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ORIGINAL PAPER

Evaluation of beta globin mRNA as an early marker of haemoglobin response to epoetin treatment

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Abstract Approximately 60% of anaemic cancer patients respond to epoetin treatment. An early marker of response would be valuable in order to avoid ineffective treatment. We have previously shown that beta globin mRNA increases rapidly after epoetin beta treatment of healthy controls. In the present study we have evaluated whether a change of this marker during the first 2 weeks of epoetin treatment could predict later Hb response in anaemic cancer patients. Twenty cancer patients with Hb <11 g/dl received epoetin beta (NeoRecormon®) 10,000 IU three times weekly during 6 weeks. Hb, reticulocytes and β-globin mRNA were followed. The latter was measured quantitatively using PCR via the 5′ nuclease assay. Eleven patients responded with a Hb increase of >1 g/dl, nine were nonresponders. All responders increased in β-globin mRNA within 2 weeks, mean 7.7× base-line. With a cut-off of an increase of 3× base-line value, we obtained a specificity of 45% and a sensitivity of 91% for the prediction of a later increase of Hb >1 g/dl. With a cut-off of 4× base-line, the specificity increased to 66%, but the sensitivity decreased to 82%. Beta globin mRNA increases before Hb in all responding patients. However, some non-responding patients also show an increase, and there is a trade-off between specificity and sensitivity as the cut-off level is set at different levels. Compared to reticulocyte count, β-globin mRNA is more reliable in the individual patient, but the clinical usefulness of the assay needs to be evaluated in further studies.

Keywords Epoetin treatment · Response marker · Response prediction · Beta-globin mRNA · Molecular markers

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Introduction

Anaemia is a very common complication of cancer [1, 2], and it has been shown that improvement of Hb levels in anaemic cancer patients increases quality of life (QoL) [3–6]. However, anaemia treatment is only given to a minority of anaemic cancer patients [7], mainly due to the high nominal cost of erythropoiesis stimulating agents (ESA). About 30–40% of cancer patients do not respond to ESA treatment, and there is no reliable way of predicting response. Base-line as well as an early increase of transferrin receptor (TfR) levels have been suggested [8] as suitable markers. Soluble TfR is a marker of erythropoietic activity, since an increased erythropoiesis needs an increased influx of iron to the erythroblasts and an over-expression of the transferrin receptor is a means to increase the import of iron. However, TfR also is a marker of true iron deficiency, and an increase during epoetin therapy may either mirror increased erythropoietic activity in the bone marrow or a

depletion of iron deposits or both. The endogenous base-line S-Epo level has been shown to correlate with response to therapy [8–11], but first S-Epo levels are below 200 U/L in most cancer patients, and second the correlation at the group level is of little use for managing the individual patient. Even if there is a significant difference between groups of patients with S-Epo below or above a chosen cut-off level (in some studies 50 U/L, in others 100, 200 and 500), a significant proportion of patients above such cut-off levels may respond to therapy. In myelodysplastic syndrome (MDS) a prediction model has been validated including S-Epo base-line level above or below 500 U/L and the frequency of transfusions [12]. The combination of base-line S-Epo and an early increase in TfR in one study gave a good predictive value [13]. Attempts have been made to define a ratio between observed and predicted S-Epo (O/P ratio), which could indicate the chance of a response to epoetin therapy. However, this has not been found to correlate with response in patients with solid tumours [14] and this variable has the same weakness as the endogenous S-Epo itself. Therefore, an early indicator of a later Hb response to epoetin therapy is still sought. Measurement of gene expression is a novel method for monitoring therapeutic response, and we have previously shown that an event preceding the Hb rise, namely the formation of beta globin mRNA, increases rapidly in response to epoetin treatment of healthy volunteers [15]. The aim of the present study was to investigate whether β-globin mRNA is an early marker of Hb response to epoetin treatment in patients with anaemia of cancer.

Study design

Any cancer anaemia population may be expected to have a response frequency of a little over 50% during 6 weeks of epoetin therapy. The study was planned before the very recent results with additional iv iron were published. Therefore, epoetin treatment of a non-selected group of patients with various cancer diagnoses could be expected to give fairly equal groups of responders versus non-responders. Epoetin treatment (NeoRecormon®) at a dose of 10,000 IU Q3/week (the standard dose at the time of the design of the study) was given during 6 weeks, and since an early marker must react within the first 2 weeks to be useful, samples were taken twice weekly during the first 2 weeks, and once weekly during week 3–6.

It should be stressed that the aim was not to achieve the highest possible response rate but to see whether responders and non-responders differed in their β-globin mRNA response.

