Early magnesium modifications as a surrogate marker of efficacy of cetuximab-based anticancer treatment in KRAS wild-type advanced colorectal cancer patients

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Received 2 April 2010; revised 5 August 2010; accepted 6 August 2010

Background: KRAS wild-type mutational status is necessary but not sufficient to get benefit from epidermal growth factor receptor inhibition. Predictive markers are currently being evaluated. In this study, we investigated early hypomagnesemia as a predictor of efficacy and outcome in terms of time to progression (TtP) and overall survival (OS) in a cohort of patients affected by advanced colorectal adenocarcinoma KRAS wild-type cetuximab-treated.

Patients and methods: One hundred and forty-three patients affected by stage IV colorectal adenocarcinoma KRAS wild type receiving cetuximab + irinotecan (CTX+IRI) as third-line anticancer treatment and resistant to oxaliplatin- and irinotecan-based chemotherapy were retrospectively included. Magnesium plasma levels were measured before the first day and 7, 14, 21 and 28 days after CTX+IRI infusion.

Results: The median magnesium basal value showed a statistically significant decrease after the start of CTX+IRI treatment (at 28 days, \( P < 0.0001 \)). Patients with an early decrease of magnesium levels >50% compared with the basal level had a higher tumor response rate (55.8% versus 16.7%, \( P < 0.0001 \)), a longer TtP (6.3 versus 3.6, \( P < 0.0001 \)) and a longer median OS (11.0 versus 8.1, \( P = 0.002 \)).

Conclusions: We have shown that early hypomagnesemia could be a predictor of efficacy and outcome in those patients. Magnesium circulating level is an easy and inexpensive biomarker to routinely be detected in patients treated with cetuximab.

Key words: cetuximab, colorectal cancer, magnesium

Introduction

Magnesium is a central electrolyte in a large number of important cellular metabolic reactions. Recent evidences suggest an Mg\(^{2+}\) role in all key events of tumor biology [1], such as the regulation of oxidative stress [2], carcinogenesis [3], tumor progression [4] and angiogenesis [5].

Recently, Harry Rubin refined his ‘membrane magnesium mitosis’ model of cell growth in which magnesium plays the pivotal role in regulating cell proliferation. According to this model, hypomagnesemia induces both growth arrest by affecting cell cycle regulatory proteins, protein synthesis, DNA duplication and increased apoptosis [4, 6].

About 70% of filtered magnesium is reabsorbed in the ascending limb of the loop of Henle because of the action of the Mg\(^{2+}\)-permeable channel TRPM6 (transient receptor potential cation channel, subfamily M, member 6). This channel is up-regulated by the epidermal growth factor receptor (EGFR) signaling, with EGF acting as an autocrine/paracrine magnesiotropic hormone [7]. Therefore, drug-induced Mg\(^{2+}\) wasting can be a consequence of indirect tubular nephrotoxicity [8] or direct inhibition of the EGFR by anti-EGFR antibodies. Cetuximab-induced hypomagnesemia has been reported since its commercial introduction [9].

Clinical trials have shown that cetuximab is synergistic with chemotherapy for patients with metastatic colorectal cancer (mCRC) [10–12] but its benefit is limited to patients with KRAS wild-type tumors [13–15]. KRAS is one of the major downstream gene of EGFR signaling and its mutation is associated with resistance to cetuximab and a shorter survival in EGFR-positive mCRC patients.
A previous study from our group reported a correlation between the reduction of Mg\(^{2+}\) plasma levels observed in patients with mCRC treated with cetuximab + irinotecan (CTX+IRI) and clinical response and survival in terms of time to progression (TtP) and overall survival (OS) independently from KRAS mutational status [16].

We aimed to assess the incidence of hypomagnesemia and ipocalcemia in mCRC wild-type KRAS cancer patients treated with a weekly combination of CTX+IRI and to evaluate the potential correlation with tumor response and outcome.

**patients and methods**

**study design and patients eligibility criteria**

We included in this retrospective study 143 patients affected by stage IV and histologically confirmed colorectal adenocarcinoma KRAS wild type receiving CTX+IRI as third-line anticancer treatment and resistant to oxaliplatin- and irinotecan-based chemotherapy. The period of accrual ranged from January 2008 to March 2009 and it was consecutive.

