ABSTRACT

Purpose: The endothelial cell-specific form of nitric oxide synthases (ecNOS) was mapped at 7q35-q36 and plays an important role in vascular development and tumor growth in human prostate cancer. Bone metastasis, clinical T-stage, tumor grade, and serum prostate-specific antigen (PSA) have been shown to have prognostic importance in the outcome of prostate cancer. The purpose of this study was to evaluate ecNOS polymorphism as a genetic indicator of the outcome of the disease.

Experimental Design: In this study, we characterized the Glu-Asp298 ecNOS polymorphism in a series of 161 prostate cancer cases. Logistic regression models were used to assess the contribution of these genotypes to prostate cancer progression.

Results: For Glu-Asp298 polymorphism, we found that GG genotype was associated to advanced disease [P = 0.020; odds ratio (OR), 2.12; 95% confidence interval (CI), 1.12–4.03] and bone metastasis [P = 0.038; OR, 2.23; 95% CI, 1.03–4.84]. Furthermore, after logistic regression analysis with step-wise routine to identify predictive parameters of metastasis, which included age at diagnosis, advanced stage, GG genotype, high grade, and high serum PSA, we observed that Glu-Asp298-GG genotype [P = 0.004; OR, 7.4; 95% CI, 1.87–29.26], high grade tumor [P = 0.009; OR, 6.15; 95% CI, 1.56–24.17], and high serum PSA [P < 0.001; OR, 245.12; 95% CI, 19.93–3013.90] were significantly associated with bone metastasis.

Conclusions: This study demonstrates a strong association between Glu-Asp298-GG genotype as a nitric oxide-related genetic factor and advanced disease and bone metastasization. The establishment of a genetic profile for each patient may be useful in the prediction of the outcome of prostate cancer patients.

INTRODUCTION

Tumor biological aggressiveness as well as the clinical outcome can present strong variations among human prostate cancers. Detection of genetic alterations may be a useful tool as a molecular indicator of prognosis.

Cytogenetic and molecular analyses have demonstrated that human chromosome arm 7q contains a gene that may play an important role in the progression of human prostate cancer (1–5). The ecNOS3 is located at 7q35-q36 and seems to have an important role in vascular development, maintenance of vascular tone, and tumor growth in human prostate cancer (6). NO synthases are a family of enzymes responsible for the generation of NO from the amino acid l-arginine (7, 8). NO produces multiple effects that can influence the outcome of advanced cancer disease and metastasis. Specifically, NO regulates vasodilation and platelet aggregation, which affect tumor cell arrest in capillaries (9). NO is also a major cytotoxic mediator secreted by activated macrophages (10–13) and endothelial cells shown to be responsible for the destruction of tumor cells passing through capillary beds. Moreover, the production of endogenous NO is associated with apoptosis of tumorigenic cells (14, 15).

Taken together, these results suggest the possibility that production of endogenous NO may be detrimental to tumor cell survival and production of metastasis (16–18). The in vitro treatment of tumor cells with cytokines such as tumor necrosis factor α, interleukin 1, and IFN-γ induces production of NO, which leads to apoptosis and both sequels could be blocked by specific inhibition of NO production (14). These data suggest that one factor contributing to the death of circulating tumor cells is the production of NO and that NO plasma levels may contribute to control the progression of the disease.

Prostate cancer is generally a slow growing tumor with a slow clinical course of the disease. Unfortunately, almost half of all prostate cancers present advanced disease at the time of diagnosis and a subset of these present metastatic spread. The risk of prostate cancer progression is related to clinical stage and grade of the disease at the time of diagnosis. Bone metastasis, T stage, and tumor grade have been shown to have prognostic importance in the outcome of prostate cancer (19).

Genetic polymorphisms in the ecNOS gene may be responsible for variations in the genetic control of plasma NO (20, 21). Recently, a point mutation of guanine to thymine at nucleotide position 1917 in the endothelial nitric oxide synthase gene

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2 To whom requests for reprints should be addressed, at Unit of Molecular Oncology, Instituto Português de Oncologia, Porto, R. Dr. Ant. Bernardino Almeida, 4200-072 Porto, Portugal. Phone: 351-22-550-2011; Fax: 351-22-502-6489; E-mail: mop06210@mail.telepac.pt.

3 The abbreviations used are: ecNOS, endothelial cell-specific form of nitric oxide synthase; NO, nitric oxide; OR, odds ratio; CI, confidence interval; PSA, prostate-specific antigen.
Table 1  Association of Glu-Asp298 polymorphism with some outcome parameters among cases of patients with prostate cancer: Gleason grade; clinical T stage; and bone metastasis

