Anti-Interferon Alpha Antibodies in Patients with High-Risk BCR/ABL-Negative Myeloproliferative Neoplasms Treated with Recombinant Human Interferon-α

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Background: The objective of this study was to characterize the incidence and impact of immunogenicity to interferon-α (IFN-α-2a, IFN-α-2b, and Peg-IFN-α-2a) over a period of 12 months in patients with BCR/ABL-negative myeloproliferative neoplasms (MPNs).

Material/Methods: A total of 131 patients from an observational prospective cohort were selected. Antidrug antibodies, in serial serum samples obtained monthly after initiation of therapy, were measured by ELISA and WISH/VSV CPE assays. The association between antidrug antibodies and treatment response and adverse effects was evaluated.

Results: Among patients who completed 12 months of follow-up, binding antibodies (BAbs) were detected in 53% of those receiving IFN-α (69 of 131) and neutralizing antibodies (NAbs) were detected in 19% (25 of 131). NAbs-positivity was correlated with poorer clinical response, and Bab-positivity was associated with more adverse events. Almost all BAbs and NAbs appeared within 8 months after treatment began (≥95%). Complete remission (CR) rate was 62% for patients who were BAbs-positive and 69% for patients who were BAbs-negative; however, the CR rate of patients with NAbs(+) (24%) was obviously lower than in patients with NAbs(−) (75%). Patients with BAbs(+) had more immune adverse effects (including fever, myalgia, skin reaction, and stomatitis) than BAbs(−) patients, and NAbs to IFN-α had no obvious influence on the adverse effects rate.

Conclusions: The development of BAbs and NAbs can adversely affect IFN-α treatment in patients with MPN.

MeSH Keywords: Immunogenicity Interferon-alpha • Interferon-alpha • Myeloproliferative Disorders

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Interferon-α (IFN-α) and its pegylated form (peg-IFNα) have a long history of efficacy in the treatment of hematological malignancies, especially in patients with high-risk BCR/ABL-negative myeloproliferative neoplasms (MPNs). Early studies demonstrated that IFN-α treatment can improve the blood count and reduce JAK2V617F mutant allele burden in patients with MPN [1–4]. Although IFN-α clearly has therapeutic efficacy in MPN, treatment with IFN-α is far from ideal, and remains an experimental MPN therapy at present [5]. Not all MPN patients achieve a complete remission (CR) in response to IFN-α treatment. One explanation for the poor efficacy of IFN-α therapy is immunogenicity leading to the development of antidrug antibodies.

Antibodies to IFN-α were originally described in 1981 by Vallbract, who observed the appearance of neutralizing anti-IFN-α antibodies (NAbs) during IFN treatment in a patient with nasopharyngeal carcinoma [6]. Since then, the development of anti-IFN antibodies has been considered a common clinical problem in patients with several diseases treated with IFN-α, including leukemia, multiple sclerosis, and chronic hepatitis B/C [7,8]. IFN antibodies are detected in patient sera either by their capacity for binding IFN (binding antibodies, BAbs) or, more importantly, by their neutralizing effects (NAbs). BAbs may bind to several different antigenic epitopes of the IFN molecule, some of which are not involved in IFN activation. Anti-IFN NAbs bind to IFN and interfere with its biological activity by inhibiting the interactions between IFN and its receptor. NAbs may be responsible for IFN treatment failure. Several studies suggest that, among chronic hepatitis C patients receiving IFN-α, anti-IFN-α NAbs develop more frequently in non-responsive patients than in the responders [9–11]. Previous studies demonstrated that the presence of antibodies against IFN reduces the response to treatment and increases the risk of treatment discontinuation. There are fewer reports about anti-IFN antibodies in MPN patients receiving IFN-α.

The aims of this study were to determine the presence of antidrug antibodies in a relatively homogeneous group of MPN patients treated with 3 different IFN-α preparations (IFN-α-2a, IFN-α-2b, and Peg-IFN-α-2a), and to gain new insight into the biological and clinical significance of antibodies to IFN-α in MPN patients.

