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Estrogen receptor-beta genetic variations and overall survival in patients with locally advanced gastric cancer

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Abstract

Estrogen has been shown not only to reduce the incidence of colorectal cancer but also gastric cancer (GC). Polymorphisms in estrogen receptor β gene, *ESR2*, correlate with colorectal cancer survival. To better understand the role of *ESR2* in GC, genomic DNA extracted from 169 Japanese patients and 172 patients from Los Angeles County (LAC) was analyzed for association of overall survival (OS) with three *ESR2* polymorphisms, which are of biological significance using multivariable Cox proportional hazard regression. *ESR2* rs1271572 (C > A) and rs3020443 (T > G) had univariate and multivariable associations with OS in the Japanese cohort, whereas the C allele of *ESR2* rs2978381 (T > C) predicted favorable OS in the Japanese cohort but worse OS in the LAC cohort. The interaction term of the *ESR2* rs2978381 and cohort group reached statistical significance. Our study provides evidence that genetic variations in *ESR2* gene are significantly associated with survival in patients with locally advanced GC.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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INTRODUCTION

Estrogen functions are mediated by two subtypes of nuclear receptors, estrogen receptor α and estrogen receptor β (ERβ), which transduce extracellular signals into transcriptional response by binding to estrogen response elements as homodimers or heterodimers either directly in promoter regions or indirectly through alternative DNA elements such as regions of activator protein 1 or specificity protein 1.[1–3] The estrogen receptor α and ERβ have different tissue distributions and are sometimes coexpressed within the same tissue and cell type. The ERβ activates transcription of various targets upon binding to 17β-estradiol or related ligands and is also the most abundantly expressed sex-steroid hormone receptor in the gut.

Loss of ERβ expression correlates with higher grades, malignant transformation and advanced Dukes stage of colorectal cancer.[4–6] Multiple review articles have indicated that the loss of ERβ expression is a common step in the development of colonic carcinoma,[7,8] suggesting that ERβ is responsible for the protective effect of estrogens against colorectal carcinogenesis. An analysis of prospective data from the Women's Health Initiative have shown that the use of postmenopausal hormone replacement therapy significantly reduces the relative risk of developing colorectal cancer.[9] Moreover, several studies support evidence that hormone therapy reduces the incidence and risk of death from colon cancer.[10–17] In gastric adenocarcinoma it has been shown that ERβ is expressed more abundantly than estrogen receptor α.[18–23] Some studies demonstrated that ERβ overexpression was associated with lower tumor stage, lack of perineural invasion and favorable survival time in gastric cancer (GC), whereas other studies reported the inverse associations.[18–20,22–30] Epidemiologic studies have indicated the reduced incidence of GC by the protective effect of estrogen.[31,32] However, despite the abundance of literature the association between estrogen and prognosis in GC remains inconclusive.

Several single-nucleotide polymorphisms (SNPs) in the ERβ gene, *ESR2*, located in a promoter region have been shown to be associated with colorectal cancer survival.[33] However, to the best of our knowledge, this is the first study evaluating the associations between the ERβ gene and prognosis in GC. We hypothesized that SNPs in *ESR2* might be related with overall survival (OS) in patients with GC. We therefore tested the prognostic value of previously reported SNPs with known biological significance within a promoter region of *ESR2*, namely rs1271572, rs2978381 and rs3020443, in two independent cohorts of patients with locally advanced GC.

MATERIALS AND METHODS

Eligible patients

This study enrolled two independent cohorts. For the Japanese cohort, a total of 169 patients with histologically confirmed (stage I to stage IV with no distant metastases; AJCC 6th) gastric adenocarcinoma treated with surgery alone or surgery plus fluoropyrimidine-based adjuvant chemotherapy in Fukushima Red Cross Hospital (Fukushima) or Kitasato University East Hospital (Sagamihara) between 1991 and 2011 were included. For the Los Angeles County (LAC) cohort, a total of 172 patients with histologically confirmed (stage I

to stage IV with no distant metastases; AJCC 6th) gastric adenocarcinoma treated with surgery alone or surgery plus fluoropyrimidine-based adjuvant (radio)-chemotherapy in multiple centers in LAC between 1992 and 1997 were included. Japanese patients were followed clinically every 3 months for the first 2 years and then every 6 months. Japanese patient data were collected retrospectively through chart review. Patients in LAC were participants in a case–control study.[34] Pathologic stage was assigned according to tumor–node–metastasis classification, sixth edition. All tissue analyses in the current study were carried out at the University of Southern California/Norris Comprehensive Cancer Center following approval by the University of Southern California Institutional Review Board of Medical Sciences. All patients signed an informed consent for the analysis of molecular correlates.