<table>
<thead>
<tr>
<th></th>
<th>Glu-Asp298 polymorphism</th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>GT/TT</td>
<td></td>
<td>G/G</td>
</tr>
<tr>
<td>Gleason grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High grade (≥7)</td>
<td>76</td>
<td>44 (57.9)</td>
<td></td>
<td>32 (42.1)</td>
</tr>
<tr>
<td>Low grade (&lt;7)</td>
<td>79</td>
<td>49 (62.0)</td>
<td></td>
<td>30 (38.0)</td>
</tr>
<tr>
<td>Clinical T stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Advanced (T1, T2)</td>
<td>75</td>
<td>37 (49.3)</td>
<td></td>
<td>38 (50.7)</td>
</tr>
<tr>
<td>Early (T3, T4)</td>
<td>86</td>
<td>58 (67.4)</td>
<td></td>
<td>28 (32.6)</td>
</tr>
<tr>
<td>Bone metastasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presence</td>
<td>34</td>
<td>15 (44.1)</td>
<td></td>
<td>19 (55.9)</td>
</tr>
<tr>
<td>Absence</td>
<td>127</td>
<td>81 (63.8%)</td>
<td></td>
<td>46 (36.2)</td>
</tr>
</tbody>
</table>

*χ² test for categorical variables.

(Glu-Asp298 polymorphism in exon 7) has been reported (22, 23).

In this study, we characterize the Glu-Asp298 genotypes in a series of prostate cancer cases and report its association with prognostic factors in prostate cancer suggesting its role as genetic indicators of the outcome of the disease.

MATERIALS AND METHODS

Selection of Patients. The polymorphism of the ecNOS gene was determined in 161 consecutive unrelated patients with histologically confirmed prostate cancer that were diagnosed and treated at the Department of Urology at the Instituto Português de Oncologia, Porto, Portugal, from 1997 to 1998. The median age at diagnosis was 66 years with a mean age of 66.6 ± 8.21. Eighty-six (53.4%) cases had early prostate disease (defined as clinical T stages 1 and 2) and 75 (46.6%) had advanced disease (defined as clinical T stages 3 and 4). The evaluation of histological grade (Gleason score) was available in 155 cases. All samples were obtained with the informed consent of the participants before their inclusion in the study.

Genotyping of Glu-Asp298 Polymorphism. DNA was extracted from peripheral blood leukocytes. For the detection of the Glu-Asp298 polymorphism in the ecNOS gene containing exon 7, we used the PCR according to a previously published protocol (23). After PCR amplification, the resulting 457-bp product was incubated at 37°C for at least 20 h with 8 units of the restriction enzyme BanII (Fermentas). BanII digested the amplified fragments into smaller fragments (137 and 320 bp) in the presence of wild-type genotypes. The restricted fragments were separated on 3% agarose gels with ethidium bromide staining. In the G to T substitution at position 1917 of the ecNOS gene, a BanII restriction site is lost.

Statistical Analysis. Analysis of data were performed using the computer software SPSS for Windows (Version 7.5) and Epi Info (Version 6.04a). χ² analysis was used to compare categorical variables. A 5% level of significance was used in the analysis. The observed number of each genotype was compared with that expected for a population in the Hardy-Weinberg equilibrium by using a goodness of fit χ² test. The Glu-Asp298 genotypes were associated with Gleason Grade (high grade, ≥7 versus low grade, <7) clinical T stage (early, T1/T2 versus advanced, T3/T4) and bone metastasis (presence versus absence). The OR and its 95% CI were calculated as a measurement of the association between Glu-Asp298 genotypes and the outcome variables already mentioned. In a secondary analysis, we performed forward step-wise logistic regression analysis (threshold for inclusion/exclusion of a variable; P = 0.10) to calculate the most important set of predictive variables for bone metastasis (as detected by radionuclide bone scan). In this analysis, we included a new variable serum PSA at time of diagnosis (high serum PSA, ≥100 ng/ml versus low serum PSA, <100 ng/ml), and the statistical models were corrected to the age at diagnosis.

RESULTS

The frequency of Glu-Asp298 genotypes was 40.4% (65 out 161) for GG, 48.4% (78 of 161) for GT, and 11.2% (18 of 161) for TT. This distribution did not differ significantly (P > 0.05) from those predicted by the Hardy-Weinberg equilibrium. In Table 1, we present the results regarding the association of Glu-Asp298 with the outcome parameters among cases of patients with prostate cancer. For Glu-Asp298 polymorphism, we found that GG genotype was present in 50.7% of cases with advanced disease and in 32.6% of cases with early disease and that this difference was statistically significant (P = 0.020; OR, 2.12; 95% CI, 1.12–4.03). Furthermore, this GG genotype was also more frequent in patients with bone metastasis (P = 0.038; OR, 2.23; 95% CI, 1.03–4.84). However, no significant associations were found between GG genotype and Gleason grade (P = 0.600; OR, 1.18; 95% CI, 0.62–2.26).

We used logistic regression analysis with step-wise routine to identify predictive parameters of metastasis, which included age at diagnosis, advanced stage, GG genotype, high grade, and high serum PSA. Table 2 displays the main results of that analysis. We observed that Glu-Asp298-GG genotype (P = 0.004; OR, 7.4; 95% CI, 1.87–29.26), high grade tumor (P = 0.009; OR, 6.15; 95% CI, 1.56–24.17), and high serum PSA (P < 0.001; OR, 245.12; 95% CI, 19.93–3013.90) were significantly associated with bone metastasis.

