Imaging, Diagnosis, Prognosis

Clinical Cancer Research

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

Yue-Feng Wen[1,2], Bin Qi[1,2], Huai Liu[1,2], Hao-Yuan Mo[1,2], Qiu-Yan Chen[1,2], Juan Li[1,2], Pei-Yu Huang[1,2], Yan-Fang Ye[1,2], Ying Zhang[1,3], Man-Quan Deng[1,2], Xiang Guo[1,2], Ming-Huang Hong[1,4], Ka-Jia Cao[1,2], and Hai-Qiang Mai[1,2]

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. *Clin Cancer Res; 17(8); 2451–8. ©2011 AACR.*

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

Authors' Affiliations: [1]State Key Laboratory of Oncology in South China, [2]Department of Nasopharyngeal Carcinoma, [3]Tumor Resources Bank, and [4]GCP Center, Sun Yat-sen University Cancer Center, Guangzhou, PR China

Note: Y.-F. Wen and B. Qi contributed equally to this article.

Corresponding Author: Hai-Qiang Mai, Department of Nasopharyngeal Carcinoma, Sun Yat-sen University Cancer Center, 651 Dongfeng Road East, Guangzhou 510060, PR China. Phone: 86-20-87343380; Fax: 86-20-87343392; E-mail: maihq@sysucc.org.cn

doi: 10.1158/1078-0432.CCR-10-2264

Translational Relevance

Accurate prognostic stratification of patients with locoregionally advanced nasopharyngeal carcinoma (NPC) at diagnosis is essential for selecting patients who are suitable for more aggressive treatment, especially when there is convincing evidence of the survival benefit of combined-modality treatment. Looking for new prognostic biomarkers that can be combined with the clinical staging may help to refine therapeutic decisions in the treatment of NPC. We found that the *EDNRA*/H323H polymorphism was independent prognostic marker for patients with locoregionally advanced NPC. The analysis of the *EDNRA*/H323H polymorphism can help identify patients at high risk for a poor disease outcome and also indicate that endothelin A receptor may represent a target for development of new therapies in NPC.

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 nonsynonymous 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.

Cell lines and culture conditions

Seven NPC cell lines (SUNE-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% CO_2 at 37°C.

Table 1. Patient demographics and treatment characteristics

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

[a]1997 American Joint Committee on Cancer/International Union Against Cancer staging system.

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*-diamminedichloroplatinum II) 100 mg/m^2 i.v. drip on day 1 and 5-FU 1,000 mg/(m^2 d) continuous i.v. for 120 hours] repeated every 3 weeks were given. As concurrent chemoradiotherapy, DDP 100 mg/m^2 on days 1, 22, and 43 during radiotherapy, or DDP 40 mg/m^2 once weekly during radiotherapy was given.

End point

The primary end point for the study was OS, defined as the time from the day of enrollment to the date of death from any cause or to last follow-up visit.

DNA extraction and genotyping

Blood for genotyping was prospectively collected at time of entry into the study. All sample collection and storage procedures were standardized. The specific polymorphic loci examined included *EDN1* G8002A (rs2071942), *EDN1* Lys198Asn (rs57072783), *EDN1*T-1370G (rs1800541), *EDNRA* H323H (rs5333), *EDNRA* C+70G (rs5335), *EDNRA* C+1222T (rs5343), and *EDNRA* G-231A (rs1801708). These SNPs were selected using the following criteria: associated with susceptibility and prognosis of other diseases (18–23) and adequate frequency in Asian populations to enable evaluation. DNA was extracted from peripheral lymphocytes using the QIAamp DNA Blood Midi Kit (QIAGEN). Polymorphisms were detected using the ligase detection reaction-PCR (LDR-PCR; refs. 28, 29) technique by Shanghai Biowing Applied Biotechnology Co., Ltd. In addition, approximately 10% of the samples were randomly selected to be genotyped again by a different investigator, and the results were 100% concordant. Only between 1 and 4 samples failed to be genotyped for each SNP (98.0%–99.5% completion rate).

Cell proliferation assay

NPC cell lines cultured in RPMI 1640 were harvested at 80% confluence and plated at 1×10^3 cells/well in 96-well plates 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 Corporationat) at 490 nm. The proliferation rate (%) was calculated using the background-corrected absorbance by the following equation: $P\% = 100 \times (A_{\text{experimental well}} - A_{\text{untreated control well}})/A_{\text{untreated control well}}$. 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 χ^2 tests (for categorical variables) and 1-way ANOVA (for continuous variables) where appropriate. Hardy–Weinberg equilibrium was tested using a goodness-of-fit χ^2 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 ($\leq$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 (III 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 $P \geq 0.05$ 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 multiple-site variation and traits and diseases within populations. According to the methods for testing associations between estimated haplotype frequencies derived from 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 stages.

