Polymorphisms in the Endothelin-1 and Endothelin A Receptor Genes and Survival in Patients with Locoregionally Advanced Nasopharyngeal Carcinoma

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Abstract

Purpose: We aimed to investigate the prognostic role of endothelin-1 (EDN1) and endothelin A receptor (EDNRA) gene polymorphisms in patients with locoregionally advanced nasopharyngeal carcinoma (NPC).

Experimental Design: Two hundred three consecutive patients with locoregionally advanced NPC were enrolled. Seven potentially functional polymorphisms in the EDN1 and EDNRA genes were determined by ligase detection reaction-PCR method from prospectively collected blood samples. The influence of the genetic polymorphisms on patient overall survival (OS) was analyzed using Cox proportional hazards model, Kaplan–Meier method, and the log-rank test.

Results: The 5-year OS in patients with EDNRA/H323H TT, TC, and CC genotypes were 81.3%, 62.1%, and 75.0%, respectively (P = 0.004). Patients carrying the heterozygous (TC) or homozygous variant (CC) genotype in EDNRA/H323H were combined for analysis, which revealed that the 5-year OS in patients with TC/CC genotypes was significantly lower than those with the wild-type TT genotype (63.2% vs. 81.3%; P = 0.002). Multivariate analysis showed that EDNRA/H323H polymorphism (HR: 1.95; 95% CI: 1.18–3.23; P = 0.009) and N classification (HR: 1.35; 95% CI: 1.03–1.79; P = 0.03) were independent significant prognostic factors for OS in patients with locoregionally advanced NPC. In contrast, the EDN1 polymorphisms revealed no prognostic value.

Conclusions: The EDNRA/H323H polymorphism was a novel and independent prognostic marker for patients with locoregionally advanced NPC. The analysis of EDNRA/H323H polymorphism may help identify patient subgroups at high risk for poor disease outcome.

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Introduction

Nasopharyngeal carcinoma (NPC) is common in Southern China, with an annual incidence of 15 to 50 cases per 100,000 people (1). Radiotherapy (RT) is the primary treatment. The prognosis of patients with stage I and II NPC generally is favorable; however, more than 50% of patients with locoregionally advanced NPC eventually will develop recurrent disease after RT alone (2). Incorporation of chemotherapy with standard RT has improved the therapeutic outcome of patients with locoregionally advanced NPC. However, the incidence of relapses remains high (3, 4). Further improvement in treatment results will probably come from more intensive multimodal therapy for high-risk patients.

Accurate prognostic stratification of patients at diagnosis is essential for selecting patients who are suitable for more aggressive treatment. The staging classification is one approach by which one can predict the likelihood of clinical outcome. However, even within the same staging category, there will be variability in patient outcome due to the heterogeneity of the tumor. Therefore, it is necessary to look for new prognostic markers that can be used in combination with clinical staging to help refine therapeutic decisions in the treatment of NPC.

The endothelin-1 (ET-1)/endothelin A receptor (ETAR) axis may directly contribute to tumor growth and indirectly modulate tumor–host interactions in various tumors (5, 6). Engagement of ETAR by ET-1 triggers activation of tumor proliferation (7, 8), VEGF-induced angiogenesis (9, 10), invasiveness (11, 12), and inhibition of apoptosis (13, 14). We reported previously that high pretreatment plasma big ET-1 levels are generally associated with post-treatment distant failure in patients with advanced-stage NPC (15). Our study also showed that ETAR was overexpressed in 73.9% of NPC, and ETAR expression was an
independent determinant of survival and a robust independent predictor of distant metastasis (16). Experimental study has shown that the ETAR antagonist ABT-627 can inhibit the growth and metastasis of NPC cells and increase sensitivity to chemotherapy (17).

