematological nor cytogenetic response. These 14 patients were switched to next-generation TKIs, and no further data could be collected due to ethical approval limitations.

AGP results.

At diagnosis. Of the 22 CP patients, 11 (P1–P4, P6–P8, and P10–P13) had elevated AGP (mean 1.2 (±0.12) g/L) and 3 (P5, P9, and P23) of the 4 AP patients had elevated AGP (mean 1.61 (±0.38) g/L; Fig. 1).

During follow-up. The mean AGP level among the 14 patients who failed treatment (resistant group) was 1.05 (±0.09) g/L, while the mean level for the 12 patients who responded optimally (optimal responders group) was nonsignificantly higher at 1.1 (±0.06) g/L (Fig. 1).

Of the 14 resistant patients who had mutations/ACAs, 8 (P3, P7, P8, P12, P19, P21, P22, and P31) showed a mean AGP level of 1.06 (±0.09) g/L, while 6 (P4, P11, P13, P15, P16, and P18) of the 14 patients with no mutations/ACAs showed no significant difference in the AGP level of 1.04 (±0.08) g/L.

There were also no significant differences between AGP levels in six patients (P7, P8, P12, P19, P21, and P3) who had ACAs and the two patients (P3 and P22) who had the mutations.

The mean AGP level of healthy individuals was 0.75 g/L (0.47–1.0 g/L). This level was significantly lower when compared with different patient groups (Fig. 1). However, among the different groups of patients, there were no significant differences.

Nonetheless, the group that failed treatment showed a strong correlation between AGP and LDH (P = 0.0001), WBCs (P = 0.002), and Abs. Baso (P = 0.03; Table 1).

PGP results. The mean PGP levels were 1.25 (±0.06), 1.17 (±0.02), 1.21 (±0.03), and 1.2 (±0.04) g/L for AP, CP, optimal responders, and resistant group, respectively. Flow cytometric analysis showed no significant difference in the fluorescence intensities of blast cells incubated with CD243 and blast cells incubated with isotropic control among the different diseased groups. (Figs. 2 and 3).

There was also no significant difference in the fluorescence intensities of blast cells between patients who had ACAs and patients who had SNPs/mutations.

Table 1. Relationship between AGP and other markers in CML patients.

| AGP LEVEL | CORRELATION COEFFICIENT | BIOMARKERS |
|-----------|--------------------------|-------------|
| CML GROUPS | SIGNIFICANT CORRELATION | PLTs | WBCs | Abs. Baso | LDH |
| CP | r = −0.56, P = 0.19 | 0.52 | 0.39 | 0.35 |
| AP | r = −0.3, P = 0.61 | 0.1 | 0.7 | 0.04 |
| Resistant group | r = 0.47 | 0.7 | 0.53 | 0.86 |
| Optimal responders | r = −0.069 | 0.47 | 0.13 | 0.07 |

Notes: Pearson correlation of AGP and PLTs, WBCs, Abs. Baso, and LDH. No correlation could be identified between AGP and other biomarkers of CML patients in the group that responded optimally to treatment (optimal responders). However, the group that failed treatment (resistant group) showed a strong correlation between AGP and LDH (P = 0.0001), WBCs (P = 0.002), and Abs. Baso (P = 0.03; Pearson correlation coefficient (r), significant correlation values (P)).
Discussion and Conclusion

AGP is an acute-phase protein that has been proposed to promote CML resistance to IM. In this study, the protein levels were correlated with different stages of the disease and did not show any significant correlation between AGP and disease response or progression. Our results comply with those reported by Jorgensen et al and le Coutre et al who showed that supraphysiological levels of AGP did not result in CML resistance to IM in animal models.

Our results showed a moderate-to-strong correlation between AGP and other CML markers, namely, WBCs, Abs. Baso, and LDH, suggesting that AGP as an acute-phase protein might behave as a marker that reflects disease status rather than being a cause of resistance to treatment. The correlation between AGP and PLTs might become significant in larger cohorts of patients.

On the other hand, PGP expression did not show any correlation with the disease stage or other markers of disease. Our results in this regard comply with the study carried out by Weisberg and Griffin.

There was a significant difference in the AGP levels between the patient group and the control group; however, there was no significant difference in AGP levels among the different groups of patients.

Combining AGP with other markers showed significant correlations during different disease stages that may be employed as a warning sign for resistance of CML to TKIs.

PGP expression, on the other hand, did not differ in patients presenting in CP or AP and showed no specific pattern among those who resist or respond to IM treatment.

Figure 2. Mean results of PGP/control ratio of different CML groups. Notes: The mean PGP expression levels were 1.25 (±0.06), 1.17 (±0.02), 1.21 (±0.03), and 1.2 (±0.04) for AP, CP, optimal responders, and resistant groups, respectively.

Figure 3. PGP expression using flow cytometry. Notes: (A) Differentiation of WBC population using CD45 ECD antibody/SSC, (B) discrimination of viable and nonviable cells by 7AAD antibody/SSC, (C) CD243 on viable blast cells, (D) isotypic control on blast cells, and (E) difference between the fluorescence intensities of blast cells incubated with CD243 and blast cells incubated with isotypic control (CD243/control ratio).
The resistance observed in our CML patient population could not be correlated with AGP and PGP levels. There was also no significant pattern of AGP and PGP expression, irrespective of the response or resistance to treatment.

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Author Contributions
Designed the project, carried out the experiments, interpreted and analyzed the results, and wrote the manuscript: NIA-D. Provided clinical data: MAY. Supervised the progress of experiments: HMM. Critically revised the manuscript: HMM, APJ and MAY. All the authors read and approved the final manuscript.

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