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).

EDNRA gene polymorphisms and OS in NPC patients

The 5-year OS in patients with *EDNRA*/H323H TT, TC, and CC genotypes were 81.3%, 62.1%, and 75.0%, respectively ($P = 0.004$; Fig. 1A). The 5-year OS in patients with *EDNRA*/H323H TC genotype was significantly lower than those with TT genotype ($P = 0.001$). 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%; $P = 0.002$; Fig. 1B). This association remained significant after applying Bonferroni adjustment for the 7 SNPs evaluated in this analysis. None of the polymorphisms in *EDNRA*/G-231A, *EDNRA*/C+70G, or *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).

Table 2. Impact of *EDN1* and *EDNRA* genotypes on OS

Genotype	No. of patients	No. of death	5-y OS, %	*P*
EDN1				
G8002A				0.88
GG	107	33	71.6	
GA	73	26	74.8	
AA	22	7	72.7	
Lys198Asn				0.52
GG	106	33	69.3	
TG	75	28	72.5	
TT	20	5	72.7	
T-1370G				0.28
TT	138	50	67.2	
TG	51	14	80.2	
GG	12	2	82.5	
EDNRA				
H323H				0.004 (0.028)[a]
TT	108	24	81.3	
TC	86	40	62.1	
CC	8	2	75.0	
G-231 A				0.16
GG	91	23	78.3	
GA	92	36	67.7	
AA	17	7	69.5	
C+70G				0.92
CC	41	14	72.0	
CG	97	33	73.0	
GG	61	19	71.9	
C+1222T				0.82
CC	106	37	71.3	
CT	75	24	74.4	
TT	19	5	70.7	

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

[a]Bonferroni correction (×7).

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

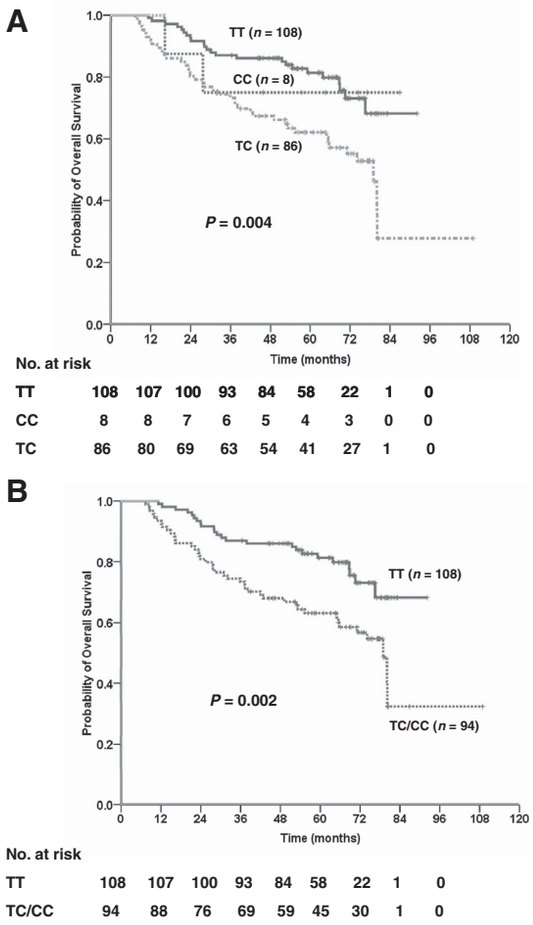

Figure 1. Kaplan–Meier OS curves according to EDNRA/H323H genotypes: A, *TT* versus TC versus CC genotype. B, TT versus TC/CC genotype.

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 5 or less years than in patients with an OS time of more than 5 years ($P \leq 0.0001$).

Table 3. Results of the multivariate analysis of OS

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

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% ± 12.1% vs. 12.0% ± 4.5%, $P < 0.0001$). Addition of EDNRA antagonist BQ-123 shows a significant decrease in cell proliferation (30.6% ± 12.1% vs. 8.1% ± 4.0%, $P < 0.0001$), indicating that the observed differences in cell proliferation rates are due to EDNRA variation.