DNA extraction and genotyping

Genomic DNA was extracted from formalin-fixed paraffin-embedded tissues of 138 out of 169 Japanese patients and from peripheral whole blood of the other patients, using the QIAmp Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol (www.qiagen.com). PCR-based direct DNA sequence analysis using ABI 3100 A Capillary Genetic Analyzer and Sequencing Scanner v1.0 (Applied Biosystems, Life Technologies, Grand Island, NY, USA) was performed for genotyping the SNPs. Both forward and reverse primers of Table 1 were used for DNA amplification for each polymorphism. A tenth of the samples in each cohort were randomly selected and examined for quality control. The genotyping quality control by direct DNA sequencing provided a genotype concordance of 99% or more. All analyzed SNPs were genotyped with a success rate of > 97%. In case of failure, extracted genomic DNA had limited quantity and/or poor quality. The investigator analyzed the SNPs blindly to the clinical data set. This study was conducted adhering to the REporting recommendations for tumor MARKer prognostic studies (REMARK).

Statistical analysis

The primary endpoint of this study was OS, which was defined as the period from the date of surgery or diagnosis to death in both cohorts. If there was no observed event, the endpoint was censored at the last time of contact or follow-up.

χ^2-tests were performed to examine differences in baseline patient characteristics between the two cohorts. Exact test was performed to test allelic distribution of all SNPs in each race/ethnic group for deviation from Hardy–Weinberg equilibrium. Linkage disequilibrium among candidate SNPs for each race/ethnic group was assessed using D' and r^2 values, and the haplotype frequencies were inferred using Haploview version 4.2 (www.broad.mit.edu/mpg/haploview).

Kaplan–Meier curves and log-rank tests were conducted for univariate analysis of the association between the *ESR2* SNPs and OS using codominant, dominant or recessive genetic models when appropriate. The baseline demographic and clinical characteristics that remained significantly associated with endpoint in the model selection procedures ($P < 0.1$) were included in multivariable analysis of the SNPs and clinical outcome. Concordant probability estimates for the Cox proportional hazards model were calculated to evaluate the

incremental contribution in discrimination provided by the *ESR2* SNPs over the baseline prognostic markers in both cohorts.[35] With the sample size of 169 patients in the Japanese cohort and 172 patients in the LAC cohort, we would have 80% power to identify the SNPs with a hazard ratio of 1.95–2.20 and 1.70–1.94, respectively, and minor allele frequency of > 10% using a two-sided log-rank test. To simplify the scenarios of power calculation, we only considered the dominant model of inheritance. All tests were two-sided at a 0.05 significance level and performed by using the SAS statistical package version 9.4 (SAS Institute, Cary, NC, USA).

RESULTS

The baseline characteristics in both cohorts were summarized in Table 2. The median follow-up periods were 4.0 years in the Japanese cohort and 8.3 years in the LAC cohort. The median OS was 5.7 years for the Japanese cohort compared with 2.6 years for the LAC cohort. The clinicopathologic characteristics and outcome in both cohorts varied considerably. In brief, compared with the Japanese cohort, the LAC cohort comprised younger patients, more advanced T- and N-categories, a higher percentage of proximal-located and poorly differentiated cancer as well as a worse general condition. In the Japanese cohort, age, performance status, stage, T-/N-category and tumor site were significantly associated with OS (Supplementary Table 1). On the other hand, in the LAC cohort, race, stage, T-/N-category and tumor differentiation grade were significantly correlated with OS (Supplementary Table 2).