Recently, various single nucleotide polymorphisms (SNP) have been identified on genes of endothelin-1 gene (EDN1) and endothelin A receptor gene (EDNRA). The SNPs in EDN1 and EDNRA genes are associated with susceptibility and prognosis of some diseases, including glaucoma, heart failure, dilated cardiomyopathy, diabetic retinopathy, and atherosclerosis (18–23). It remains unclear, however, whether gene polymorphisms of the EDN1 and EDNRA are associated with the prognosis of NPC patients. SNP in the coding region, especially non-synonymous SNPs, may influence protein activity and thus may be associated with cancer development and progression. On the basis of the biological and pathologic significance of ET-1 and ETAR in NPC, we postulated that functional genetic variation in the EDN1 and EDNRA genes contributes to the clinical outcomes of NPC. Therefore, we evaluated the effects of SNPs of EDN1 and EDNRA genes on the survival of patients with locoregionally advanced NPC.

Materials and Methods

Patient selection

Between October 2000 and August 2005, 203 consecutive patients with locoregionally advanced NPC at the Department of NPC, Sun Yat-sen University Cancer Center, were enrolled prospectively in this study. Patients with biopsy proven, previously untreated NPC with 1997 American Joint Committee on Cancer (AJCC)/International Union Against Cancer (UICC) stages III and IV(A–B) were eligible for this study (24, 25). Other criteria included age greater than 18 years, ethnic Han Chinese, and an Eastern Cooperative Oncology Group performance status of 0 or 1. The exclusion criteria included presence of distant metastasis and other concomitant malignant disease. The study was approved by the Clinical Research Ethics Committee of the Sun Yat-sen University Cancer Center, and written informed consent was obtained from all patients.

Patient characteristics are summarized in Table 1. There were 151 male patients and 52 female patients, with a male-to-female ratio of 2.9:1, and their median age was 44 years (range, 18–77 years). Two hundred two (99.5%) patients had World Health Organization (WHO) grade 2 or 3 NPC (26). One hundred forty-three had stage III disease, and 60 had stage IV disease.

Table 1. Patient demographics and treatment characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Number of patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>18–77</td>
</tr>
<tr>
<td>Median age</td>
<td>44</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>151 (74.4)</td>
</tr>
<tr>
<td>Female</td>
<td>52 (25.6)</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
</tr>
<tr>
<td>WHO type 1</td>
<td>1 (0.5)</td>
</tr>
<tr>
<td>WHO type 2</td>
<td>8 (3.9)</td>
</tr>
<tr>
<td>WHO type 3</td>
<td>194 (95.6)</td>
</tr>
<tr>
<td>Overall stagea</td>
<td>143 (70.4)</td>
</tr>
<tr>
<td>III</td>
<td>60 (29.6)</td>
</tr>
<tr>
<td>T classificationa</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>5 (2.5)</td>
</tr>
<tr>
<td>T2</td>
<td>70 (34.5)</td>
</tr>
<tr>
<td>T3</td>
<td>85 (41.9)</td>
</tr>
<tr>
<td>T4</td>
<td>43 (21.2)</td>
</tr>
<tr>
<td>N classificationa</td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>41 (20.2)</td>
</tr>
<tr>
<td>N1</td>
<td>78 (38.4)</td>
</tr>
<tr>
<td>N2</td>
<td>62 (30.5)</td>
</tr>
<tr>
<td>N3</td>
<td>22 (10.8)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>167 (82.2)</td>
</tr>
<tr>
<td>No</td>
<td>36 (17.7)</td>
</tr>
</tbody>
</table>

*a1997 American Joint Committee on Cancer/International Union Against Cancer staging system.

Cell lines and culture conditions

Seven NPC cell lines (SUPE-1, HONE-1, 6-10B, 5-8F, C666-1, CNE-1, and CNE-2) were obtained from the Department of Experimental Research, Sun Yat-sen University Cancer Center. CNE-1 was derived from WHO grade 1 NPC, whereas the others were from WHO grade 2 or 3 NPC. These cell lines were cultured in RPMI 1640 (Invitrogen) with 10% FBS in a humidified incubator with 5% CO2 at 37°C.
Pretreatment evaluation
All patients were evaluated by complete physical examination, fiberoptic nasopharyngoscopy, MRI of the head and neck, chest X-ray, abdominal imaging with ultrasound, and bone scan. All patients were prospectively included in a disease-specific database.