Table 1 shows baseline characteristics of patient population. Standard criteria for anticancer treatment suitability were used. Serum creatinine 0.8–1.44 mg/dl reference range was taken.

Patients were considered ineligible for accrual when they had reported fever (>38.0°C) during the last week or had received any radiotherapy, chemotherapy, immunotherapy or growth factors during the last 4 weeks before study entry. Were excluded from the final evaluation patients who received radiotherapy or growth factors during the study period. Patients recently (<1 week) or simultaneously treated with chronic steroid-based therapy and with acute or chronic infections or inflammatory diseases were considered ineligible. Malabsorption syndromes, uncontrolled diabetes, genetic magnesium-wasting syndromes, drugs and toxins intake (such as ethanol, loop diuretics, thiazide, cisplatin, cyclosporine) were also established criteria for ineligibility.

Before being considered for accrual, all patients had a documented disease progression after two standard anticancer regimens: one oxaliplatin-based chemotherapy regimen (capecitabine + oxaliplatin or FOLFOX IV regimen as first line) and one irinotecan-based chemotherapy folinic acid + fluorouracil + irinotecan (FOLFIRI) regimen as second line for at least 2 months.

Ethical approval for the study was obtained and informed consent was signed by all the subjects enrolled in the study.

**treatment plan**

Cetuximab was given at a loading dose of 400 mg/m\(^2\), followed by weekly infusions of 250 mg/m\(^2\). Irinotecan was administered weekly at the dose of 90 mg/m\(^2\). All the patients were treated until disease progression or until unacceptable toxic effects occurred. Modifications to cetuximab dose were made only in cases of toxic effects to the skin and to irinotecan dose in cases of hematologic or non-hematologic toxic effects. The reduction of both cetuximab and irinotecan dose was of 25% of the starting dose. Cetuximab dose adjustment was made in 14 (%) patients because of skin toxicity.

Thirty-seven (%) patients required reduction of irinotecan dose, mainly because of grade ≥3 diarrhea or persistent (longer than 2 weeks) neutropenia (with or without fever). No statistically significant differences in the rate of reduction were observed between patients who developed magnesium reduction or in cetuximab or in irinotecan doses. Tumor response was evaluated every 8 weeks by the use of consistent imaging techniques (computed tomography or magnetic resonance imaging).

Assessment was done by the investigators according to RECIST. Adverse events were recorded according to the National Cancer Institute—Common Toxicity Criteria version 2.0.

**KRAS status**

Formalin-fixed and paraffin-included tumor samples were analyzed for KRAS exon 2 mutations, located within the codons 12 and 13 (Gly12Asp, Gly12Ala, Gly12Val, Gly12Ser, Gly12Arg, Gly12Cys and Gly13Asp). For each tumor sample, the neoplastic area was selected and DNA was extracted using the DNA extraction kit from QIAGEN (QIAamp DNA Blood Mini Kits: Hilden Germany). The DNA extracted was PCR amplified using the following sense and antisense primer: 5’-AAGGCTCTGCTGAAATGACTG-3’ and 5’-CAAAGATGGTCTCCTGACCAG-3’. PCR products were purified using QiAquick® PCR Purification kit, and the PCR products were direct sequenced with Big Dye V1.1 Terminator Kit (Applied Biosystems, Foster City, CA) and an ABI Prism 3100 DNA sequencer (Applied Biosystems).

**electrolyte evaluation**

Venous peripheral blood was drawn before the 1st day and 7, 14, 21 and 28 days after CTX+IRI infusion. Samples were centrifuged for 10 min at 600 g and plasma stored at −80°C until tested. Standard biochemical tests were carried out before every single course.

Plasma magnesium and calcium concentrations were measured by use of a colorimetric assay (sulfaclid blue and arsenazo III method, respectively) in a Konelab 30i automatic analyzer (Dasit) in a single reference laboratory (University Campus Bio-Medico, Rome, Italy). The reference values and standard laboratory deviations for magnesium and calcium were as follows: Mg\(^{2+}\) 1.9–2.5 mg/dl, standard deviation (SD) 0.15; Ca\(^{2+}\) 8.1–10.4 mg/dl, SD 0.575. Serum calcium concentrations were corrected for hypoalbuminemia.
statistical analysis

Basal magnesium levels were compared with those observed after the start of CTX+IRI treatment using Wilcoxon’s test for nonparametric-dependent continuous variables. A linear regression model was used to carry out the correlation between magnesium and calcium levels at each time points.