**Material and Methods**

**Patients**

Patients diagnosed with polycythemia vera (PV), essential thrombocythemia (ET), and myelofibrosis (MF) according to World Health Organization criteria [12], with normal cardiac, renal, and liver function, and without history of autoimmune disease, were eligible for this study. The serum levels of anti-IFN antibodies were measured in 131 patients with MPN who received treatment with IFN-α from January 2015 to May 2017 at Inner Mongolia People’s Hospital. Of these patients, 49 patients were treated with IFN-α-2a, 45 were treated with IFN-α-2b, and 37 were treated with Peg-IFN-α-2a. The patients agreed to participate in the research studies and provided written informed consent. The measurements of serum anti-IFN-α antibodies and blood cell counts in MPN patients were approved by the Ethics Committees of Inner Mongolia People’s Hospital review boards in accordance with the Declaration of Helsinki.

Prognosis and response were assessed using the International Working Group for MPN criteria. Response classifications included: complete response (CR), partial response (PR), or no response (NR) [13,14]. Toxicity was evaluated using the National Cancer Institute Common Terminology Criteria for Adverse Events (version 4.0).

**Study procedures**

IFN-α-2a and IFN-α-2b were administered at 3×10⁶ IU subcutaneously 3 times a week, and Peg-IFN-α-2a was administered at 90–180 micrograms per week subcutaneously. Before starting therapy and monthly thereafter for at least 12 months, patient sera were obtained and monitored for the presence of anti-IFN-α antibodies. Sera were collected 36–48 h after IFN-α administration to eliminate any residual IFN activity. Sera were stored at −20°C in small aliquots until analysis.

**Detection of binding antibodies against IFN-α**

Anti-IFN BAbs were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) Kit (Invitrogen, ThermoFisher Scientific, CA), following the manufacturer’s instructions. The test consists of a solid-phase enzyme immunoassay-based sandwich system. Briefly, recombinant IFN-α was adsorbed onto microwell surfaces. The anti-IFN-α present in the sample or standard bound to the capture protein adsorbed in the microwells. A HRP-conjugated recombinant IFN-α protein was added and the captured antibodies from the samples (or standards) bound to the conjugate by their remaining free combining site. After washing, enzyme substrate solution reactive with HRP was added to the wells. A color product was formed in proportion to the amount of anti-IFN-α present in the sample or standard. The reaction was terminated by addition of acid and absorbance was measured at 450 nm.

**Detection of neutralizing antibodies against IFN-α**

The anti-IFN NAbs in patient sera were measured by antiviral cytopathic effect (CPE) using WISH cells and the vesicular stomatitis...
virus (WISH/VSV CPE) according to previously reported methods [11,15]. A positive result was defined as the ability to neutralize the antiviral activity of 10 IU of recombinant IFN-α2a or IFN-α2b. Sera were inactivated for 30 min at 56°C. IFN-α was added at 37°C for 1 h. After 18 h, cells were exposed to VSV at 37°C for 24 h. Absorbance was measured at 590 nm to determine cell viability [16]. We used the dilution of serum that reduces 10 laboratory units (LU) per mL of IFN to 1 LU/mL [17].

Statistical analysis

Differences were analyzed by nonparametric tests (Pearson chi-squared test). Analyses were performed using SPSS (Version 19.0.0, IBM Corp®). Significance was set at p<0.05.

