The Number of Lymph Node Metastases in Gastric Cancer Correlates with the Angiotensin I–Converting Enzyme Gene Insertion/Deletion Polymorphism

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Abstract

Purpose: In the present study, we aimed to substantiate the putative significance of angiotensin I–converting enzyme (ACE) on gastric cancer biology by investigating the influence of its gene polymorphism on gastric cancer progression.

Experimental Design: Genomic DNA was purified from peripheral blood mononuclear cells or tissue specimens. Amplified *ACE* gene fragments were separated on agarose gels. *D* or *I* alleles were identified by the presence of 190- or 490-bp fragments, respectively. Local expression of ACE was investigated by immunohistochemistry.

Results: Twenty-four of 113 (21%) gastric cancer patients had the *II*, 57 (51%) the *ID*, and 32 (28%) the *DD* genotype. The distribution of the *ACE* genotypes did not differ significantly from the control group of 189 patients without gastric cancer. However, the *ACE* genotypes correlated with the number of lymph node metastases and the Unio Internationale Contra Cancrum (UICC) tumor stage. Patients with the *II* genotype had a highly significantly smaller number of lymph node metastases ($P < 0.001$) and a significantly lower UICC tumor stage ($P = 0.01$) than patients with the *DD* genotype. No correlation was found between tumor type, tumor location, local tumor growth, distant metastases, and the *ACE* genotype. The expression of ACE in gastric cancer was investigated by immunohistochemistry in 100 of 113 patients. ACE was expressed by endothelial cells in all (100%) specimens and by tumor cells in 56 (56%) specimens.

Conclusions: Our study shows that ACE is expressed locally in gastric cancer and that the gene polymorphism influences metastatic behavior.

Angiotensin I–converting enzyme (ACE, CD143) is a type I cell surface zinc metallopeptidase, expressed by many cell types of various organs and tissue types, including vascular endothelial cells, epithelial cells of the small intestine, kidney tubular cells, mononuclear cells, and fibroblasts (1). ACE cleaves C-terminal dipeptides from oligopeptide substrates with an unhindered C terminus. It generates angiotensin II, the major effector of the renin-angiotensin system. Angiotensin II is a potent vasoconstrictor and activator of aldosterone and induces reabsorption of sodium and raises the blood pressure. ACE inhibitors are used in the treatment of heart failure, hypertension, and coronary heart disease.

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There is mounting evidence that ACE also participates locally in the pathology of carcinomas (1, 2). ACE is differentially expressed in several malignancies (1) and influences tumor cell proliferation, tumor cell migration, angiogenesis, and metastatic behavior (2–4). Inhibition of ACE activity suppresses tumor growth and angiogenesis *in vitro* and *in vivo* in animal models (5–9), and epidemiologic studies have provided evidence that ACE inhibitors may decrease the risk and mortality rate of cancer (10, 11). ACE inhibitors are currently under consideration as "novel" antineoplastic treatment and cancer prevention strategies (2, 11). A polymorphism in the *ACE* gene, consisting of the insertion (*I*) or deletion (*D*) of a 287-bp DNA fragment in intron 16, accounts for 20% to 50% of the variance in ACE expression or activity in blood and tissues among individuals (12–14). Medeiros et al. (15) showed that this *ACE* gene polymorphism is significantly associated with advanced disease in prostate cancer, and we recently found a significant up-regulation of ACE mRNA and protein levels in gastric cancer (16). Intrigued by these observations, we aimed to elucidate the putative significance of ACE on gastric cancer biology by investigating the influence of the gene polymorphism on gastric cancer progression.

Materials and Methods

Patient populations and samples. Samples from 113 gastric cancer patients operated on between 1995 and 2002 were retrieved from the archive of the Department of Pathology (Table 1). Sera or tissue