Treatment
All recruited patients were treated according to the treatment policy for NPC in Sun Yat-sen University Cancer Center. The patients were treated with a uniform RT protocol as described previously (27). Megavoltage photons (6 MV) were used to treat the primary tumor and neck lymph nodes. RT was given 5 times a week at 2 Gy/d. The accumulated radiation dose to the primary tumor was 68 to 72 Gy. The accumulated dose was 60 to 62 Gy to the involved areas of the neck and 50 Gy to the uninvolved areas.

Concurrent chemoradiotherapy was given to 26 patients, and induction plus concurrent chemoradiotherapy to 141 patients. As induction chemotherapy, 2 cycles of PF chemotherapy [DDP (cis-diaminedichloroplatinum II) 100 mg/m² i.v. drip on day 1 and 5-FU 1,000 mg/m² d] continuous i.v. for 120 hours] repeated every 3 weeks were well tolerated and allowed to attach overnight. The medium was changed to RPMI 1640 without serum. The cells were incubated in serum-free medium for 72 hours within the absence or presence of 10 nmol/L ET-1 (Sigma). One group of cells was also pretreated with a selective ETAR antagonist, 100 nmol/L BQ123 (Sigma) for 2 hours, and then cells were stimulated with 10 nmol/L ET-1 for 72 hours. The cell numbers were measured using the MTT (Sigma) assay (30). Absorbance was determined with a SpectraMax M5 Plate Reader (Molecular Devices Corporation) at 490 nm. The proliferation rate (%) was calculated using the background-corrected absorbance by the following equation: \( P\% = 100 \times \left( A_{\text{experimental well}} - A_{\text{untreated control well}} \right) / A_{\text{untreated control well}} \). All experiments were carried out in quintuplicate and repeated 3 times. Data represent the average of quintuplicate determinations of 3 separate experiments; mean ± SD. Statistical analysis was carried out by the unpaired t-test.

Follow-up
The follow-up ended on May 31, 2010, with a median follow-up of 62.4 (range, 7–109) months. After completion of treatment, patients were followed up at least every 3 months during the first 3 years, and then every 6 months thereafter until death. All local recurrences were diagnosed by fiberoptic endoscopy and biopsy and/or MRI of the nasopharynx and the skull base showing progressive bone erosion and/or soft tissue swelling. Regional recurrences were diagnosed by clinical examination of the neck and, in doubtful cases, by fine needle aspiration or MRI of the neck. Distant metastases were diagnosed by clinical symptoms, physical examination, and imaging methods including chest radiography, abdominal sonography, whole body bone scan, CT scan, and MRI. In cases of death, the reasons for death were verified from the medical records, death certificates, and from either relatives or the primary physicians who had witnessed the death. During follow-up, 33 (16.3%) patients had locoregional relapse, 46 (22.7%) had distant metastasis, and 66 (32.5%) had died. The 5-year OS was 73.0%.

Statistical analysis
Demographic and clinical information was compared across genotype, using Pearson χ² tests (for categorical variables) and 1-way ANOVA (for continuous variables) where appropriate. Hardy–Weinberg equilibrium was tested using a goodness-of-fit χ² test with 1 degrees of freedom. Each genotype was independently analyzed for correlation with survival times. The Kaplan–Meier method was adopted to estimate survival curves, and the log-rank test was used to compare patients’ survival time between genotype groups. Multivariate analyses using Cox regression were used to assess the importance of genotypes with adjustment for age (≤45 years vs. >45 years), gender (male vs. female), T classification (T1–2 vs. T3–4), N classification (N0–1 vs. N2–3), overall stage (II vs. IV), and chemotherapy (without vs. with chemotherapy). Analyses were carried out using the statistical software package SPSS 16.0 (SPSS). All statistical tests were two-sided, and a value of...
of $P \geq 0.05$ was considered statistically significant. The Bonferroni correction was applied to adjust the primary analysis for 7 comparisons.