The allelic frequencies for all SNPs were within the probability limits of Hardy–Weinberg equilibrium (Exact test, $P > 0.05$) in each race/ethnic group. No significant linkage disequilibrium was found in the Japanese cohort. In the LAC cohort, linkage disequilibrium was found between *ESR2* rs1271572 and *ESR2* rs2978381 in Caucasians ($D' = 0.90$, $r^2 = 0.55$). Haplotype analysis was constructed for those two polymorphisms; however, it showed no significant results. In addition, significantly different allelic distributions in *ESR2* rs2978381 and *ESR2* rs3020443 were found between the two cohorts (Supplementary Table 3).

Univariate and multivariable analyses in the Japanese cohort

In the Japanese cohort, all of the three *ESR2* SNPs were significantly associated with OS in univariate analysis. *ESR2* rs1271572 (C > A) and rs3020443 (T > G) remained statistically significant in multivariable analysis when adjusted by age, performance status, stage and tumor site. The A allele of *ESR2* rs1271572 predicted shorter OS, whereas the G allele of *ESR2* rs3020443 was significantly associated with longer OS. The C allele of *ESR2* rs2978381 (T > C) correlated with significantly favorable OS in univariate analysis but this was not statistically significant in multivariable analysis. Concordant probability estimates was 0.712 (s.e., 0.024) in the multivariable model for OS when only baseline tumor characteristics were included. The Concordant probability estimates increased to 0.736 (SE, 0.022) and 0.726 (SE, 0.023) when *ESR2* rs1271572 or *ESR2* rs3020443 was added to the model, respectively.

Univariate and multivariable analyses in the LAC cohort

In the LAC cohort, only the *ESR2* rs2978381 was significantly associated with OS. Patients with the C allele of *ESR2* rs2978381 had significantly worse OS than those with T/T genotype in both univariate and multivariable analyses (Table 3). Concordant probability estimates were 0.683 (SE, 0.022) and 0.705 (SE, 0.023) in the multivariable model for OS, including baseline tumor characteristics only and after adding *ESR2* rs2978381 into the model, respectively. Interestingly, the C allele was associated with worse OS in the LAC cohort but with better OS in the Japanese cohort (Figure 1).

Interaction test between *ESR2* polymorphism and cohort

We performed an exploratory interaction test between the three *ESR2* SNPs and cohort group based on a likelihood ratio test in a multivariable Cox proportional hazards regression model adjusting for T- and N-categories and stratified by race. All three SNPs had a significantly different effect on survival in the Japanese and LAC cohorts. The Interaction term of the *ESR2* rs2978381 and cohort group reached statistical significance (adjusted $P = 0.021$). Moreover, *ESR2* rs1271572 and rs3020443 also showed a significant interaction with OS (adjusted $P = 0.023$ and 0.015, respectively).

DISCUSSION

Our study demonstrates that genetic variations in a promoter region of the *ESR2* gene may predict prognosis in patients with locally advanced GC. These results also provide preliminary evidence, suggesting that the prognostic value of *ESR2* gene variations may vary in an ethnic-dependent manner.

We found that all analyzed *ESR2* SNPs were significantly associated with OS in univariate analysis among Japanese patients with locally advanced GC. Moreover, *ESR2* rs1271572 and rs3020443 remained statistically significant in multivariable analysis. Functional variations in the promoter region of the *ESR2* gene encoding ERβ have been reported to influence the expression of the gene and function of the protein. The 5′-UTR of the *ESR2* gene is plentiful in CpG islands and permits several ERβ transcript variants.[36] It has been shown that hypermethylation of the CpG islands near the untranslated exon 0 N of the *ESR2* gene is linked to transcriptional inactivation of ERβ in several cancers.[1,37,38] Moreover, SNPs in the promoter 0 N region of the *ESR2* gene may affect cancer risk.[19,39] The functionality of *ESR2* rs3020443 has not been well characterized. However, the T/T genotype of *ESR2* rs1271572 was reported to inhibit expression of its gene by downregulating transcriptional activity of the promoter 0 N,[40] suggesting that the T allele of *ESR2* rs1271572 may lead to a decrease in the transcriptional activity of the ERβ gene promoter 0 N. The functional mechanism of *ESR2* rs2978381 is still not well understood. However, this SNP is located near *ESR2* rs2987983, which has been identified as a putative susceptibility SNP for cancers and is also located among binding sites for a number of transcription factors.[41,42] Given the location of the SNP, it is biologically plausible that this variation may have an impact on the gene transcription. Further mechanistic studies confirming the functional role of these polymorphisms are highly warranted.