Table 1. Patient characteristics

			I/D genotype			
		Total	***II***	***ID***	***DD***	***P***
Controls						
Patients	[*n* (%)]	189	41 (22)	95 (50)	53 (28)	
Age	[mean ± SD]	67.7 ± 6.1	69.2 ± 6.2	67.9 ± 6.4	65.8 ± 5.0	n.s.
Gender	Men [*n* (%)]	75 (40)	17 (42)	38 (40)	20 (38)	n.s.
	Women [*n* (%)]	114 (60)	24 (59)	57 (60)	33 (82)	
Gastric cancer patients						
Patients	[*n* (%)]	113	24 (21)	57 (50)	32 (28)	n.s.
Age	[mean ± SD]	63.8 ± 12.6	64.1 ± 12.1	64.5 ± 11.6	62.3 ± 14.7	n.s.
Gender	Men [*n* (%)]	82 (73)	15 (63)	43 (75)	24 (75)	n.s.
	Women [*n* (%)]	31 (27)	9 (38)	14 (25)	8 (25)	
Tumor type	Intestinal [*n* (%)]	68 (60)	14 (58)	38 (67)	16 (50)	n.s.
	Diffuse [*n* (%)]	33 (29)	4 (17)	17 (30)	12 (38)	
	Mixed [*n* (%)]	8 (7)	4 (17)	2 (4)	2 (6)	
	Indeterminate [*n* (%)]	4 (4)	2 (8)	0	2 (6)	
Localization	Gastroesophageal junction [*n* (%)]	19 (17)	5 (21)	8 (14)	6 (19)	n.s.
	Corpus/Fundus [*n* (%)]	75 (66)	15 (63)	41 (72)	19 (59)	
	Antrum/Pylorus [*n* (%)]	19 (17)	4 (17)	8 (14)	7 (22)	
T category	pT_1 [*n* (%)]	15 (13)	6 (25)	7 (12)	2 (6)	n.s.
	pT_{2a} [*n* (%)]	18 (16)	4 (17)	8 (14)	6 (19)	
	pT_{2b} [*n* (%)]	37 (33)	5 (21)	19 (33)	13 (41)	
	pT_3 [*n* (%)]	33 (29)	7 (29)	19 (33)	7 (22)	
	pT_4 [*n* (%)]	10 (9)	2 (8)	4 (7)	4 (13)	
Mean number of lymph nodes	studied [mean ± SD]	26.5 ± 12.5	26.9 ± 10.8	25.7 ± 14.3	27.7 ± 10.5	n.s.
	with metastases [mean ± SD]	6.9 ± 9.0	3.0 ± 5.3	7.0 ± 9.6	10.0 ± 9.1	0.002
N category	pN_0 [*n* (%)]	32 (28)	13 (54)	15 (26)	4 (13)	0.004
	pN_1 [*n* (%)]	43 (38)	8 (33)	24 (42)	11 (34)	
	pN_2 [*n* (%)]	21 (19)	2 (8)	12 (21)	7 (22)	
	pN_3 [*n* (%)]	17 (15)	1 (4)	6 (11)	10 (31)	
M category	pM_0 [*n* (%)]	86 (76)	21 (88)	44 (77)	21 (66)	n.s.
	pM_1 [*n* (%)]	25 (24)	3 (13)	13 (23)	11 (34)	
UICC tumor stage	Stage IA [*n* (%)]	10 (9)	5 (21)	3 (5)	2 (6)	0.02
	Stage IB [*n* (%)]	23 (20)	7 (29)	14 (25)	2 (6)	
	Stage II [*n* (%)]	18 (16)	2 (8)	13 (23)	3 (9)	
	Stage IIIA [*n* (%)]	18 (16)	4 (17)	8 (14)	6 (19)	
	Stage IIIB [*n* (%)]	6 (5)	1 (4)	4 (7)	1 (3)	
	Stage IV [*n* (%)]	38 (34)	5 (21)	15 (26)	18 (56)	
ACE inhibitor	No [*n* (%)]	38	10 (91)	18 (82)	10 (77)	n.s.
	Yes [*n* (%)]	8	1 (9)	4 (18)	3 (23)	
Immunohistochemistry	ACE^- tumor cells [*n* (%)]	44 (44)	8 (38)	22 (43)	14 (50)	n.s.
	ACE^+ tumor cells [*n* (%)]	56 (56)	13 (62)	29 (57)	14 (50)	
	ACE^- endothelial cells [*n* (%)]	0 (0)	0 (0)	0 (0)	0 (0)	
	ACE^+ endothelial cells [*n* (%)]	100 (100)	21 (100)	51 (100)	28 (100)	

NOTE: n.s. denotes statistically not significant.

samples used in the present study were obtained from patients who had undergone either complete (85 of 113 cases) or partial (28 of 113 cases) gastrectomies as well as from 189 control patients without gastric cancer (Table 1). Criteria of exclusion were the confirmation of gastric cancer only by biopsy and the diagnosis of adenocarcinoma arising from Barrett's esophagus. This study was carried out in accordance with the guidelines of the Ethics Committee of the University of Magdeburg. Data were encoded to ensure patient protection.

