Genetic variation in the bleomycin hydrolase gene and bleomycin-induced pulmonary toxicity in germ cell cancer patients

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Objective Use of bleomycin as a cytotoxic agent is limited by its pulmonary toxicity. Bleomycin is mainly excreted by the kidneys, but can also be inactivated by bleomycin hydrolase (BMH). An 1450A \textgreater G polymorphic site in the BMH gene results in an amino acid substitution in the C-terminal domain of the protein. Deletion of this domain, including the polymorphic site, reduces enzymatic activity. We investigated the relationship between the BMH genotype and the risk of bleomycin-induced pneumonitis (BIP).

Methods From male germ cell cancer patients, treated with bleomycin-containing chemotherapy at the University Hospital Groningen, The Netherlands, between 1977 and 2003, data were collected on age, cumulative bleomycin dose, pretreatment creatinine clearance, pulmonary metastases, lung function parameters, and occurrence of BIP. BIP was defined as: death due to BIP, or presence of clinical and/or radiographic signs of BIP during or following treatment. Polymerase chain reaction and restriction fragment length polymorphism were used to determine the BMH genotype.

Results BIP developed in 38 (11\%) of 340 patients; four of these cases were fatal. BMH genotype distribution did not differ between patients with and those without BIP. Patients with BIP were older and had a lower pretreatment creatinine clearance. Changes in pulmonary function tests were similar in patients with different genotypes.

Conclusions The BMH genotype was not associated with the development of BIP nor with changes in pulmonary function tests. Since renal function is important for bleomycin pharmacokinetics, variations in renal clearance may have obscured significant effects of the BMH genotype. Pharmacogenetics and Genomics 15:399–405 © 2005 Lippincott Williams & Wilkins.

Keywords: germ cell cancer, bleomycin, bleomycin hydrolase, pulmonary toxicity, polymorphism

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Introduction

Bleomycin, a glycopeptide antibiotic derived from \textit{Streptomyces verticillus}, is an antitumour agent that induces reactive oxygen radicals by forming a complex with Fe\textsuperscript{2+} [1]. It is used in the chemotherapeutic regimen of patients with a disseminated germ cell tumour, who have a high cure rate following bleomycin- and cisplatin-containing chemotherapy. Bleomycin has been shown to be important for this high treatment efficacy [2]. However, use of bleomycin is limited by its potentially life-threatening pulmonary toxicity [3].

Bleomycin-induced pneumonitis (BIP) is initiated by damage to the lung vasculature by cytokines and free radicals followed by influx of inflammatory cells and fibroblasts resulting in pulmonary fibrosis [4]. In germ cell cancer patients, who are treated with bleomycin in combination with etoposide and cisplatin, bleomycin causes signs and symptoms of BIP in 3 to 14\% of cases [5–8], while fatal BIP develops in 1 to 3\% of bleomycin-treated patients [5–7,9,10]. Known risk factors for BIP are a high cumulative bleomycin dose, a decreased glomerular filtration rate, older age, supplemental oxygen exposure and, possibly, cigarette smoking and the use of...
Bleomycin is mainly excreted by the kidneys. However, it can also be inactivated by bleomycin hydrolase (BMH), a neutral cysteine protease that is present in many tissues, but has relatively low expression in lung and skin [11–13]. Decreased BMH activity has been associated with susceptibility to bleomycin-induced pulmonary fibrosis in mice [14–16]. Furthermore, BMH knockout mice show increased sensitivity to the development of pulmonary fibrosis [17].

An 1450A > G polymorphic site has been identified in exon 11 of the BMH gene, leading to the presence of either Val443 or Ile443 in the carboxy terminus of the protein [11,18]. This C-terminal domain has been suggested to be a key regulatory region, since deletion of the C-terminal 18 amino acids of human BMH, which includes the polymorphic residue 443, has been demonstrated to reduce aminopeptidase activity [19]. The polymorphic genotype of the BMH gene may lead to decreased inactivation of bleomycin and may, thus, influence sensitivity to bleomycin and the risk of BIP.

We investigated the relation between the BMH genotype and the development of BIP in patients with a disseminated germ cell tumour following bleomycin-containing chemotherapy in an attempt to identify a novel tool for predicting an increased risk of BIP in the individual patient.

Methods
Patients
We reviewed the medical records of all patients who had been treated for a disseminated germ cell tumour at the University Hospital Groningen, The Netherlands, between January 1977 and January 2003. A total of 418 consecutive patients had been treated with a bleomycin-containing chemotherapeutic regimen during this period. The study protocol was approved by the local ethics committee.