The degree of linkage disequilibrium (LD) between polymorphisms measured as $D'$ and $r^2$, and haplotypes were assessed via the SHEsis software (31). Because it is not possible to unambiguously assign the individual haplotype in a population of unrelated subjects (e.g., a patient with EDN1 G8002A GA, Lys198Asn GG, and T-1370G TG genotypes may have 2 of 4 possible haplotypes, GGG and AGT or GGT and AGG), Fallin and colleagues (32) proposed methods to overcome the lack of phase information usually associated with samples of unrelated individuals and provide a comprehensive way of assessing the relationship between sequence or multilocus genotype data and disease endpoints assuming a simple case/control sampling design, the study population was dichotomized into good and bad survivors on the basis of the observed OS times, and haplotype frequencies were estimated and compared between the 2 groups (32, 33).

Results

Relationship between distribution of EDN1 and EDNRA genotypes and clinical features in NPC patients

Genotype frequencies for both EDN1 and EDNRA polymorphisms were found to be in Hardy–Weinberg equilibrium. No associations were detected between genotype and age, sex, T classifications, N classifications, or overall survival. No associations were detected between genotype and clinical features in NPC patients (Table 2). Multivariate analysis showed that EDNRA/C-1222T influenced OS in patients with locoregionally advanced NPC (Table 3).

EDN1 gene polymorphisms and OS in NPC patients

No significant influence in OS was seen for EDN1/G8002A, EDN1/Lys198Asn, and EDN1/T-1370G genotypes in NPC patients (Table 2).

Table 2. Impact of EDN1 and EDNRA genotypes on OS

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of patients</th>
<th>No. of death</th>
<th>5-y OS, %</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDN1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G8002A</td>
<td></td>
<td></td>
<td></td>
<td>0.88</td>
</tr>
<tr>
<td>GG</td>
<td>107</td>
<td>33</td>
<td>71.6</td>
<td></td>
</tr>
<tr>
<td>GA</td>
<td>73</td>
<td>26</td>
<td>74.8</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>22</td>
<td>7</td>
<td>72.7</td>
<td></td>
</tr>
<tr>
<td>Lys198Asn</td>
<td></td>
<td></td>
<td></td>
<td>0.52</td>
</tr>
<tr>
<td>GG</td>
<td>106</td>
<td>33</td>
<td>69.3</td>
<td></td>
</tr>
<tr>
<td>TG</td>
<td>75</td>
<td>28</td>
<td>72.5</td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>20</td>
<td>5</td>
<td>72.7</td>
<td></td>
</tr>
<tr>
<td>T-1370G</td>
<td></td>
<td></td>
<td></td>
<td>0.28</td>
</tr>
<tr>
<td>TT</td>
<td>138</td>
<td>50</td>
<td>67.2</td>
<td></td>
</tr>
<tr>
<td>TG</td>
<td>51</td>
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<td></td>
</tr>
<tr>
<td>GG</td>
<td>12</td>
<td>2</td>
<td>82.5</td>
<td></td>
</tr>
<tr>
<td>EDNRA</td>
<td></td>
<td></td>
<td></td>
<td>0.004 (0.028)*</td>
</tr>
<tr>
<td>H323H</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>TT</td>
<td>108</td>
<td>24</td>
<td>81.3</td>
<td></td>
</tr>
<tr>
<td>TC</td>
<td>86</td>
<td>40</td>
<td>62.1</td>
<td></td>
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<tr>
<td>CC</td>
<td>8</td>
<td>2</td>
<td>75.0</td>
<td></td>
</tr>
<tr>
<td>G-231A</td>
<td></td>
<td></td>
<td></td>
<td>0.16</td>
</tr>
<tr>
<td>GG</td>
<td>91</td>
<td>23</td>
<td>78.3</td>
<td></td>
</tr>
<tr>
<td>GA</td>
<td>92</td>
<td>36</td>
<td>67.7</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>17</td>
<td>7</td>
<td>69.5</td>
<td></td>
</tr>
<tr>
<td>C-70G</td>
<td></td>
<td></td>
<td></td>
<td>0.92</td>
</tr>
<tr>
<td>CC</td>
<td>41</td>
<td>14</td>
<td>72.0</td>
<td></td>
</tr>
<tr>
<td>CG</td>
<td>97</td>
<td>33</td>
<td>73.0</td>
<td></td>
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<tr>
<td>GG</td>
<td>61</td>
<td>19</td>
<td>71.9</td>
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<tr>
<td>C-1222T</td>
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<td>0.82</td>
</tr>
<tr>
<td>CC</td>
<td>106</td>
<td>37</td>
<td>71.3</td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>75</td>
<td>24</td>
<td>74.4</td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>19</td>
<td>5</td>
<td>70.7</td>
<td></td>
</tr>
</tbody>
</table>