Determination of the ACE genotype. Genomic DNA was purified from peripheral blood mononuclear cells or tissue specimens by using the QIAamp DNA blood kit (Qiagen) or the E.Z.N.A. Tissue DNA Mini Kit (PEQLAB Biotechnologie GmbH, Elangen, Germany). DNA was dissolved at 100 ng/μL in 10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0. The *ACE* genotype of patients and healthy controls was determined by PCR according to Yoshida et al. (17). A typical 50-μL reaction mixture consisted of 25 μL of HotStarTaq Master Mix (Qiagen), 100 ng of genomic DNA, 250 pmol of each primer (ACE-US: 5′-CTggAgAC-

CACTCCCATCCTTTCT; ACE-DS: 5′-gATgTggCCATCACATTCgTCAgAT), and 5% (v/v) DMSO. An initial 15-minute denaturation at 95°C was followed by 40 cycles for 1 minute at 64°C, 1 minute at 72°C, and 0.6 minute at 94°C. Amplified *ACE* gene fragments were separated on 1.6 % agarose gels and visualized by ethidium bromide staining. *D* or *I* alleles were identified by the presence of 190- or 490-bp fragments, respectively (Fig. 1). An independent PCR analysis was carried out for each sample.

Histology and immunohistochemistry. For histology, tissue samples were fixed in 10% neutralized formalin and embedded in paraffin. Deparaffinized sections were stained using H&E. Gastric cancer was classified according to Laurén (18) into diffuse, intestinal, and mixed type (Table 1). The tumor-node-metastasis (TNM) stage was determined according to the Unio Internationale Contra Cancrum (UICC) guidelines (19) and was based on histologic confirmation. Immunostaining was done as described elsewhere (16). Omission of primary antibodies served as negative controls.

Statistical analysis. Differences between populations were evaluated using a χ^2 test or one-way ANOVA for comparison of group means. Continuous variables were compared by means of the unpaired Student's *t* test. A *P* value less than 0.05 was considered to be "significant". However, for the comparison of subgroups, an α correction of the *P* value was carried out and a value of 0.016 was then considered to be significant.

Results

ACE gene polymorphism. Table 1 summarizes the clinical characteristics of the patients. The mean age of the gastric cancer patients was 63.8 ± 12.6 years, including 82 men and 31 women, and the mean age of the control patients was 67.7 ± 6.1 years, including 75 men and 114 women. Although the control group included significantly more women than the gastric cancer group, the gene polymorphism was not associated with patient gender. Twenty-four of 113 (21%) patients with gastric cancer had the *II*, 57 (50%) the *ID*, and 32 (28%) the *DD* genotype. The distribution of the *ACE* genotypes did not differ significantly from the control group or from the distribution predicted by the Hardy-Weinberg equilibrium (Table 1).

Univariate analyses showed that the *ACE* genotype correlated highly significantly with the number of lymph node metastases ($P < 0.002$) and nodal involvement in terms of N category ($P = 0.004$), and significantly with the UICC tumor stage (Table 1; $P = 0.02$). Comparing the group of tumors comprised of UICC stages IA, IB, and II with those of stages IIIA, IIIB, and IV results in a significant correlation between the *ACE* genotype and the UICC tumor stages ($P = 0.006$). Note that the UICC tumor stage is also dependent on the N category. On average, 26.5 ± 12.5 lymph nodes were studied per patient with no significant differences in the number of lymph nodes investigated in each *ACE* genotype group (i.e., *II* versus *ID* versus *DD*; Table 1). Thus, the total number of lymph nodes investigated did not influence the number of lymph node metastases observed (Table 1).

We then compared gastric cancer patients with the *II* and the *DD* genotype. In these two subgroups, 21 (88%) patients with the *II* genotype were found to have either no ($n = 0$) or less than six ($n = 1$) lymph node metastases, whereas 17 (53%) patients with the *DD* genotype were found to have more than six ($n = 2$ or 3) lymph node metastases ($P < 0.001$; odds ratio, 7.9; 95% confidence interval, 1.97-32.01). Similarly, 14 (58%) patients with the *II* genotype were found to be in stage I or II gastric cancer, whereas 25 (78%) patients with the *DD* genotype were found to be in stage III to IV gastric cancer ($P = 0.01$; odds ratio, 5.0; 95% confidence interval, 1.56-16.06). Tumor type (classification according to Laurén), tumor location (gastroesophageal junction, corpus/fundus, and antrum/pylorus), or local tumor growth (T category) was not associated with the *ACE* genotype.