Bleomycin-induced pneumonitis
Data on the occurrence of BIP were derived from medical records. Since no well-defined severity score exists for BIP, we used the following classification: (a) death due to BIP; (b) clinical and/or radiographic signs of BIP resulting in hospitalization and/or premature cessation of bleomycin administration; (c) clinical and/or radiographic signs of BIP after completion of treatment; or (d) no signs of BIP. In categories b and c, we also recorded use of steroids for treatment of BIP and/or postponement or cancellation of postchemotherapy surgery because of BIP. All patients were classified according to this scoring system by one person who was ignorant of genotype status.

Data on age at the start of chemotherapy, cumulative bleomycin dose, renal function before the start of chemotherapy (creatinine clearance calculated using the formula by Cockcroft and Gault), and presence of pulmonary metastases were also recorded as known and possible risk factors for BIP. Data on smoking status during treatment were incomplete and were, therefore, not assessed in this study.

Pulmonary function tests
According to frequently used treatment protocols between 1978 and 1993, pulmonary function tests were performed in patients receiving bleomycin-containing chemotherapy to investigate the development of BIP. Measurements of the following parameters were performed before and at three-week intervals during chemotherapy: transfer factor of the lungs for carbon monoxide (TlCO), transfer factor of the lungs for carbon monoxide per unit alveolar volume (KlCO), diffusing capacity of the alveolo-capillary membrane for carbon monoxide (Dm), pulmonary capillary blood volume (Vc), and slow inspiratory vital capacity (VC). Decreases in VC and Vc have been shown to be most specific for bleomycin-induced pulmonary alterations [20].

DNA isolation
After patients had given informed consent, blood samples were taken at the general practitioner’s or at our outpatient clinic and DNA was isolated from lymphocytes according to standard protocols [21]. From deceased patients, serum samples that had been routinely stored after follow-up visits or, when these were also not present, paraffin-embedded histologic material were collected. Preferably, histologic material derived from normal tissue was used, but genotyping for polymorphic enzymes has also been reported to generate reliable results when tumour samples are used [22]. DNA was isolated from serum and paraffin-embedded material using Qiagen Mini Kits (Qiagen GmbH, Hilden, Germany) according to only slightly modified protocols that had been provided by the manufacturer.

Polymerase chain reaction and restriction
BMH genotypes were determined using polymerase chain reaction (PCR) and a restriction fragment length polymorphism technique, as previously described [23]. PCR was carried out using a reverse primer and a forward mismatch primer (5’-CGA CGT TG AAA ACG ACG GCC AGT AGT GC TGT TTA GAG CAG GAA CCA TATT-3'; Invitrogen, Merelbeke, Belgium) to create a MunI restriction site. A universal M13 primer extension was used at the 5’ end of the forward primer to create sufficient restriction fragment length (underlined part of
the above sequence). PCR was performed in a total volume of 50 µl consisting of 5 µl DNA, 1.5 mM MgCl₂, 0.25 mM dinucleotide triphosphate, 1.25 U Taq polymerase (Invitrogen), and 15 pmol primers in 10 × PCR buffer (Invitrogen). Primers were used to amplify a DNA fragment of 150 bp using the following PCR program: one denaturing cycle at 94°C for 3 min; 35 annealing cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min; and one elongation cycle at 72°C for 5 min. In order to obtain sufficient PCR product for the restriction reaction (see below), DNA from serum and paraffin-embedded material was amplified twice using PCR with 25 and 20 annealing reactions under the above-mentioned conditions. After digestion with MmuI (Roche Diagnostics GmbH, Mannheim, Germany) and electrophoresis on a 2.5% agarose gel, the BMH A/A genotype was identified by a 150 bp fragment, the A/G genotype by 150, 111, and 39 bp fragments, and the G/G genotype by 111 and 39 bp fragments. DNA samples for which the three respective genotypes had been confirmed by sequencing were used as controls in each restriction reaction. Whenever the genotype could not be accurately determined using enzyme restriction and electrophoresis, the corresponding DNA sample was sequenced. In six serum samples, the BMH genotype could not be accurately determined by neither enzyme restriction nor sequencing.

**Statistical analysis**

Categorical variables (such as genotype distribution, or prevalence of pulmonary metastases) were compared between the BIP and non-BIP groups using the Pearson Chi-square test. Continuous variables were examined using the Mann–Whitney test or, when data were paired, the Wilcoxon test. Logistic regression analysis was used to examine associations between BIP and its possible risk factors.