NOTE. The numbers of patients for each genotype do not add up to 203 because of failure of genotyping assays in some patients.

*Bonferroni correction ($\times 7$).

EDNRA/C+70G and EDNRA/C+1222T influenced OS in NPC patients (Table 2). Multivariate analysis showed that EDNRA/H323H polymorphism (HR: 1.95; 95% CI: 1.18–3.23; $P = 0.009$) and N classification (HR: 1.35; 95% CI: 1.03–1.79; $P = 0.03$) were independent significant prognostic factors for OS in patients with locoregionally advanced NPC (Table 3).

EDN1/EDNRA haplotypes and their effects on survival

There was LD between alleles of the 7 loci with the following $D'$ and $r^2$ values: EDN1/G8002A and EDN1/Lys198Asn ($D' = 0.97$, $r^2 = 0.92$); EDNRA/C-70G and EDNRA/C+1222T ($D' = 0.82$, $r^2 = 0.35$). Haplotype frequencies were estimated and compared between patients with an OS time of 5 or less years versus patients with an OS time of more than 5 years. We examined the effect of EDN1 haplotypes composed of 3 SNPs on NPC survival. Four haplotypes were inferred (Table 4). The distribution of
EDN1 haplotypes was not associated with OS (global $P = 0.09$). We also examined the effect of EDNRA haplotypes composed of 4 SNPs on NPC survival. Eleven haplotypes were inferred (Table 4). Consistent with the results of the genotype analyses, the distribution of EDNRA haplotypes was significantly associated with OS (global $P = 0.0001$). Both the TCCG (EDNRA H323H, C+70G, C+1222T, and G-231A wild-type alleles) and TCTG (EDNRA H323H, C+70G, and G-231A wild-type alleles with +1222T allele) haplotypes were significantly more frequent in patients with an OS time of more than 5 years ($P = 0.04$ and $P = 0.03$, respectively). The frequency of the CCTA haplotype was significantly higher in patients with an OS time of less than 5 years ($P \leq 0.0001$).

**Cell proliferation studies**

The genotypes of polymorphisms in the EDNRA gene in 7 human NPC cell lines were shown in Table 5. We compared cell proliferation rates from human NPC cells (CNE-1, CNE-2, and C666-1) with EDNRA/H323H TT genotype to those (SUNE-1, HONE-1, 6-10B, and 5-8F) with EDNRA/H323H TC/CC genotype. After 72 hours of ET-1 stimulation, the proliferation rate of the cell lines with EDNRA/H323H TC/CC genotype was significantly higher than the cells with EDNRA/H323H TT genotype (30.6% vs. 12.1% vs. 12.0% vs. 4.5%, $P < 0.0001$). Addition of EDNRA antagonist BQ-123 shows a significant decrease in cell proliferation (30.6% vs. 12.1% vs. 8.1% vs. 4.0%, $P < 0.0001$), indicating that the observed differences in cell proliferation rates are due to EDNRA variation.