A drug history was available from 46 gastric cancer patients. Only eight patients had received ACE inhibitors for reasons unrelated to gastric cancer, resulting in a sample size too small to study any correlations between the administration of ACE inhibitors and the TNM categories or the UICC tumor stage.

Immunohistochemistry. Immunohistochemistry was done on paraffin-embedded tissue sections from 100 of 113 patients with gastric cancer. We examined the expression of ACE in the nontumorous foveolar and surface epithelium, any occurrences of intestinal metaplasia, endothelial cells, and the carcinomas. ACE was expressed in tumor epithelial cells of 56 (56%) and in endothelial cells of all (100%) patients (Fig. 2). Intestinal metaplasia was found in 29 patients and showed immunoreactivity at the brush border in 28 (97%) cases. ACE was not expressed by nonneoplastic and nonmetaplastic gastric foveolar and surface epithelium. The expression of ACE in gastric cancer cells did not correlate with the genotype (Table 1), the TNM categories, or the UICC tumor stage.

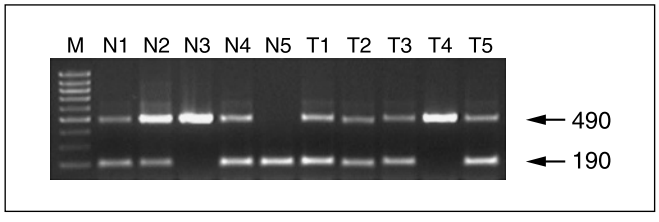

Fig. 1. Determination of *ACE* genotypes by PCR amplification. Amplified *ACE* gene fragments were separated on 1.6 % agarose gels and visualized by ethidium bromide staining. *D* or *I* alleles were identified by the presence of 190- or 490-bp fragments, respectively. *M,* molecular weight markers; *N1-N5,* control group; *T1-T5,* patients diagnosed with gastric cancer.

Discussion

Epidemiologic studies have investigated the association between the administration of ACE inhibitors and cancer risk, with no two studies reporting significant reductions in risk for the same type of cancer among patients taking ACE inhibitors (10, 11, 20–26). In contrast, experimental studies, investigating the influence of the renin-angiotensin system and ACE inhibitors on tumor growth, neoangiogenesis, and metastatic behavior, provide mounting evidence for the involvement of the renin-angiotensin system and ACE in tumor biology (5, 7–9). Despite the up-regulation of ACE observed in different solid human tumors (1), which underlines its potential significance for tumor biology, the discrepancy between the epidemiologic and the experimental studies has, until now, not been resolved.

The experimental studies have shown that the local renin-angiotensin system can influence tumor biology in different ways: (*a*) by promoting neoangiogenesis and by enhancing microvessel density in solid tumors, which is critical for tumor