The TTP analysis was calculated as the period from the date of starting the treatment to the first observation of disease progression or to death from any cause within 60 days after the start of treatment or the most recent tumor assessment. The OS time was calculated as the period from the date of starting treatment until death from any cause or until the date of the last follow-up, at which point data were censored. TTP and OS were both determined by Kaplan–Meier product-limit method [17].

Stratified permutation tests were carried out to explore the association between tumor response and acne-like rash and between tumor response and magnesium modifications. The differences in terms of TTP and OS according to the presence and severity of acne-like rash and magnesium reduction were evaluated by the log-rank test [18]. The Cox proportional hazards model was applied to the multivariate survival analysis [19].

Patients were stratified according to magnesium reduction into two groups (>]50% or £50% versus basal level within the first 4 weeks after the start of cetuximab-based treatment) to carry out the survival analysis and the correlation with clinical response. A reduction of >50% in one time point was sufficient to include a patient in that category. The median value of percentage magnesium reduction was calculated and the cut-off point fixed.

To evaluate the correlation between acne skin rash and survival and clinical response, we compared patients who developed a rash 0–1–2 versus 3 grade. The median value of rash grade in all the cohort was calculated and clinical response, we compared patients who developed a rash 0–1–2 versus 3 grade. The median value of rash grade in all the cohort was calculated and patients’ population separated into two equivalent groups.

The cut-off point for safety data and survival data was 1 December 2009. SPSS software (version 17.00, SPSS, Inc., Chicago, Illinois) was used for statistical analysis. A P value of <0.05 was considered to indicate statistical significance.

results

univariate analysis

One hundred and forty-three consecutive patients (81 males, 62 females), aged 31–83 years (median age, 62 years), with advanced colorectal cancer KRAS wild type were included in the study. All patients matched all the inclusion criteria. Patients’ characteristics are shown in Table 1.

The median magnesium basal value showed a statistically significant decrease 7 days after the start of CTX+IRI antitumor treatment (P = 0.04). The highest statistical significance was recorded 28 days after the start of treatment (P < 0.0001). Eight patients during any of the programmed time points developed a hypomagnesemia G1 according to the CTC criteria and only one patient a G2 hypomagnesemia (all of them at the last time point).

Calcium levels progressively decreased during cetuximab-based therapy, maintaining the correlation at any time points (data not shown). The reduction of calcium serum levels at the planned time points was not clinically relevant. In details, the highest median reduction (at any of the planned time points) was 13%. However, a statistical significant correlation of magnesium and calcium circulating levels was detected at any points (data not shown), suggesting that magnesium could play a key role in the regulation of calcium metabolism also in our patients’ population.

Patients were stratified according to magnesium reduction into two groups (>] or £50% compared with basal value within the first 4 weeks from the start of treatment) in order to carry out survival analysis and correlation with clinical response. Those patients who developed an early decrease of magnesium serum levels >50% showed a higher response rate with respect to patients who did not show an equal reduction (55.8% versus 16.7%, P < 0.0001).

Median TTP was statistically significant longer (6.3 months, 5.65–6.57) in patients with an early magnesium reduction superior of 50% compared with TTP (3.6 months, 3.15–4.04) of those with a reduction inferior to 50%, P < 0.0001 (Figure 1).

Median OS was also longer (11.0 months, 10.53–12.45) in patients with >50% of magnesium reduction compared with the OS of 8.1 (7.69–9.22) months of patients with a lower magnesium reduction (P = 0.002) (Figure 2).

The statistical analysis confirmed the impact of magnesium reduction also classifying patients according to magnesium reduction level as following: <20%, 20%–50%, 50%–70% and >70%. However, no statistical differences have been recorded in terms of both TTP and OS between patients who developed magnesium reduction <20% versus 20%–50%, as shown in Table 2.