Assuming an overall BIP prevalence of 10% in our group of 340 patients, we would have been able to detect an approximately three times higher prevalence of BIP in patients with the A/G or G/G genotype compared with patients with the A/A genotype (BIP prevalence 13.6% vs. 4.7%) with a power of 85% and α = 0.05 using a one-sided test.

**Results**

From 340 patients (81% of cohort), data were available on both BMH genotype and occurrence of BIP. The median age of this group was 29 years with ages varying between 16 and 78 years. Most patients had received four courses of a combination of bleomycin, etoposide, and cisplatin (BEP) or of bleomycin, vinblastine, and cisplatin (PVB). The median cumulative bleomycin dose was 270 mg with 83% of patients receiving 270 mg bleomycin or more (range 60–480 mg). Pulmonary metastases were present in 43% of patients. Pretreatment creatinine clearance varied between 58 and 225 ml/min (Table 1).

In the total cohort of 418 bleomycin-treated patients, BIP developed in 50 (12%) patients. Eight of these patients died of BIP; while bleomycin administration was stopped prematurely in 24 patients. In the 340 patients for whom both clinical data and BMH genotype status were available, BIP was present in 38 (11%) and was fatal in four of these cases (Table 1). Bleomycin administration was stopped prematurely in 21 patients; in 10 of these patients postchemotherapy surgery was postponed and/or treatment with steroids was started. In 13 patients, BIP occurred after completion of the intended treatment, while postchemotherapy surgery was postponed or steroids were started in two of these patients.

DNA was isolated from lymphocytes, serum or paraffin-embedded material in 243 (72%), 59 (17%), and 38 (11%) patients, respectively, A/A homozygote, A/G heterozygote and G/G homozygote genotype frequencies were 45.9%, 43.5%, and 10.6%. Allele frequencies were in Hardy–Weinberg equilibrium.

Genotype distribution did not differ significantly between patients with BIP and those without (P = 0.288;
The frequencies of BIP were 13.5%, 8.1% and 13.9% in patients with the A/A, A/G or G/G genotype, respectively.

Patients with BIP were older and had a lower pretreatment creatinine clearance than patients without BIP, while the cumulative bleomycin dose and the frequency of pulmonary metastases were similar between groups (Table 3).

In logistic regression analysis, only pretreatment creatinine clearance was independently associated with the occurrence of BIP ($P = 0.004$). The BMH genotype was not associated with BIP.

Data on changes in pulmonary function tests were available for subgroups of patients only. First, we investigated the relation between the BMH genotype and changes in lung function parameters after at least three courses of chemotherapy compared with the pretreatment value. Since the number of patients with a G/G genotype and available lung function parameters was very small, patients with A/G and G/G genotypes were grouped. BMH genotype status did not influence the maximal changes in $T_{LCO}$, $V_c$, and $D_m$ after at least three courses of chemotherapy (cumulative bleomycin dose 270 mg or more) compared with the pretreatment value (Table 4). Patients with the A/G or G/G genotype had a significantly larger decrease in $K_{CO}$ after completion of at least three courses of chemotherapy than patients with the A/A genotype.

Second, we investigated the relation between the BMH genotype and changes in pulmonary function in patients for whom lung function data were complete, i.e. data on lung function parameters were present before and after each course of chemotherapy. Data on genotype and the lung function parameter VC were complete in 47 patients (median age 28 years, range 17–53 years; median cumulative bleomycin dose 360 mg, range 270–360 mg); data on genotype and the lung function parameter $V_c$ were complete in 32 patients (median age 29 years, range 17–53 years; median cumulative bleomycin dose 360 mg, range 270–360 mg). Compared with the pretreatment value, VC was significantly decreased after four cycles of chemotherapy in patients with the A/A genotype and after three cycles in patients with the A/G or G/G genotype (Fig. 1a); $V_c$ was decreased after two cycles of chemotherapy in patients in both genotype categories (Fig. 1b). Mean VC and $V_c$ and percent decrease in VC and $V_c$ did not differ between patients with A/A and patients with A/G or G/G genotypes at any moment during treatment. No significant differences were found in $T_{LCO}$, $K_{CO}$, and $D_m$ between patients with different BMH genotypes.