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

Haplotypes	OS ≤ 5 y, %	OS > 5 y, %	*P*
EDN1[a]			
ATG	7.0	12.7	0.06
ATT	21.0	14.9	0.06
GGG	6.6	6.8	0.98
GGT	62.3	64.8	0.84
EDNRA[b]			
CCCA	6.2	8.4	0.25
CCCG	3.1	3.9	0.64
CCTA	14.9	1.2	<0.0001
CGCA	7.8	3.5	0.07
CGCG	5.4	3.0	0.26
TCCA	3.4	1.0	0.10
TCCG	3.7	8.8	0.04
TCTA	2.0	3.4	0.40
TCTG	12.8	21.3	0.03
TGCA	8.5	5.0	0.63
TGCG	31.2	37.1	0.08

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

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

NPC cell lines	*EDNRA* genotype			
	H323H	C+70G	C+1222T	G-231A
SUNE-1	TC	CC	CC	GG
HONE-1	CC	GG	CT	GG
6-10B	TC	GG	CC	GG
5-8F	TC	GG	CC	GG
C666-1	TT	CC	TT	GG
CNE-1	TT	GG	CT	GG
CNE-2	TT	GG	CT	GG

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 outcome 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 *ENDRB* 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 hypermethylation. Lo and colleagues (37) found the hypermethylation 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 polymorphism 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 consequences 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 distance 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 circumstantial evidence suggesting that EDN1 Lys198Asn polymorphism 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 expression 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 nevertheless 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 selectively promote tumor perfusion and oxygenation, and consecutively increase the effectiveness of tumor radiotherapy and chemotherapy (45, 46). Third, the association 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 associated 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.

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 locoregionally 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.

Acknowledgments

The authors thank Hai-De Qin for statistical assistance and the Tumor Resources Bank of Sun Yat-sen University Cancer Center for providing blood samples.

Grant Support

National Natural Science Foundation of China (No.: 81072226); 863 Project (No.: 2006AA02A404); Sci-Tech Project Foundation of Guangdong province (No.: 2007B060401064); Guangdong Provincial Medical Research Foundation (No.: B2006063); Sci-Tech Project Foundation of Guangzhou city (No.: 2007Z1-E4022); and the Fundamental Research Funds for the Central Universities (2009).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 25, 2010; revised January 1, 2011; accepted January 10, 2011; published online April 12, 2011.

References

1. Min HQ. Nasopharyngeal carcinoma. In: Gun GY, editor. Epidemiology. Beijing, China: People's Medical Press; 1996. p. 280–5.
2. Altun M, Fandi A, Dupuis O, Cvitkovic E, Krajina Z, Eschwege F. Undifferentiated nasopharyngeal cancer (UCNT): current diagnostic and therapeutic aspects. Int J Radiat Oncol Biol Phys 1995;32:859–77.
3. Huncharek M, Kupelnick B. Combined chemoradiation versus radiation therapy alone in locally advanced nasopharyngeal carcinoma: results of a meta-analysis of 1,528 patients from six randomized trials. Am J Clin Oncol 2002;25:219–23.
4. Langendijk JA, Leemans CR, Buter J, Berkhof J, Slotman BJ. The additional value of chemotherapy to radiotherapy in locally advanced nasopharyngeal carcinoma: a meta-analysis of the published literature. J Clin Oncol 2004;22:4604–12.
5. Grant K, Loizidou M, Taylor I. Endothelin-1: a multifunctional molecule in cancer. Br J Cancer 2003;88:163–6.
6. Nelson J, Bagnato A, Battistini B, Nisen P. The endothelin axis: emerging role in cancer. Nat Rev Cancer 2003;3:110–6.
7. Grant K, Knowles J, Dawas K, Burnstock G, Taylor I, Loizidou M. Mechanisms of endothelin 1-stimulated proliferation in colorectal cancer cell lines. Br J Surg 2007;94:106–12.
8. Zhang WM, Zhou J, Ye QJ. Endothelin-1 enhances proliferation of lung cancer cells by increasing intracellular free Ca^{2+}. Life Sci 2008;82:764–71.
9. Wülfing P, Kersting C, Tio J, Fischer RJ, Wülfing C, Poremba C, et al. Endothelin-1-, endothelin-A-, and endothelin-B-receptor expression is correlated with vascular endothelial growth factor expression and angiogenesis in breast cancer. Clin Cancer Res 2004;10:2393–400.
10. Knowles J, Loizidou M, Taylor I. Endothelin-1 and angiogenesis in cancer. Curr Vasc Pharmacol 2005;3:309–14.
11. Wilson JL, Burchell J, Grimshaw MJ. Endothelins induce CCR7 expression by breast tumor cells via endothelin receptor A and hypoxia-inducible factor-1. Cancer Res 2006;66:11802–7.
12. Rosanò L, Cianfrocca R, Masi S, Spinella F, Di Castro V, Biroccio A, et al. Beta-arrestin links endothelin A receptor to beta-catenin signaling to induce ovarian cancer cell invasion and metastasis. Proc Natl Acad Sci U S A 2009;106:2806–11.
13. Nelson JB, Udan MS, Guruli G, Pflug BR. Endothelin-1 inhibits apoptosis in prostate cancer. Neoplasia 2005;7:631–7.
14. Del Bufalo D, Di Castro V, Biroccio A, Varmi M, Salani D, Rosanò L, et al. Endothelin-1 protects ovarian carcinoma cells against paclitaxel-induced apoptosis: requirement for Akt activation. Mol Pharmacol 2002;61:524–32.
15. Mai HQ, Zeng ZY, Zhang CQ, Feng KT, Guo X, Mo HY, et al. Elevated plasma big ET-1 is associated with distant failure in patients with advanced-stage nasopharyngeal carcinoma. Cancer 2006;106:1548–53.
16. Mai HQ, Zeng ZY, Zhang HZ, Hou JH, Mo HY, Guo X, et al. Correlation of endothelin A receptor expression to prognosis of nasopharyngeal carcinoma. Chin J Cancer 2005;24:611–5.
17. Mai HQ, Zeng ZY, Feng KT, Ye YL, Zhang CQ, Liang WJ, et al. Therapeutic targeting of the endothelin a receptor in human nasopharyngeal carcinoma. Cancer Sci 2006;97:1388–95.
18. Kim SH, Kim JY, Kim DM, Ko HS, Kim SY, Yoo T, et al. Investigations on the association between normal tension glaucoma and single nucleotide polymorphisms of the endothelin-1 and endothelin receptor genes. Mol Vis 2006;12:1016–21.