Results

Clinical characteristics of patients

The characteristics of the 131 patients at baseline are shown in Table 1. The male (73)-to-female (58) ratio was 1.25 to 1. The median time from diagnosis to beginning of treatment with IFN-α was 26 months (range 0–65). A large proportion of patients were Jak-2 mutation-positive (87 patients, 66%), with the largest contribution from the PV subgroup (60 patients, 95%). None of 63 PV patients, 9 of 43 (21%) ET patients, and 8 of 25 (32%) MF patients carried mutations in the calreticulin (CALR) gene. Only 1 MF patient had MPL mutation. Splenomegaly was present in 43 patients (33%) of the total MPN cohort, with 68% of MF patients presenting this clinical characteristic. Using the International Prognostic Scoring System (IPSS) Risk Group for MF patients, 8 patients (32%) were low risk, 12 patients (48%) were intermediate risk, and 5 patients (20%) were high risk. Five patients (20%) developed MF after PV/ET. At baseline, hemoglobin in the PV group (mean ±SD, 178±22.3) was higher than in other groups; platelet counts in the ET group (mean ±SD, 772±390.3) were higher than in the PV and MF groups; but there were no significant differences in the leukocyte counts among the MPN subgroups. Some of the patients previously underwent phlebotomy or were treated with hydroxyurea.

The relationship between baseline characteristic and the presence of antibodies

Of the 131 MPN patients treated with IFN-α, 69 (52.3%) developed BAbs, whereas 25 out of 69 BAbs-positive patients tested NAbs-positive. NAbs was not detected in BAbs-negative patients. Patients treated with IFN-α2a (71.4%) tested positive for BAbs more than patients treated with IFN-α2b (33.3%) or Peg:IFN-α2a (51.4%) (p<0.01). However, no differences in the incidence of NAbs were detected among the 3 treatment groups (Table 2). IFN-α treatment duration in each group was not significantly different. Thus, the differences in frequency of antibody production during therapy cannot be attributed to the amount of IFN-α given. Baseline clinical characteristics did not differ between antibody-positive and -negative groups (p>0.05). Thus, antibody development was not predictable on the basis of baseline characteristics.

Time pattern of antibodies production by IFN-α

Antibodies to IFN-α were not present in any patients before initial treatment (0 month). As time from beginning of treatment increased, more and more patients developed antibodies. Almost all BAbs (Figure 1A) and NAbs (Figure 1B) appeared within 8 months (±95%), with the fastest growth occurring between 3 to 6 months, and no new antibody development from 10 to 12 months after treatment began. The presence of new antidrug antibodies was rare after 12 months without further monitoring. There was no significant difference in the time of BAbs and NAbs development among the 3 drug treatment groups.

Association between hematologic response and antidrug antibodies to IFN-α

The median follow-up time for the entire cohort was 16 months (range, 12–23 months). All patients were evaluated for response. Similar rates of hematologic responses were observed in patients with BAbs(+) and BAbs(–) (Figure 2A). The overall CR rate was 62% for patients with BAbs(+) and 69% for patients with BAbs(–), respectively. However, there were significant differences in the effect of IFN-α treatment between NAbs(+) and NAbs(–) (Figure 2B). The CR rate of the NAbs(+) patients (24%) was obviously lower than patients that were NAbs(–) (75%). The presence of BAbs had less influence on the CR rate, but the prevalence of NAbs resulted in poor clinical response to IFN-α treatment.

Association between molecular response rates and antidrug antibodies to IFN-α

Unlike hematologic response, which was rapidly achieved in most patients, the achievement of meaningful molecular responses required exposure to IFN-α for at least 6 months. The median follow-up time for the entire cohort was 16 months (range, 12–23 months). JAK2V617F was detected in 87 of the 131 patients treated (60 with PV, 17 with ET and 10 with MF). The overall molecular response rate was 59% for BAbs(+) and 37% for NAbs(+) (Figure 3). The NR rate of the NAbs(+) patients (63%) was significantly higher than patients that were NAbs(–) (34%). The presence of BAbs had less influence on the JAK2V617F mutant allele burden, but the prevalence of NAbs resulted in poor clinical response to IFN-α treatment.
The relationship between the frequency of antibody development and the adverse effects of IFN-α

Eighty-five percent of patients developed some adverse effects in response to IFN-α treatment, but the adverse effects were generally grade 1 or 2. No patient had a grade 4 adverse event. The most frequent adverse event was fever, which occurred in 40% of patients (Table 3). Patients with BAbs showed more immune adverse effects, including fever, myalgia, skin reaction, and stomatitis. The occurrence of antidrug antibodies had no significant effect on hematologic and other non-hematologic toxicity. Interestingly, the NAbS to IFN-α had no obvious influence on the incidence of adverse effects.