### Table 3 Characteristics of patients with and without bleomycin-induced pneumonitis

<table>
<thead>
<tr>
<th>Variable</th>
<th>No pulmonary toxicity (N=302)</th>
<th>Bleomycin-induced pneumonitis (N=38)</th>
<th>$P$-value$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>29 (16–78)</td>
<td>31 (20–59)</td>
<td>0.054</td>
</tr>
<tr>
<td>Cumulative bleomycin dose (mg)Median (range)</td>
<td>270 (60–480)</td>
<td>285 (150–360)</td>
<td>0.307</td>
</tr>
<tr>
<td>Pretreatment creatinine clearance (ml/min)Median (range)</td>
<td>124 (58–225)</td>
<td>111 (62–155)</td>
<td>0.001</td>
</tr>
<tr>
<td>Pulmonary metastases, N (%)</td>
<td>133 (44%)</td>
<td>14 (37%)</td>
<td>0.399</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>169 (56%)</td>
<td></td>
</tr>
</tbody>
</table>

$^*$Pulmonary metastases: Chi-square test; other variables: Mann–Whitney test.

### Table 4 Maximal changes in pulmonary function tests after completion of at least three courses of chemotherapy in patients with the A/A genotype versus patients with the A/G or G/G genotype

<table>
<thead>
<tr>
<th>Variable</th>
<th>A/A genotype</th>
<th>A/G or G/G genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{LCO}$ (mmol/min/kPa)</td>
<td>Before chemotherapy</td>
<td>After at least three courses</td>
</tr>
<tr>
<td>28</td>
<td>11.20 ± 2.07</td>
<td>9.17 ± 1.93</td>
</tr>
<tr>
<td>32</td>
<td>11.64 ± 2.32</td>
<td>8.87 ± 2.28</td>
</tr>
<tr>
<td>$K_{CO}$ (mmol/min/kPa/L)</td>
<td>Before chemotherapy</td>
<td>After at least three courses</td>
</tr>
<tr>
<td>25</td>
<td>1.75 ± 0.33</td>
<td>1.49 ± 0.24</td>
</tr>
<tr>
<td>31</td>
<td>1.70 ± 0.25</td>
<td>1.32 ± 0.23</td>
</tr>
<tr>
<td>$D_m$ (mmol/min/kPa)</td>
<td>Before chemotherapy</td>
<td>After at least three courses</td>
</tr>
<tr>
<td>25</td>
<td>18.85 ± 5.32</td>
<td>15.16 ± 5.01</td>
</tr>
<tr>
<td>30</td>
<td>20.32 ± 7.19</td>
<td>14.17 ± 4.71</td>
</tr>
<tr>
<td>VC (l)</td>
<td>Before chemotherapy</td>
<td>After at least three courses</td>
</tr>
<tr>
<td>35</td>
<td>4.96 ± 1.10</td>
<td>4.54 ± 1.09</td>
</tr>
<tr>
<td>36</td>
<td>5.31 ± 1.00</td>
<td>4.76 ± 1.09</td>
</tr>
<tr>
<td>$V_c$ (ml)</td>
<td>Before chemotherapy</td>
<td>After at least three courses</td>
</tr>
<tr>
<td>30</td>
<td>86.97 ± 16.76</td>
<td>72.40 ± 18.50</td>
</tr>
</tbody>
</table>

Values are means ± standard deviations.

$^*$Absolute change in patients with A/A genotype versus absolute change in patients with A/G or G/G genotype, Mann–Whitney test.

$T_{LCO}$, transfer factor of the lungs for carbon monoxide; $K_{CO}$, $T_{LCO}$ per unit alveolar volume; $D_m$, diffusing capacity of the alveolo-capillary membrane for carbon monoxide; $V_c$, pulmonary capillary blood volume; VC, slow inspiratory vital capacity.
The use of bleomycin as an antitumour agent is limited by its potentially fatal pulmonary toxicity. We investigated whether the BMH genotype is associated with the risk of BIP in a cohort of male germ cell tumour patients following bleomycin administration.

BIP developed in 38 (11%) patients and was fatal in 11% of these cases, which is in agreement with previous studies [5,7,24]. Age, renal function, cumulative bleomycin dose, and pulmonary metastases have been previously described as risk factors for BIP [6]. We found a higher age and a lower pretreatment creatinine clearance in patients with BIP than in those without, but no differences in cumulative bleomycin dose nor in the occurrence of pulmonary metastases. After adjusting for age, only creatinine clearance before the start of treatment remained significantly associated with BIP.