In this study, the *ESR2* rs2978381 predicted OS in both Japanese and US patients with GC. Furthermore, the effect of C allele of the SNP on survival was statistically significant in both cohorts and showed an inverse correlation. This phenomenon may result from the diversity in allele frequency that produces different patterns of survival association of the allele across different ethnic groups.[5] In our study, a significant difference in allele frequency of the SNP was observed (Supplementary Table 3). On the other hand, this finding may be attributed to regional differences not only in biology but also in etiology of gastric carcinogenesis underlying the different GC subtypes observed in Japan and the United States. Preliminary interaction tests revealed that all three candidate SNPs showed significant interactions between the cohorts, indicating that *ESR2* gene variations may affect prognosis of GC patients in a regional- or ethnic-dependent manner. GC can be classified at least into three principal subtypes based on clinical and epidemiological data in addition to gene expression analysis.[43] Distal intestinal GC is strongly associated with chronic inflammation related to *Helicobacter pylori* infection.[7,8] For gastroesophageal junction or proximal GC, inflammation due to chronic gastric acid secretion may be the driving force in carcinogenesis.[44,45] In Asians, the intestinal type is more prevalent than the diffuse type,[43,45] which is associated with higher rate of ERβ expression.[20,22,23] Expression rate of ERβ by immunohistochemistry in GC has been shown to be higher in Asians than in Western populations.[20,22,27] In our study, Japanese patients had a significantly higher incidence of more differentiated cancer than US patients, implying that the influence of estrogen may differ according to the cohorts. These GC-specific diversities based on physiologic and clinicopathologic backgrounds among regions may account for the different effect of the *ESR2* gene in this study.

Our findings may also have clinical implications for future treatment insofar that the estrogen pathway could serve as a potential target for adjuvant treatment strategies to improve survival in patients with GC. A meta-analysis of epidemiologic studies has shown that postmenopausal hormone replacement therapy reduces the incidence of GC.[46] It is likely that the use of estrogen before activation of aberrant pathways will protect against developing GC; however, it is uncertain whether estrogen will be able to restrain tumor progression when precancerous processes are already initiated. In addition, given the opposite effect of ERβ gene variation observed in our study, GC patients may respond differently to estrogen pathway-targeted therapy according to their race. Moreover, genetic variations in *ESR2* may also be of predictive significance enabling us to select individuals who achieve maximum benefit from intensive chemotherapies. Further clinical validation studies not only in adjuvant GC but also in metastatic GC are needed to confirm these presumptions.

In an exploratory subgroup analysis, the worse survival associated with the C allele of the *ESR2* rs2978381 SNP was also observed among Caucasian patients ($N = 90$) in the LAC cohort (adjusted hazard ratio 1.51, $P = 0.23$). However, this association did not differ significantly compared with the association in the Japanese cohort, which is likely due to reduced sample size. Body mass index (BMI) had a positive association with plasma estradiol (E2) levels.[3] Moreover, the plasma E2 levels in postmenopausal women can be affected by adiposity, ethnicity and lifestyle factors such as diet and smoking.[3,47,48] It is well established that plasma E2 levels are positively associated with BMI in Asian and Caucasian

populations. The higher E2 levels in the US population in each gender may be related to the higher BMI compared with those in Japanese.[49,50] The difference of E2 levels derived from environmental and non-genetic factors may impact the protective effect of estrogen in GC, probably explaining the findings in our study. In addition, estrogen is considered to have a protective role against development of GC, leading to a globally significant lower incidence and delayed onset in females compared with males.[51,52] We therefore performed a sub-analysis by gender to evaluate a gender-specific difference in the prognostic impact of 40 the ERβ gene SNPs. However, no clear gender-related effect of *ESR2* variations could be observed in the Japanese and US cohorts (Supplementary Table 4).