Table 1. Baseline characteristics in 131 myeloproliferative neoplasm patients treated with interferon-α.

| Characteristic                  | PV (n=63) | ET (n=43) | MF (n=25) |
|--------------------------------|-----------|-----------|-----------|
| **Age (years)**                | 53.4±13.5 | 47.2±11.2 | 53.0±11.7 |
| Mean ±SD (range)               | (19–73)   | (29–68)   | (40–72)   |
| **Gender**                     |           |           |           |
| Male                           | 37 (59%)  | 25 (58%)  | 11 (44%)  |
| **Time from diagnosis (months)** | 24 (0–52) | 36 (0–65) | 20 (0–36) |
| **Positive JAK2V617F**         | 60 (95%)  | 17 (40%)  | 10 (40%)  |
| **JAK2V617F allele burden, %** |           |           |           |
| Median                         | 56 (18.1–82.3) | 26 (2.8–52.0) | 29 (4.9–48.4) |
| Range                          |           |           |           |
| **Positive CALR**              | 21 (21%)  | 18 (18%)  | 8 (32%)   |
| **Positive MPL**               | 0 (0%)    | 0 (0%)    | 1 (4%)    |
| **Hemoglobin (g/L)**           | 178±22.3  | 124±17.1  | 116±29.0  |
| Mean ±SD (range)               | (154–212) | (96–142)  | (82–147)  |
| **WBC (×10⁹)**                 | 8.2±6.3   | 7.8±5.6   | 7.9±7.1   |
| Mean ±SD (range)               | (3.6–18.4)| (3.5–15.2)| (3.0–20.0)|
| **Platelets (×10⁹)**           | 387.1±96.3| 772±390.3| 403±132.3|
| Mean ±SD (range)               | (102–673)| (470–2002)| (109–1014)|
| **Presence of splenomegaly**   | 19 (30%)  | 7 (16%)   | 17 (68%)  |
| **International Prognostic Scoring System (IPSS)** |           |           |           |
| Risk Group                     | NA        | NA        | Low: 8 (32%) |
|                                |           |           | Intermediate: 12 (48%) |
|                                |           |           | High: 5 (20%) |
| **Post PV/ET**                 | NA        | NA        | 5 (20%)   |
| IFN-α type                     |           |           |           |
| IFN-α-2a                       | 21 (10)   | 18 (10)   | 10 (10)   |
| IFN-α-2b                       | 18        | 16        | 11        |
| Peg-IFN-α-2a                   | 24        | 9         | 4         |
| Prior therapy                  |           |           |           |
| Phlebotomy                     | 42        | 0         | 0         |
| Hydroxyurea                    | 0         | 5         | 5         |
| Previously untreated           | 21        | 39        | 20        |

PV – polycythemia vera; ET – essential thrombocytosis; MF – myelofibrosis; WBC – white blood cell; Peg-IFN-α-2a – pegylated interferon-α-2a; Mean ±SD or patient numbers are indicated in each category.

Discussion

This study shows the prevalence of antibody development in response to IFN-α therapy in patients with MPN. Both binding and neutralizing antibodies were observed during the course of the
clinical trials. We report here that approximately 53% of patients with MPN treated by IFN-α were positive for anti-IFN-α BAbs and 19% of patients were positive for NAbs. This fraction of patients is not negligible. The results of published immunogenicity studies show a wide variation (0–50%) in the reported antidrug antibody frequency, depending on the IFN antibody detection methods and the dose, duration of treatment, underlying disease, and several intrinsic patient factors studied [18]. Furthermore, the presence of free drug may mask the detection of antidrug antibodies due to drug interference. The latter concern may be addressed by performing radioimmunoassay (RIA), which is less susceptible to drug interference compared with ELISAs [19]. At present, an international standard for IFN antibodies does not exist. Therefore, the different incidences of anti-IFN antibody-positive patients in various IFN trials are difficult to compare. Interestingly, the BAbs produced by Peg-IFN-α-2a and IFN-α-2b were lower than that of IFN-α-2a. This seems to explain the data showing that Peg-IFN-α-2a and IFN-α-2b have less toxicity and enhanced tolerability in conjunction with significant hematologic and molecular remission rates [20]. Pegylated products may reduce immunogenicity, as the PEG moiety can mask immunoreactive sites on the native molecule [21,22]. However, several examples of immunogenic responses to pegylated drugs have been reported, including Peg-IFN-α used in the treatment of hepatitis C [23–25]. The development of