Moreover, as previously reported, patients were stratified into two groups according to skin toxicity (grade 0–1–2 and 3, respectively). A correlation between the presence and severity of the acne-like rash cetuximab-related and tumor response was demonstrated by applying stratified permutation tests. Patients with a grade 3 rash showed an higher response rate (44%)
versus those patients with a grade 0, 1 and 2 (38.2%) \((P = 0.012)\). Comparing patients who developed a grade 3 acne-like rash with the others, a statistical significant correlation was recorded between the skin toxicity and TtP: 5.7 (5.21–6.06) versus 4.4 (3.91–4.78) months, \(P = 0.04\). No significant changes were detected in terms of OS between patients with skin toxicity grade 3 compared with 0–1–2 grades (10.71 versus 9.03, \(P = 0.10\)). Tables 3 and 4 summarize the influence of magnesium reduction and skin toxicity cetuximab-related on tumor response, TtP and OS in our cohort of patients.

### multivariate analysis

Multivariate analysis was carried out taking into account skin toxicity and magnesium reduction levels because these are the recognized parameters according to literature data. Performing a multivariate analysis of TtP, the early magnesium reduction preserved the statistical significance while the acne-like skin rash lost its significance. In details, the calculated relative risk of progression in the group of patients with a 50% early magnesium modification is 0.143 [95% confidence interval (CI) 0.111–0.279] with a \(P<0.001\). The relative risk of progression for the group of patients with G3 skin rash was 0.850 (95% CI: 0.629–1.450) if compared with patients without this reduction \((P = 0.181)\).

These results suggest that magnesium reduction seems to act as a stronger prognostic factor than skin toxicity in our patients’ population.

### Table 3. Influence of magnesium reduction on tumor response, TtP and OS

<table>
<thead>
<tr>
<th>Tumor response rate</th>
<th>Number of patients (%)</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤50% reduction of magnesium levels</td>
<td>8/48 (16.7)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>&gt;50% reduction of magnesium levels</td>
<td>53/95 (55.8)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TtP</th>
<th>Median TtP (95% CI) in months</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤50% reduction of magnesium levels</td>
<td>3.6 (3.15–4.04)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>&gt;50% reduction of magnesium levels</td>
<td>6.3 (5.65–6.57)</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>OS</th>
<th>Median OS (95% CI) in months</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤50% reduction of magnesium levels</td>
<td>8.1 (7.69–9.22)</td>
<td>0.002</td>
</tr>
<tr>
<td>&gt;50% reduction of magnesium levels</td>
<td>11.0 (10.53–12.45)</td>
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</table>

TtP, time to progression; OS, overall survival; CI, confidence interval.

### Table 4. Influence of skin toxicity on tumor response, TtP and OS

<table>
<thead>
<tr>
<th>Tumor response rate</th>
<th>Number of patients (%)</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 0–1–2</td>
<td>10/34 (38.2)</td>
<td>0.012</td>
</tr>
<tr>
<td>Grade 3</td>
<td>51/109 (44.0)</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>TtP</th>
<th>Median TtP (95% CI) in months</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 0–1–2</td>
<td>4.4 (3.91–4.78)</td>
<td>0.04</td>
</tr>
<tr>
<td>Grade 3</td>
<td>5.7 (5.21–6.06)</td>
<td></td>
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<table>
<thead>
<tr>
<th>OS</th>
<th>Median OS (95% CI) in months</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 0–1–2</td>
<td>9.03 (8.56–9.91)</td>
<td>0.101</td>
</tr>
<tr>
<td>Grade 3</td>
<td>10.71 (10.01–11.30)</td>
<td></td>
</tr>
</tbody>
</table>

TtP, time to progression; OS, overall survival; CI, confidence interval.

Figure 2. Correlation of magnesium reduction (< versus >50%) with overall survival.
There is ample evidence that benefit of cetuximab is limited to patients with KRAS wild-type tumors [13]. KRAS is actually considered the predictor response to treatment [14, 15].

In the present study, we reported early (within the first 4 weeks) hypomagnesemia as a predictor of efficacy and outcome in terms of TTP and OS in a selected cohort of 143 patients affected by advanced colorectal adenocarcinoma KRAS wild-type cetuximab-treated.

The primary objective of the study was the sequential evaluation of plasma magnesium concentrations in patients affected by wild-type KRAS mCRC during EGFR-targeting antibody therapy. The secondary composite end point was the correlation between magnesium concentrations at different time points with clinical response and outcome during treatment.

We provide further evidence of an early magnesium reduction in mCRC KRAS wild-type cancer patients following CTX+IRI treatment. The median magnesium basal value showed a statistically significant decrease 7 days after the start of CTX+IRI anticancer treatment with the highest statistical significance 28 days after the start of treatment.