This study is hypothesis-generating and has a number of limitations that need to be considered. The number of patients in each cohort is modest for a polymorphism study and may lack the adequate statistical power to investigate the associations with clinical outcome. In addition, selection bias cannot be excluded due to the retrospective study design. One hundred and nine (64%) out of 169 Japanese patients received adjuvant chemotherapy, which was not associated with OS. Information about adjuvant chemotherapy was missing in approximately half of the patients of the US cohort. Nevertheless, our analysis allowed us to assume that *ESR2* gene variations may serve as prognostic markers in an ethnic-dependent manner although we cannot exclude that the results were attributable to chance. Owing to small sample size of Asian patients in the LAC cohort, we were not able to validate the associations between LAC Asian patients and Japanese patients (data not shown). Therefore, translational studies including larger patient cohorts with same ethnicity are necessary to validate our findings.

In conclusion, our results provide evidence that genetic variations in a promoter region of the *ESR2* gene are significantly associated with survival in patients with locally advanced GC. Our data also suggest that the prognostic value of *ESR2* gene variations may vary in an ethnic-dependent manner. Further functional correlative preclinical analyses and external clinical validation studies are needed to confirm our results.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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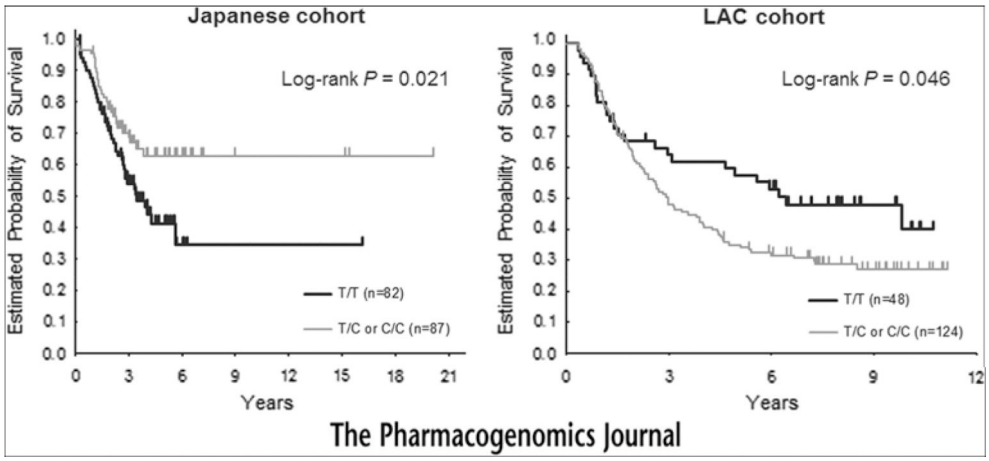

Figure 1.

Probability of overall survival by *ESR2* rs2978381 in the Japanese and LAC cohorts, (left) Japanese cohort, (right) LAC cohort.

Table 1.

Analyzed polymorphisms in genes encoding ERβ and their functional significance

rs number	Location of polymorphism	Base exchange	Published data	Forward primer (5′–3′) Reverse primer (5′–3′)	MAF[a]
rs1271572	Promoter chromosome 14:64295199	C > A	Associated with breast and prostate cancer risk (J Biomed Sci 2013;20:32; Cancer Res 2000;60:702) T/T genotype inhibits ESR2 expression (J Biomed Sci 2013;20:32)	GCAGCTGTTGCTGATGAAAA CCCCCTCGTCTTCCTCTATT	0.46
rs2978381	Promoter chromosome 14:64299934	T > C	Associated with colorectal cancer survival (Cancer Res 2013;73:767)	GCCAGGATCTCTGCATTCTC AGGCTGAGGAAGCACTTGAC	0.50
rs3020443	Promoter chromosome 14:64325622	T > G	Associated with colorectal cancer survival (Cancer Res 2013;73:767)	GGAGAAAAAGGTTTAGGATGTGA TTATCAGTGGACCCATGCAA	0.19

Abbreviation: MAF, Minor allele frequency.

[a]Frequency from Ensemble database: http://www.ensembl.org/index.html.

Table 2.