Table 2. The characteristics of patients grouped according to the presence of binding and neutralizing antibodies to IFN-α.

| Characteristics            | BAbs                  | NAbs                  |                  |
|----------------------------|-----------------------|-----------------------|------------------|
|                            | Positive (n=69) | Negative (n=62) | P               | Positive (n=25) | Negative (n=106) | P               |
| Gender                     |                       |                       |                 |                |                        |                 |
| Female                     | 30                    | 28                    | 0.85            | 13              | 43                    | 0.30            |
| Male                       | 39                    | 34                    |                 | 12              | 63                    |                 |
| Age (years)                | 50.6±12.2             | 53.6±14.1             | 0.19            | 49.3±11.5       | 52.2±14.7             | 0.36            |
| (31–68)                    | (19–72)               |                       |                 | (31–66)         | (19–72)               |                 |
| Time from diagnosis (months)| 25±10.9              | 27±17.5               | 0.43            | 22±11.3         | 27±21.7               | 0.27            |
| (0–43)                     | (0–65)               |                       |                 | (0–40)          | (0–65)               |                 |
| Treatment duration (months)| 15.5±2.2             | 16.3±3.8              | 0.14            | 15.3±2.1        | 17.0±4.8              | 0.09            |
| IFN-α type                 |                       |                       | 0.001           |                |                        | 0.87            |
| IFN-α-2a (49)              | 35                    | 14                    | 10              | 39              |                        |                 |
| IFN-α-2b (45)              | 15                    | 30                    | 9               | 36              |                        |                 |
| Peg-IFN-α-2a (37)          | 19                    | 18                    | 6               | 31              |                        |                 |

BAbs – binding antibodies; NAbs – neutralizing antibodies; Peg-IFN-α-2a – pegylated interferon-α-2a; mean ±SD or patient numbers are indicated in each category.

Figure 1. Kinetics of cumulative cases of patients who developed binding antibodies (BAbs) (A) or neutralizing antibodies (NAbs) (B).
NAbs after treatment with Peg-IFN-α-2a (16%) was no different than after treatment with the 2 non-Peg-IFN-α (20%). At present, it is difficult to explain the different immunogenicity in response to the different types of IFNs used in this study.

For each type of antibody (BAbs and NAbs), results for active treatment groups over the entire 12-month treatment period remained consistent with 9-month results (Figure 1); therefore, we believe that 1 year was an adequate duration to characterize immunogenicity. Ongoing monitoring will reveal whether there is a longer-term trend for increasing antibody incidence. Previous studies have shown that patients who have remained NAbs-negative during the first 24 months of IFN beta therapy rarely develop NAbs with continuing treatment [26]. A longer duration of treatment in the clinical setting may be required to reveal any effect of antibody status on clinical efficacy, particularly given the small number of patients developing NAbs, which may lead to disease relapse.

A significant association between the occurrence of NAbs and the emergence of clinical resistance to IFN-α was noted [27]. The NAb assay measures ability to neutralize IFN activity in vitro, which is anticipated to predict potential in vivo neutralization [28]. We report here that approximately 75% of NAbs-negative patients with MPN were in CR after IFN-α treatment (Figure 2); however, only 24% of NAbs-positive patients were in CR. It is worth noting that 48% of NAbs-positive MPN patients were PR, and the incidence of PR may be correlated with titer and persistence of antibodies [29,30]. High-titer, persistent NAbs have been consistently shown to impair the activity of IFN, which can result in a clinical relapse despite continuous IFN therapy [31,32].