Patients

Twenty patients with malignant disease and Hb <10.5 g/dl, who received no anti-tumoural therapy within 6 weeks before entering the study, were treated with epoetin beta (NeoRecormon®) 10,000 IU s.c. three times per week during 6 weeks. No anti-tumour therapy was given during the study. Other causes of anaemia than the cancer itself were excluded by normal levels of S-B12, S-folate, S-ferritin, S-haptoglobin, S-Fe, S-Tf and Tf saturation. Microcytosis was not allowed and two F-Hb samples were negative. The diagnoses included 4 biliary, 5 pancreatic, 3 gynaecologic, 2 breast-, 1 gastric and 1 prostate cancer, 3 multiple myelomas and 3 MDS patients.

Screening samples were taken no more than 2 weeks before start of treatment. Samples were then taken twice weekly during the first 2 weeks of treatment, once weekly during week 3–6 for measurement of Hb, reticulocytes and β-globin mRNA.

The study was performed according to the rules of the Helsinki declaration. Permission was obtained by the Ethics committee of Uppsala University. All patients gave their informed written consent.

Methods

All laboratory variables except β-globin mRNA were measured in the routine hospital laboratory. β-globin mRNA was measured quantitatively using a PCR via the 5′ nuclease pathway as follows.

cDNA synthesis

Whole blood was diluted 10 times in 10 mM Tris–acetate pH 7.5, 10 mM magnesium acetate, and 50 mM potassium acetate. The sample was then diluted an additional 10 times in 1 × OPA the same buffer with 2 U/µl RNase Inhibitor (Fermentas). About 1 µl of the diluted samples was transferred to tubes containing 1 × First strand buffer (Amersham Pharmacia Biotech), 10 mmol/l DTT, 1.25 mmol/l dNTP, 10 U/µl MMLV reverse transcriptase (Amersham Pharmacia Biotech), 2.5 U/µl RNase Inhibitor (Fermentas) and 2.5 µmol/l random hexamers (Biomers). The 20 µl reactions were incubated at 37°C for 60 min. Finally, 30 µl H_2O was added to each sample giving a final volume of 50 µl.

PCR conditions

About 5 µl of the cDNA was used for quantitative analysis by PCR with real-time detection via the 5′ nuclease assay

using the MX3000P instrument from Stratagene [15]. The amplification mix contained 1× PCR Rxn Buffer (Invitrogen), 1.5 mmol/l $MgCl_2$, 0.4 mmol/l dNTP, 0.2 μmol/l of β-globin primers (5′-AGTCTGCGGTTACTGCCCTG-3′ and 5′-ACCAGCAGCCTGCCCAG-3′), 0.1 μmol/l β-globin TaqMan probe (5′-CCACCAACTTCATCCACGTTCACCTTGT-3′) (SGS, Köping, Sweden) labelled with FAM and TAMRA at the 5′ and 3′ ends, respectively, and 1 U of Platinum *Taq* DNA Polymerase (Invitrogen) in a total volume of 25 μl. After a preincubation of 50°C for 2 min and 95°C for 10 min the reactions were subject to a temperature profile of 95°C for 15 s and 58°C for 1 min, cycled 50 times. Results are presented as change from base-line.

Results

Eleven patients (55%) responded with a Hb increase of >1 g/dl within 6 weeks, nine were non-responders. There was a wide variation in Hb increase among the responders, range 1–4 g/dl. The maximum Hb in responders was usually observed in week 6. There was no correlation between diagnosis and response patterns, except that both MM patients responded but none of the two MDS patients. Results are shown in Table 1.

All patients with a Hb response ≥1 g/dl increased in β-globin mRNA, mean 7.7 (range 1.4–13.8) × base-line value. However, so did 6/9 of the non-responders (range 2.2–8 × base-line). Among the non-responders there were 3 patients who increased their Hb 0.9 g/dl or very near the response limit. Two of these had an mRNA increase of 6 vs. 8 times base-line, the third a decrease to –7× base-line. There was no significant correlation between the increase in Hb and mRNA.

The specificity and sensitivity of β-globin mRNA measurement as a response marker depended on the cut-off limits. With a cut-off of 3× base-line the sensitivity was 91% and the specificity 45% (1 responder missed, 5 false positives), with a cut-off of 4× base-line the sensitivity was 82% and the specificity 66% (2 responders missed, 3 false positives).

The reticulocyte count increased during the first 2 weeks in 7 out of the 11 responders, (mean for the group 62 ± 62.9 (0–160) × 10(9)) but also in three of the non-responders (mean for the group 19.3 ± 26.9 (0–65) × 10(9)). Four of the Hb responders had no reticulocyte increase at all during the first 2 weeks (range 0–4 × 10(9)) but saw a significant increase in week 4. This gives very low figures for sensitivity and specificity. With a cut-off of 2× base-line the sensitivity was 27%, specificity 50%, and with 3× base-line sensitivity only 18%. There

Table 1 Base-line data, Hb, reticulocyte (Ret) and β-globin mRNA responses during 6 weeks of epoetin therapy