Baseline clinical characteristics of Japanese and LAC patient cohorts

	Japanese (N = 169)		*LAC (N = 172)*		P-*value*[a]
	N	***%***	**N**	***%***	
Gender					
Male	109	64	115	67	0.65
Female	60	36	57	33	
Age (year)					
Median (range)	67 (31–88)		61 (26–74)		< 0.001
< 65	65	38	101	59	< 0.001
⩾ 65	104	62	71	41	
Stage					
I – II	81	48	84	49	0.34
III–IV	88	52	74	43	
Unknown[b]			14	8	
T-category					
T1 – T2	78	46	54	31	0.017
T3 – T4	91	54	108	63	
Unknown[b]			10	6	
N-category					
N0 – N1	121	72	82	48	0.003
N2 – N3	48	28	66	38	
Unknown[b]			24	14	
Primary tumor site					
Proximal	46	27	77	45	< 0.001
Distal	123	73	95	55	
Tumor differentiation					
Differentiated	68	40	44	26	0.018
Undifferentiated	101	60	114	66	
Unknown[b]			14	8	
Ethnicity					
Asian	169	100	30	17	NA
Caucasian			90	52	
Hispanic			38	22	
African American			14	8	

[a] Based on χ^2-test or Wilcoxon test, whichever was appropriate.

[b] Excluded in χ^2-test.

Table 3.

Associations between *ESR2* SNPs and overall survival in the Japanese and LAC cohorts

Genotype	Japanese cohort (N = 169)						LAC cohort (N = 172)					
	Univariate analysis				Multivariable analysis[a]		Univariate analysis				Multivariable analysis[a]	
	N	5-year survival rate ± SE	HR (95% CI)	P-value	HR (95% CI)	P-value	*N*	5-year survival rate ± SE	HR (95% CI)	P-value	HR (95%CI)	P-value
ESR2 rs1271572				**0.0051**		**0.025**				0.82		0.36
C/C	59	0.73 ± 0.06	1		1		59	0.42 ± 0.06	1		1	
C/A	73	0.44 ± 0.07	2.59 (1.40, 4.81)		2.32 (1.22, 4.40)		74	0.38 ± 0.06	0.96 (0.63, 1.45)		1.03 (0.62, 1.70)	
A/A	35	0.40 ± 0.10	2.44 (1.23, 4.87)		2.34 (1.14, 4.78)		34	0.51 ± 0.09	0.84 (0.50, 1.43)		0.68 (0.37, 1.27)	
C/A or A/A*	108	0.41 ± 0.06	2.54 (1.41, 4.57)	**0.0012**	2.33 (1.27, 4.27)	**0.007**	108	0.42 ± 0.05	0.92 (0.62, 1.36)	0.67	0.89 (0.56, 1.43)	0.64
ESR2 rs2978381				0.065		0.40				0.084		0.077
T/T	82	0.41 ± 0.06	1		1		48	0.58 ± 0.07	1		1	
T/C	69	0.64 ± 0.06	0.55 (0.33, 0.93)		0.75 (0.44, 1.27)		86	0.30 ± 0.05	1.67 (1.05, 2.65)		1.80 (1.08, 2.99)	
C/C	18	0.57 ± 0.13	0.66 (0.30, 1.48)		0.64 (0.28, 1.47)		38	0.46v0.08	1.34 (0.76, 2.34)		1.41 (0.71, 2.81)	
T/C or C/C*	87	0.63 ± 0.06	0.58 (0.36, 0.93)	**0.021**	0.72 (0.44, 1.18)	0.19	124	0.35 ± 0.04	1.56 (1.00, 2.44)	**0.046**	1.71 (1.04, 2.81)	**0.036**
ESR2 rs3020443				**0.0040**		**0.043**				0.54		0.090
T/T	119	0.44 ± 0.05	1		1		97	0.45 ± 0.05	1		1	
T/G*	42	0.75 ± 0.07	0.39 (0.20, 0.76)		0.50 (0.25, 0.98)		62	0.37 ± 0.06	1.12 (0.77, 1.64)		1.46 (0.94, 2.26)	
G/G*	7						9					

Abbreviations: CI, confidence interval; HR, hazard ratio; LAC, Los Angeles County. Based on the log-rank test in the univariate analysis and Wald test in the multivariable analysis within Cox regression model.

[a]Adjusted for age, performance status, stage and tumor site in the Japanese cohort; adjusted for stage, T-category, N-category, and tumor differentiation and stratified by race in the LAC cohort. Bold characters; significant.

[b]Combined in the analysis in the dominant genetic model.