The high levels of BAbs that developed during IFN-α treatment may correlate with more adverse events and predict the subsequent development of NAbs [33]. The BAbs, therefore, may be related to intolerance of treatment, leading to poor clinical responses.
prognosis. The ELISA used for BAbs is a commercially available assay that is easier to perform, less difficult to interpret, and less expensive than the NAbs assay under most circumstances. The BAbs ELISA is suitable for automation and allows straightforward interpretation if adequate diagnostic thresholds are used. Measurement of BAbs by ELISA can provide useful prognostic information that allows for treatment stratification in future studies.

Conclusions

IFN-α is still a mainstay of therapy for MPN. We have provided a thorough characterization of the immunogenic profile of IFN-α, evaluating immune responses to IFN-α-2a, IFN-α-2b, and Peg-IFN-α-2a treatment. We suggest that the development of BAbs and NAbs can adversely affect IFN-α treatment in patients with MPN. Furthermore, our study revealed that the emergence of anti-IFN-a NAbs is a candidate causal factor for NR in a considerable number of MPN patients. These findings suggest a need to routinely monitor patients receiving IFN-α for development of antibodies. Despite limitations on the interpretation of results regarding the impact of antidrug antibodies on treatment effect, the low incidence of immunogenicity, including extremely low frequency of NAbs, means that patients are still at risk of experiencing impaired efficacy during treatment with IFN-α due to immunogenicity. Although our findings provide an important suggestion for the clinical setting, the role of BAbs and NAbs in MPN therapy needs to be demonstrated by a clinical study with a larger number of patients and the introduction of more advanced analytical methods.

Table 3. Adverse effects of patients grouped according to the presence of antibodies to IFN-α.

| Adverse effects          | BAbs(+) (n=69) | BAbs(-) (n=62) | NAbs(+) (n=25) | NAbs(-) (n=106) |
|--------------------------|----------------|----------------|----------------|-----------------|
|                          | No. (%)        | No. (%)        | No. (%)        | No. (%)         |
| Hematologic              |                |                |                |                 |
| Thrombocytopenia         | 7 (10%)        | 10 (16%)       | 2 (8%)         | 15 (14%)        |
| Anemia                   | 6 (9%)         | 5 (8%)         | 2 (8%)         | 5 (6%)          |
| Leukopenia               | 7 (10%)        | 4 (6%)         | 3 (12%)        | 8 (8%)          |
| Non hematologic          |                |                |                |                 |
| Fever                    | 38 (55%)       | 14 (23%)       | 11 (44%)       | 41 (39%)        |
| Myalgia                  | 18 (26%)       | 3 (5%)         | 5 (20%)        | 16 (15%)        |
| Fatigue                  | 17 (25%)       | 15 (24%)       | 5 (20%)        | 23 (22%)        |
| Skin/allergic reaction   | 11 (16%)       | 2 (3%)         | 3 (12%)        | 10 (9%)         |
| Stomatitis               | 7 (10%)        | 2 (3%)         | 2 (8%)         | 7 (7%)          |
| Alopeaia                 | 6 (9%)         | 5 (8%)         | 3 (12%)        | 8 (8%)          |
| LFT elevation            | 5 (7%)         | 9 (15%)        | 3 (12%)        | 11 (10%)        |
| Mood disorder            | 5 (7%)         | 5 (8%)         | 1 (4%)         | 9 (8%)          |
| Diarrhea                 | 5 (7%)         | 3 (5%)         | 2 (8%)         | 6 (6%)          |
| Nausea                   | 6 (9%)         | 4 (6%)         | 2 (8%)         | 6 (6%)          |
| Headache                 | 4 (6%)         | 6 (10%)        | 2 (8%)         | 7 (7%)          |
| Thyroiditis              | 3 (4%)         | 1 (2%)         | 2 (8%)         | 2 (2%)          |

BAbs – binding antibodies; NAbs – neutralizing antibodies; No. – number of patients.
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