**Table 3.** Results of the multivariate analysis of OS

<table>
<thead>
<tr>
<th>Variables</th>
<th>HR</th>
<th>95% CI</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDNRA/H323H polymorphism (TT vs. TC/CC)</td>
<td>1.95</td>
<td>1.18-3.23</td>
<td>0.009</td>
</tr>
<tr>
<td>N classification (N0–1 vs. N2–3)</td>
<td>1.35</td>
<td>1.03–1.79</td>
<td>0.03</td>
</tr>
<tr>
<td>Age (&lt;45 y vs. &gt;45 y)</td>
<td>1.62</td>
<td>0.99–2.65</td>
<td>0.05</td>
</tr>
<tr>
<td>Gender (male vs. female)</td>
<td>0.81</td>
<td>0.44–1.51</td>
<td>0.52</td>
</tr>
<tr>
<td>T classification (T1–2 vs. T3–4)</td>
<td>1.10</td>
<td>0.75–1.60</td>
<td>0.62</td>
</tr>
<tr>
<td>Chemotherapy (without vs. with)</td>
<td>0.82</td>
<td>0.44–1.53</td>
<td>0.55</td>
</tr>
</tbody>
</table>

*The allelic sequence in the haplotypes is in the following order: G8002A, Lys198Asn, T-1370G; global $P = 0.09$.*  
*The allelic sequence in the haplotypes is in the following order: H323H, C+70G, C+1222T, and G-231A; global $P = 0.0001$.*

**Table 4.** Haplotype frequencies in patients with an OS time 5 or less years versus patients with an OS time of more than 5 years

<table>
<thead>
<tr>
<th>Haplotypes</th>
<th>OS ≤ 5 y, %</th>
<th>OS &gt; 5 y, %</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDN1*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATG</td>
<td>7.0</td>
<td>12.7</td>
<td>0.06</td>
</tr>
<tr>
<td>ATT</td>
<td>21.0</td>
<td>14.9</td>
<td>0.06</td>
</tr>
<tr>
<td>GGG</td>
<td>6.6</td>
<td>6.8</td>
<td>0.98</td>
</tr>
<tr>
<td>GGT</td>
<td>62.3</td>
<td>64.8</td>
<td>0.84</td>
</tr>
<tr>
<td>EDNRA*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCCC</td>
<td>6.2</td>
<td>8.4</td>
<td>0.25</td>
</tr>
<tr>
<td>CCGG</td>
<td>3.1</td>
<td>3.9</td>
<td>0.64</td>
</tr>
<tr>
<td>CCTA</td>
<td>14.9</td>
<td>1.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CGCA</td>
<td>7.8</td>
<td>3.5</td>
<td>0.07</td>
</tr>
<tr>
<td>CGGG</td>
<td>5.4</td>
<td>3.0</td>
<td>0.26</td>
</tr>
<tr>
<td>TCCA</td>
<td>3.4</td>
<td>1.0</td>
<td>0.10</td>
</tr>
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<td>TCCG</td>
<td>3.7</td>
<td>8.8</td>
<td>0.04</td>
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<td>TCTA</td>
<td>2.0</td>
<td>3.4</td>
<td>0.40</td>
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<tr>
<td>TCTG</td>
<td>12.8</td>
<td>21.3</td>
<td>0.03</td>
</tr>
<tr>
<td>TGCA</td>
<td>8.5</td>
<td>5.0</td>
<td>0.63</td>
</tr>
<tr>
<td>TGCC</td>
<td>31.2</td>
<td>37.1</td>
<td>0.08</td>
</tr>
</tbody>
</table>

*The allelic sequence in the haplotypes is in the following order: G8002A, Lys198Asn, T-1370G; global $P = 0.09$.*  
*The allelic sequence in the haplotypes is in the following order: H323H, C+70G, C+1222T, and G-231A; global $P = 0.0001$.*
methylation. Lo and colleagues (37) found the hyper-
gene expression in NPC is associated with promoter hyper-
has been reported in NPC (37, 38). The silencing of
allele of
outcome. Mechanisms that could explain how the minor
polymorphism substitutes a thymine (T) for a cytosine (C),
domain and the third extracellular loop of the protein. The
morphism was significantly associated with OS in the
EDNRA
The
unknown since functional studies are not yet available.
sequences of
associated with OS in NPC patients. The functional con-
EDNRA
H323H TC/CC genotypes are lower than the patients with
primary tumors and all 4 NPC cell lines.