A previous study from our group already described early magnesium reduction as a new predictor factor of efficacy and outcome in colorectal cancer patients treated with CTX+IRI but not selected for KRAS mutational status [16].

Most importantly, in the present work we have shown that hypomagnesemia could be a predictor of outcome in those patients harboring a wild-type KRAS status.

By stratifying patients into two groups according to the early magnesium reduction percentage compared with the basal level, we described a longer median TTP and OS in those patients with magnesium reduction superior of 50%. Our present results also suggest that magnesium reduction seems to act as a stronger prognostic factor than skin toxicity in our patients’ population.

There is growing evidence that magnesium not only plays a key role in many cellular physiologic functions, such as DNA and protein synthesis and energy production, but also seems to play an important role in tumor biology [1].

Because EGFR is strongly expressed in the ascending limb of the loop of Henle, EGFR blockade may interfere with renal magnesium transport [8]. As a consequence, inhibition of the EGFR by anti-EGFR antibodies (e.g. cetuximab or panitumumab) might lead to renal Mg²⁺ wasting and therefore to hypomagnesemia, as reported in patients with colorectal cancers treated with anti-EGFR antibodies [9, 20].

Furthermore, severe hypomagnesemia is associated with treatment duration [21].

Clinical studies of cetuximab in mCRC failed to reveal an association between clinical outcome and EGFR protein expression as measured by immunohistochemistry [10, 12]. Furthermore, clinical response has been shown in patients with undetectable EGFR protein expression [14] and somatic mutations in the EGFR tyrosine kinase domain are associated with sensitivity to the tyrosine kinase inhibitors but not with cetuximab [22–24].

Nevertheless, an intact KRAS is necessary but not sufficient to derive benefit from EGFR inhibition. Furthermore, positive predictive markers are currently being evaluated including other EGFR downstream pathways such as the Phosphatidylinositol 3-kinase/phosphatase and tensin homolog gene (PTEN)/protein kinase B/mammalian target of rapamycin [25], B-type Raf mutational status [26] and some other EGFR downstream genes seem to be related to sensitivity to anti-EGFR-based anticancer therapy. The overexpression of EGFR ligands epiregulin and amphiregulin seems to predict antitumor activity resulting from cetuximab therapy [27].

Moreover, tumors that constitutively and aberrantly express nuclear factor-κB are more likely to refractory to cetuximab and to irinotecan than those that do not show nuclear expression of this transcriptional factor [28]. Also, the loss of PTEN protein expression is associated with no responsiveness to cetuximab [29].

The effects of Mg²⁺ on microvascular endothelial cell functions that could contribute to the regulation/deregulation of angiogenesis are rather intricate. A recent study reported that tumors grown in magnesium-deficient mice showed essentially no up-regulation of vascular endothelial growth factor [30]. Moreover, the reduction of magnesium circulating levels could justify the previous reported reduction of vascular endothelial growth factor serum levels in colorectal cancer patients [31, 32]. Consequently, magnesium reduction could be involved in the complex cross-talk between EGFR pathway and angiogenesis.

Low magnesium availability therefore jeopardizes the angiogenic switch which is necessary to trigger new vessels formation.

In conclusion, the present study provides evidence that magnesium decrease could be considered as a new prognostic factor of efficacy and outcome in colorectal wild-type KRAS cancer patients treated with CTX+IRI. We also suggest that magnesium decrease ‘could be a marker in patients who receive’ single anti-EGFR treatment because EGFR inhibition by anti-EGFR antibodies is the key cause of hypomagnesemia ‘pathophysiology’.

Further studies are needed to deeply understand the biological reasons for these findings and to apply a comprehensive assessment of genetic alterations in EGFR signaling pathways.

Accurately predicting drug response and efficacy factors is the ultimate goal of personalized medicine. Criteria in selecting patients who are likely to benefit from cetuximab-based therapies are increasing and the predictive algorithm to adopt is becoming more multifarious. Magnesium circulating level is an easy and economically inexpensive biomarker to routinely be detected in patients treated with cetuximab. Magnesium levels also enable an useful prediction of patient benefit from anti-EGFR treatment. In the future is ausplicable to design prospective studies with the objective to evaluate magnesium circulating levels as predictive markers of cetuximab efficacy.
references