19. Colombo MG, Ciofini E, Paradossi U, Bevilacqua S, Biagini A. ET-1 Lys198Asn and ET(A) receptor H323H polymorphisms in heart failure. A case-control study. Cardiology 2006;105:246–52.
20. Herrmann S, Schmidt-Petersen K, Pfeifer J, Perrot A, Bit-Avragim N, Eichhorn C, et al. A polymorphism in the endothelin-A receptor gene predicts survival in patients with idiopathic dilated cardiomyopathy. Eur Heart J 2001;22:1948–53.
21. Telgmann R, Harb BA, Ozcelik C, Perrot A, Schönfelder J, Nonnenmacher A, et al. The G-231A polymorphism in the endothelin-A receptor gene is associated with lower aortic pressure in patients with dilated cardiomyopathy. Am J Hypertens 2007;20:32–7.
22. Li H, Louey JW, Choy KW, Liu DT, Chan WM, Chan YM, et al. EDN1 Lys198Asn is associated with diabetic retinopathy in type 2 diabetes. Mol Vis 2008;14:1698–704.
23. Yasuda H, Kamide K, Takiuchi S, Matayoshi T, Hanada H, Kada A, et al. Association of single nucleotide polymorphisms in endothelin family genes with the progression of atherosclerosis in patients with essential hypertension. J Hum Hypertens 2007;21:883–92.
24. Fleming ID, Cooper JS, Henson DE, et al. editors. AJCC Cancer Staging Manual. 5th ed. Philadelphia: Lippincott-Raven; 1997. p. 31–9.
25. Sobin LH, Wittekind C, editors. UICC TNM Classification of Malignant Tumours. 5th ed. New York: Wiley-Liss; 1997. p. 26–30.
26. Shanmugaratnam K, Sobin LH, editors. World Health Organization. International Histological Classification: Histological Typing of Tumors of the Upper Respiratory Tract and Ear. 2nd ed. Berlin, Germany: Springer-Verlag; 1991.
27. Ma J, Mai HQ, Hong MH, Cui NJ, Lu TX, Lu LX, et al. Is the 1997 AJCC staging system for nasopharyngeal carcinoma prognostically useful for Chinese patient populations?Int J Radiat Oncol Biol Phys 2001;50: 1181–9.
28. Khanna M, Cao W, Zirvi M, Paty P, Barany F. Ligase detection reaction for identification of low abundance mutations. Clin Biochem 1999;32: 287–90.
29. Consolandi C, Busti E, Pera C, Delfino L, Ferrara GB, Bordoni R, et al. Detection of HLA polymorphisms by ligase detection reaction and a universal array format: a pilot study for low resolution genotyping. Hum Immunol 2003;64:168–78.
30. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.
31. Shi YY, He L. SHEsis, a powerful software platform for analyses of linkage disequilibrium, haplotype construction, and genetic association at polymorphism loci. Cell Res 2005;15:97–8.
32. Fallin D, Cohen A, Essioux L, Chumakov I, Blumenfeld M, Cohen D, et al. Genetic analysis of case/control data using estimated haplotype frequencies: application to APOE locus variation and Alzheimer's disease. Genome Res 2001;11:143–51.
33. Graziano F, Ruzzo A, Santini D, Humar B, Tonini G, Catalano V, et al. Prognostic role of interleukin-1beta gene and interleukin-1 receptor antagonist gene polymorphisms in patients with advanced gastric cancer. J Clin Oncol 2005;23:2339–45.