Discussion

We investigated for an association of polymorphisms of
EDN1 and EDNRA genes with clinical outcome in 203
patients with locoregionally advanced NPC. Our study
suggests that the variant EDNRA/H323H genotype was
associated with an increased progression risk in NPC
patients. To the best of our knowledge, this is the first
study to investigate whether ET-1/ETAR pathway gene
variants may have prognostic effects on the survival out-
come of patients with cancer.

Endothelin B receptor (EDNRB) gene variants have been
associated with anorectal malformations in South African
patients (34), in Korean patients with sporadic Hirschsprung’s disease (35), as well as in adult asthmatic
patients with airway obstruction (36). Polymorphisms in
the ENDRA gene were not determined in the present study
because hypermethylation of the 5’ CpG island of EDNRB
has been reported in NPC (37, 38). The silencing of EDNRB
gene expression in NPC is associated with promoter hyper-
methylation. Lo and colleagues (37) found the hyper-
methylation of the 5’ CpG island of EDNRB in 90.5%
primary tumors and all 4 NPC cell lines.

The results revealed that the EDNRA/H323H poly-
morphism was significantly associated with OS in the
NPC patients. The 5-year OS in patients with EDNRA/
H323H TC/CC genotypes are lower than the patients with
wild-type genotype. In the multivariate analysis, the
EDNRA/H323H genotype was the most important factor
associated with OS in NPC patients. The functional con-
sequences of EDNRA/H323H polymorphisms are
unknown since functional studies are not yet available.
The EDNRA/H323H polymorphism is situated in exon 6 of
the EDNRA gene, encoding the sixth membrane-spanning
domain and the third extracellular loop of the protein. The
polymorphism substitutes a thymine (T) for a cytosine (C),
which does not alter the amino acid sequence of the
receptor protein. It is not clear yet how a synonymous
SNP might have such a pronounced effect on treatment
outcome. Mechanisms that could explain how the minor
allele of EDNRA/H323H might alter ETAR function include
alterations in RNA stability, folding, or splicing; differences
in tRNA selection; or binding of noncoding RNAs (39–41),
thereby modulating the influence of ET-1 action on the
progression of NPC. Indeed, this polymorphism might be
nonfunctional in itself but may be closely linked to a
presently uncharacterized functional mutation modifying
the expression of the gene. Another possibility might be
that the polymorphism is in linkage disequilibrium with
another locus, with the causal variant being a small dis-
tance away in adjacent regulatory regions or in a nearby
gene.

We did not find an association between the variants of
EDN1 and NPC outcome. Although there was circumstan-
tial evidence suggesting that EDN1 Lys198Asn polymor-
phism affects plasma endothelin-1 level and is associated
with diabetic retinopathy (22, 42), the functional significance
of this SNP under physiologic and pathologic conditions
remains to be determined in NPC progression. ET-1 expres-
sion may therefore not be an adequate indicator of the
activity of the ETAR signaling. In view of the literature,
ETAR activation rather than ET-1 itself promotes tumor
progression by means of various mechanisms (6).