We determined BMH genotypes using PCR and restriction fragment length polymorphism, as described previously [23]. The frequency of the polymorphic G/G genotype was lower in two (6.6% and 4.0%) [25,26], but similar (9.8%) in one of three previously conducted studies in healthy subjects [27]. This latter study comprised a much larger population than the two studies in which low G/G genotype frequencies were found and may more accurately reflect the BMH genotype distribution in healthy individuals.

The frequency of BIP was similar in patients with the G/G genotype compared with patients with the A/A genotype, making an important role for this BMH polymorphism in the development of BIP less likely. Genotype status did not consistently influence alterations in pulmonary function. Although a decrease in VC occurred earlier during chemotherapy in patients with an A/G or G/G genotype, mean values of and percent decreases in VC and \( V_c \) did not differ between these groups at any stage. Although it was previously shown that the adverse effects of bleomycin on pulmonary function (VC specifically) are enhanced by an impaired renal function [28], no correlation was found between pretreatment creatinine clearance and decreases in VC and \( V_c \) in our study. The significantly larger decrease in \( K_{CO} \) in patients with the A/G or G/G genotype compared with patients with the A/A genotype (Table 4) does probably not reflect an increased toxic effect of bleomycin, since no effects of BMH genotype on changes in VC and \( V_c \) were found, while these lung function parameters have been shown to be most specific for bleomycin-induced pulmonary alterations [20].

Various explanations can be given for the apparent lack of effect of BMH genotype on BIP risk. Renal function is important for bleomycin pharmacokinetics and, as a result, bleomycin exposure, since approximately 60% of bleomycin is excreted by the kidneys during the first 24 h after administration [29]. In blood, the terminal half-life of bleomycin is 2–4 h. However, in patients with a creatinine clearance below 25 to 35 ml/min, half-life exponentially increases with decreases in creatinine clearance [30]. In the present study, none of the patients had a pretreatment creatinine clearance less than 35 ml/min, and only 6% had a creatinine clearance equal to or below 80 ml/min. Therefore, in patients with variations in renal function that were still within the normal range, differences in bleomycin concentrations in blood may have been only minor. As a result, potential effects of the polymorphic phenotype on bleomycin clearance may have been negated by rapid elimination of the agent by the kidneys. On the other hand, other studies have suggested enhanced toxic effects of bleomycin on pulmonary function after smaller decreases in renal function already [10,28]. In the present study, pretreatment creatinine clearance...
clearance, although still within the normal range, was clearly associated with the development of BIP. No data were available on changes in renal function during treatment. Therefore, we cannot exclude the possibility that decreases in renal function have influenced the occurrence of BIP.

Cumulative bleomycin dose has also been described as a risk factor for BIP. Collis et al. reported that BIP occurs in 3 to 10% of patients who receive a cumulative bleomycin dose of 300 mg, while this risk increases to 20% in patients who have been treated with a cumulative dose of 500 mg [31]. In order to reduce the risk of pulmonary toxicity, bleomycin dose was brought back to 360 mg with the introduction of PVB and is even limited to 270 mg in the current BEP regimen [32]. As a result, most patients in our study have been exposed to a relatively low cumulative dose of bleomycin, influencing the relation between bleomycin dose and BIP, but perhaps also between BMH genotype and BIP.

Unfortunately, data on both BMH genotype and BIP were not available for the total cohort. In our group of 340 patients for whom the BMH genotype could be determined, BIP was present in 38. However, another 12 patients of the total cohort of 418 patients had experienced bleomycin-induced pulmonary toxicity, resulting in death in four additional cases. Unfortunately, DNA was not available for these patients and, accordingly, the BMH genotype could not be determined. The missing of a quarter of the affected population, including four patients who died from severe pulmonary toxicity, may have influenced the frequencies of the polymorphic genotype in the BIP group and, thus, obscured significant associations.

Finally, the 1450A > G polymorphic site is located in the C-terminal domain of the BMH protein. Deletion of the last 18 amino acids of this domain, including the polymorphic site, reduces enzymatic activity [19]. However, data on the pharmacokinetics of bleomycin in patients with different BMH genotypes are lacking. Therefore, it is still not clear whether the BMH polymorphism affects bleomycin metabolism in patients.

In conclusion, the BMH genotype was not associated with the development of BIP nor with changes in pulmonary function tests in bleomycin-treated germ cell cancer patients. Since renal function is important for bleomycin pharmacokinetics, significant effects of BMH genotype may have been obscured by variations in renal clearance of bleomycin.

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References