DISCUSSION

At the time of diagnosis, ~60% of newly diagnosed patients with prostate cancer have established locally extensive or
metastatic disease (24). The overall prognosis is poor when the cancer has metastasized to distant sites.

Although clinical T stage (TNM) correlates with outcome in a large percentage of patients, a more modern concept of risk assessment methods that analyzes many criteria and combines them with clinical staging can better predict the likelihood of disease recurrence or clinical progression. In addition to a description of the anatomical extent of the primary tumor (clinical T stage), patient outcome may be influenced by characteristics of the host, factors intrinsic to the neoplasm (e.g., histological grade and genetic alterations), results of serological tests (e.g., serum PSA level), and the effects of the treatment technique. When included in cancer staging systems, tumor grade (Gleason score) and pretherapy serum PSA level substantially improve prognostic estimation and appraisal of therapeutic outcome.

It has been proposed that alterations at chromosome 7 may influence the progression of prostate cancer and patient outcome (1–5). Aneusomy (including trisomy of chromosome 7) and its association with higher tumor grade, advanced pathological stage, metastasis and early prostatic carcinoma death have been reported by several groups using FISH (1–5). Numerical aberration of chromosome 7 have been significantly associated with higher Gleason score and metastasis (1–5). Another study indicated allelic imbalance in 46% of cases on 7q (25).

We analyzed Glu-Asp298 polymorphisms at ecNOS located on 7q and its association with three outcome parameters: histological grade (Gleason score); clinical T stage; and bone metastasis. Our results indicate (Table 1) that prostate cancer patients carriers of the Glu-Asp298-GG genotype are more likely to have an advanced stage (OR, 2.12) and to develop bone metastasis (OR, 2.23). A forward step-wise routine analysis demonstrated that Glu-Asp298-GG genotype (OR, 7.40), high grade (OR, 6.15), and very high levels of serum PSA (OR, 245.12) were the most important sets of predictors of metastasis (Table 2).

Prostate cancer may spread locally or distantly. High grade and very high levels of serum PSA have been reported to be associated to tumor progression and cancer metastasis (26). For instance, 71% of patients that present pretreatment serum PSA levels > 100 ng/ml have positive bone scan, indicating metastatic prostate cancer (26). Hematogenous metastasis primarily affects the bones and occurs in up to 85% of patients who die from prostate cancer (27). Cancer patients with bone metastasis have a 5-year survival of only 25% (24, 27).

NO plays an important role in tumor growth and angiogenesis. Endogenous NO production causes oxidative DNA damage as a result of stoichiometric fluxes of NO and superoxide that generate peroxynitrite (28, 29). NO production may modify DNA directly or it may inhibit DNA repair activities such as the recently described human thymine-DNA glycosylase, which has been shown to repair G-T mismatches at CpG dinucleotides (30). This is consistent with the hypothesis that there may be synergy between the ability of NO to stimulate DNA damage through formation of peroxynitrite and to inhibit repair of that damage. The importance of NO synthases in the prostate gland pathophysiology has been demonstrated (31–33). It has been reported by Klotz et al. (34) that a selective expression of inducible NO synthase in human prostate carcinoma and NOS activity has been shown to be influenced by androgens (35).

Our results demonstrate a possible role for ecNOS genotypes in the definition of the outcome of prostate cancer. The levels of plasma NO level may be in dependence of the genetic characteristics of each individual (21).

The majority of tumor cells that enter the circulation die rapidly (9, 36, 37). This cell death can be attributed to various tumor cell characteristics such as deformability (38), aggregation (39, 40), and cell surface adhesion molecules (41). Host factors such as blood turbulence (37), natural killer cells (42, 43), macrophages (44), and platelets (45, 46), all influence the survival of blood-borne tumor emboli. Furthermore, passage of tumor cells through capillaries leads to cell lyses by shear forces (37) and by NO produced by cytokine-activated endothelial cells (47, 48). These data suggest that the production of NO is a factor that contributes to the death of circulating tumor cells and that NO plasma levels may contribute to control the disease progression.

Regarding Glu-Asp 298 genotypes in the general population, the frequencies reported are 42.5% (GG), 45.2% (GT), and 15.0% (TT) in a French study (49) and 82.6% (GG), 17.4% (GT), and 0% (TT) in a Japanese study (23). Studies are necessary to characterize the frequencies of these genotypes in the Portuguese general population and to understand the real meaning of these genotypes in prostate cancer etiopathology.

In conclusion, this study identified Glu-Asp298-GG genotype as a NO-related genetic factor predictive of advanced disease and bone metastasis. Additional studies with larger groups of subjects are needed to confirm these findings to establish a genetic profile that may be useful in the prediction of the outcome of prostate cancer patients.

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Outcome in Prostate Cancer: Association with Endothelial Nitric Oxide Synthase Glu-Asp298 Polymorphism at Exon 7

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