Validation of genotype–phenotype association studies
requires replication using an independent data set. Although
our finding of significant associations of the
EDNRA/H323H polymorphism with survival outcomes
was not examined in an independent sample, it is never-
theless supported by several lines of evidence. First, from
the evidence to date, it appears that ET-1 and ETAR play
a predominant role in malignancies (43). Our previous
studies found that the ET-1/ETAR axis is closely associated
with progression and prognosis of NPC (15–17). Second,
the ET-1/ETAR axis is related to resistance to chemotherapy
or radiotherapy. Increased expression of ETAR in breast
carcinomas is associated with resistance to chemotherapy
(44). Radiotherapy and chemotherapy can, indeed, take
advantage of better tumor oxygenation and drug delivery,
respectively, both partly dependent on the tumor blood
supply. Higher density of the ETARs was expressed in
tumor vessels, and the myogenic tone of the tumor
vascular bed was exquisitely dependent on the ET-1/ETAR
pathway (45). The use of an ETAR antagonist can selec-
tively promote tumor perfusion and oxygenation, and
consecutively increase the effectiveness of tumor radio-
therapy and chemotherapy (45, 46). Third, the associa-
tion between EDNRA/H323H genotype and worse
prognosis in our cohort is in keeping with the functional
consequences of this polymorphism: cell proliferation
studies using human NPC cells showed that cells with
EDNRA/H323H TC/CC genotypes proliferate at a faster
rate than those with wild-type TT when stimulated with
ET-1. Finally, the EDNRA/H323H polymorphism has
been reported to be associated with susceptibility and
prognosis of some diseases. Colombo and colleagues (19)
reported that EDNRA/H323H polymorphism was asso-
ciated with a substantially increased risk of heart failure.
Herrmann and colleagues (20) found that the EDNRA/
H323H polymorphism predicts survival in patients with
dilated cardiomyopathy.

Table 5. Genotypes of polymorphisms in the
EDNRA gene in 7 human NPC cell lines

<table>
<thead>
<tr>
<th>NPC cell lines</th>
<th>H323H</th>
<th>C-70G</th>
<th>C-1222T</th>
<th>G-231A</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUNE-1</td>
<td>TC</td>
<td>CC</td>
<td>CC</td>
<td>GG</td>
</tr>
<tr>
<td>HONE-1</td>
<td>CC</td>
<td>GG</td>
<td>CT</td>
<td>GG</td>
</tr>
<tr>
<td>6-10B</td>
<td>TC</td>
<td>GG</td>
<td>CC</td>
<td>GG</td>
</tr>
<tr>
<td>5-8F</td>
<td>TC</td>
<td>CC</td>
<td>CC</td>
<td>GG</td>
</tr>
<tr>
<td>C666-1</td>
<td>TT</td>
<td>CC</td>
<td>TT</td>
<td>GG</td>
</tr>
<tr>
<td>CNE-1</td>
<td>TT</td>
<td>GG</td>
<td>CT</td>
<td>GG</td>
</tr>
<tr>
<td>CNE-2</td>
<td>TT</td>
<td>GG</td>
<td>CT</td>
<td>GG</td>
</tr>
</tbody>
</table>

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There are limitations to this study. Firstly, we only studied a few selected candidate polymorphisms: pathway-based genotyping of more SNPs in EDN1 and EDNRA genes, and haplotype analysis are warranted to confirm and extend our findings. Functional studies are needed to measure phenotypes, and evaluate genotype and phenotype correlation in the context of NPC progression. Secondly, due to the lack of available tissue samples, we were unable to correlate the EDNRA genotype with ETAR mRNA or protein expression within tumors. The mechanism by which altered ETAR expression from germ line variation affects outcome may arise early in the disease process through the promotion of metastases, or reflect an interaction between the tumor and the cellular environment, which also bears the same germ line variation. This may not be reflected in the assessment of ETAR expression from available primary tumor samples. Finally, as with any study of modest size, this one may lack some generalizability. The results of this study will need to be validated in a larger cohort of patients. Additional prospective multicenter study is under way to further validate the prognostic significance of EDN1/EDNRA polymorphisms within the entire NPC patient population.

In summary, the EDNRA/H323H polymorphism was an independent prognostic marker for OS in patients with loco-regionally advanced NPC. The ETAR blocker atrasentan has shown certain efficacy in treating hormone-refractory prostate cancer in both phase II and phase III clinical trials (47, 48). In addition to the TNM (tumor node metastasis) stage, testing for the presence of EDNRA/H323H polymorphism may help identify patient subgroups at high risk for poor disease outcome and also indicate that ETAR antagonists might be beneficial for NPC patients. However, since this is the first study showing a correlation between ENDRA gene polymorphism and survival outcomes in NPC patients, further validation of this molecular marker will be required before the results of this test can be used for treatment stratification.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed. The manuscript contains original material. We did not report any similar work that has appeared in previous articles.

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