Pat	Ca diagnosis	Hb resp ≥1 g/dl	Hb init (g/dl)	Hb diff (g/dl)	Hb max (g/dl)	Ret init ×10(9)	Ret diff ×10(9)	Ret-max within 2 weeks ×10(9)	Max mRNA level × base-line within 2 weeks
1	NHL	+	10.4	4.0	14.4	58	134	192	13.8
2	Gyn	+	8.8	3.4	12.2	116	0	116	6
3	Pancreas	+	10.2	3.2	13.4	18	160	178	12
4	Biliary	+	10.1	2.7	12.8	109	0	109	12.6
5	Pancreas	+	11.3	2.6	13.9	117	98	215	4.4
6	Biliary	+	10.8	2.3	13.1	103	149	152	1.4
7	Biliary	+	10.1	2.2	12.3	39	38	77	5.8
8	Pancreas	+	9.8	1.8	11.6	95	1	96	2.6
9	Prostate	+	9.4	1.6	11.0	59	58	117	5
10	MM	+	10.3	1.6	11.9	78	4	82	8.2
11	MM	+	10.6	1.0	11.6	39	40	79	8.2
12	Gastric	–	9.3	–1.1	8.2	93	3	96	3.6
13	Gyn	–	10.3	0	10.3	91	0	91	–4.4
14	Biliary	–	9.8	0.3	10.1	179	0	179	–14
15	Breast	–	10.6	0.9	11.5	60	50	110	6
16	Breast	–	8.8	0.6	9.4	45	65	110	3.2
17	MDS	–	9.4	0.4	9.8	58	3	61	2.2
18	MDS	–	10.7	0.9	11.6	43	49	92	8
19	Pancreas	–	10.8	0.9	11.7	82	0	82	–7
20	Pancreas	–	10.7	0.5	11.2	53	4	57	8

NHL = Non-Hodgkin lymphoma, MM = multiple myeloma, MDS = myelodysplastic syndrome, Ret = reticulocytes

was no correlation between Hb increase and reticulocyte increment and no correlation between reticulocyte and mRNA increase.

Discussion

A response marker that enables the clinician to decide in individual cases within the first 2 weeks of epoetin treatment whether to continue or to stop would undoubtedly be of great value. It would save cost and relieve patients from ineffective injections. Such a marker must have a high specificity and sensitivity, and the change within the first treatment period must be of such a magnitude that reliable conclusions can be drawn for the individual patient. Many of the previously investigated markers have shown a significant difference between responders and non-responders at the group level but have been difficult to apply to individual patients. For instance, a patient with a S-Epo between 100 and 200 U/L still has a 40% chance of responding to epoetin therapy, even if patients with levels <100 U/L have a significantly greater chance of responding. It would seem very natural that the reticulocyte count would be a reliable indicator, since an increased erythropoietic activity in the bone marrow that will eventually lead to increased Hb should be mirrored by an increased influx of newly produced red cells into the circulation. However, patients with no significant increase in reticulocyte counts sometimes exhibit a good Hb response to epoetin therapy, and non-responders sometimes show an increased reticulocyte count during the first weeks (without haemolysis). This was confirmed in the present study where 4 responders had no reticulocyte increase at all (range 0–4 $\times$ 10(9)) during the first 2 weeks. A reticulocyte increase in week 4 is of less value as an early marker, since by that time the Hb itself is more valid. Also, 3 of the non-responders had a reticulocyte increase around 50 $\times$ 10(9). This is in agreement with previous studies and underscores that the reticulocyte count is not a very useful early response marker.

In a previous study [15] we have shown that β-globin mRNA increases rapidly after start of epoetin therapy in healthy volunteers. The present study shows that the same is true for patients with anaemia of cancer. There is a severalfold increase of β-globin mRNA levels over base-line within the first 2 weeks in most patients who respond to epoetin treatment with a Hb increase >2 g/dl within 6 weeks. However, there was a wide variation in β-globin mRNA increase, 1.4–13.8$\times$ base-line value, and a majority of the non-responders (6/9) also had an increase. Three of these were just below the response criterion limit, and a longer treatment time may have resulted in a Hb response also in these patients. The maximum value within 2 weeks was chosen for comparison with the base-line, and this was mostly the last value. Therefore, the optimal time for measuring β-globin mRNA seems to be after 2 weeks. As argued above, an early response marker ideally should show such a substantial change during the first weeks of epoetin treatment that reliable decisions can be made in individual patients. A high sensitivity is important to avoid that a treatment that will eventually result in a Hb increase is stopped prematurely. On the other hand, a high specificity is important in order to avoid ineffective treatment. For β-globin mRNA, a 3-fold increase cut-off gave a high sensitivity for prediciting later Hb response, but a poor specificity (91 and 45%, respectively). A use of this cut-off limit would not give a substantial change from the present routine, where Hb increase at 6 weeks is usually decisive. Using a higher cut-off limit, 4$\times$ base-line, the specificity was increased to 66%, but at the same time the sensitivity decreased to 82%. This cut-off limit would be more acceptable in terms of saving costs for ineffective treatment without risking that treatment is stopped prematurely. Further studies will show if these figures can be improved to allow clinical use of this interesting gene expression marker.

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