34. Moore SW, Zaahl MG. Association of endothelin-beta receptor (EDNRB) gene variants in anorectal malformations. J Pediatr Surg 2007;42:1266–70.
35. Kim JH, Yoon KO, Kim H, Kim JK, Kim JW, Lee SK, et al. New variations of the EDNRB gene and its association with sporadic Hirschsprung's disease in Korea. J Pediatr Surg 2006;41:1708–12.
36. Taillé C, Guénégou A, Almolki A, Piperaud M, Leynaert B, Vuillaumier S, et al. ETB receptor polymorphism is associated with airway obstruction. BMC Pulm Med 2007;7:5.
37. Lo KW, Tsang YS, Kwong J, To KF, Teo PM, Huang DP. Promoter hypermethylation of the EDNRB gene in nasopharyngeal carcinoma. Int J Cancer 2002;98:651–5.
38. Zhou L, Feng X, Shan W, Zhou W, Liu W, Wang L, et al. Epigenetic and genetic alterations of the EDNRB gene in nasopharyngeal carcinoma. Oncology 2007;72:357–63.
39. Komar AA. Silent SNPs: impact on gene function and phenotype. Pharmacogenomics 2007;8:1075–80.
40. Hoffmeyer S, Burk O, von Richter O, Arnold HP, Brockmöller J, Johne A, et al. Functional polymorphisms of the human multidrug-resistance gene: multiple sequence variations and correlation of one allele with P-glycoprotein expression and activity in vivo. Proc Natl Acad Sci U S A 2000;97:3473–8.
41. Kimchi-Sarfaty C, Oh JM, Kim IW, Sauna ZE, Calcagno AM, Ambudkar SV, et al. A "silent" polymorphism in the MDR1 gene changes substrate specificity. Science 2007;315:525–8.
42. Barden AE, Herbison CE, Beilin LJ, Michael CA, Walters BN, Van Bockxmeer FM. Association between the endothelin-1 gene Lys198Asn polymorphism blood pressure and plasma endothelin-1 levels in normal and pre-eclamptic pregnancy. J Hypertens 2001;19: 1775–82.
43. Bagnato A, Rosano L. The endothelin axis in cancer. Int J Biochem Cell Biol 2008;40:1443–51.
44. Wülfing P, Tio J, Kersting C, Sonntag B, Buerger H, Wülfing C, et al. Expression of endothelin-A-receptor predicts unfavourable response to neoadjuvant chemotherapy in locally advanced breast cancer. Br J Cancer 2004;91:434–40.
45. Sonveaux P, Dessy C, Martinive P, Havaux X, Jordan BF, Gallez B, et al. Endothelin-1 is a critical mediator of myogenic tone in tumor arterioles: implications for cancer treatment. Cancer Res 2004;64: 3209–14.
46. Yang KM, Russell J, Lupu ME, Cho H, Li XF, Koutcher JA, et al. Atrasentan (ABT-627) enhances perfusion and reduces hypoxia in a human tumor xenograft model. Cancer Biol Ther 2009;8:1940–6.
47. Carducci MA, Padley RJ, Breul J, Vogelzang NJ, Zonnenberg BA, Daliani DD, et al. Effect of endothelin-A receptor blockade with atrasentan on tumor progression in men with hormone-refractory prostate cancer: a randomized, phase II, placebo-controlled trial. J Clin Oncol 2003;21:679–89.
48. Nelson JB, Love W, Chin JL, Saad F, Schulman CC, Sleep DJ, et al. Phase 3, randomized, controlled trial of atrasentan in patients with nonmetastatic, hormone-refractory prostate cancer. Cancer 2008; 113:2478–87.