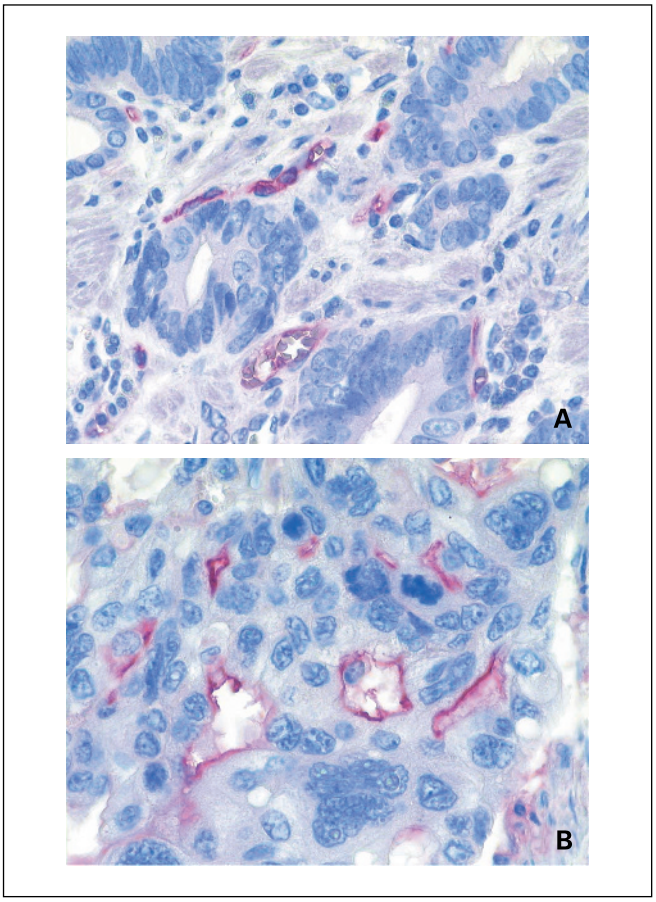

Fig. 2. Expression of ACE in gastric cancer. The distribution and expression pattern of ACE in gastric carcinomas was investigated by immunohistochemistry. ACE was found in endothelial cells (*A*) and tumor epithelial cells (*B*). Monoclonal anti-ACE antibody; hematoxylin counterstain; original magnification, ×400.

growth and is mediated in part by the vascular endothelial growth factors (3–5, 8, 9); (*b*) by promoting tumor cell proliferation (7, 28); and (*c*) by promoting the remodeling of the interstitial matrix (i.e., the mould in which tumor cells grow; ref. 29). Although these functions may be important for the biology of the tumor after induction, none of these putative pathophysiologic functions are initiating events in cancer development and biology, and there is no evidence to suggest that ACE functions either as a tumor suppressor gene or as an oncogene. The great majority of epidemiologic studies provide evidence that ACE inhibitors have no or only a minor effect on cancer risk (10, 20–26), and it is therefore probable that ACE activity affects tumor progression and disease outcome rather than incidence rate or tumor prevalence. The results of the epidemiologic study reported by Lever et al. (11) support this hypothesis, in that a significant inverse correlation was found between cancer mortality and the treatment with and the duration of administration of ACE inhibitors. Given the longer follow-up time period, a significant reduction in the risk of death from cancer in the patient group taking ACE inhibitors was able to be detected (11).

The serum and tissue expression, and hence the activity of ACE, is influenced by the insertion/deletion gene polymorphism, with an increased amount of ACE mRNA originating from the *D* allele (30). Recently, several studies have investigated the influence of this *I/D* gene polymorphism on cancer risk (15, 31–34), again with contradictory results. Cheon et al. (31) and Usmani et al. (34) did not find any correlation for renal and lung cancer patients. Haiman et al. (32) found a modest positive association between the *II* genotype and breast cancer risk. Koh et al. (33) found a significant link between the occurrence of the *DD ACE* gene polymorphism and the incidence of breast cancer. However, none of these studies considered tumor progression (i.e., tumor stage or mortality rates). Only two studies considered tumor stage or patient survival. Medeiros et al. (15) correlated the *ACE* gene polymorphism with the progression of prostate cancer and found a significant association between the *DD* genotype and the advancement of the disease. In a pilot study, Hajek et al. (35) correlated the *ACE* gene polymorphism with patient survival in leukemia and found that patients with the *II* genotype survived longer than those with the *DD* genotype.

In keeping with these observations, the distribution of different *ACE* gene alleles in our gastric cancer patients does not differ from the distribution in the general population, indicating that the risk to develop gastric cancer is not linked to a specific *ACE* genotype and hence enzyme expression or activity. Additionally, because patients with the *DD* genotype were significantly more commonly associated with a greater number of lymph node metastases and advanced UICC tumor stage, these findings support the hypothesis that ACE activity influences tumor progression, as opposed to tumor development. However, the pathophysiologic mechanism of ACE activity is not directly evident from our study.

Despite the local expression of the ACE protein observed in endothelial and tumor cells in the gastric cancer specimens, the ACE polymorphism was not linked to the T or M categories. It is therefore unlikely that ACE influences gastric cancer biology exclusively through variables that are known to have an effect on local tumor growth or distant metastases, such as tumor cell proliferation, neoangiogenesis of blood vessels, or tumor cell migration. Instead, the highly significant correlation between the *ACE* genotype and the number of lymph node metastases indicates that the *D* allele–related higher expression of ACE may promote the establishment of lymphatic metastases. However, the actual biological mechanism(s) need to be further investigated.

In summary, we provide strong evidence that ACE influences tumor progression and metastatic behavior but not the prevalence or incidence of gastric cancer. Recently, it was shown experimentally that a combination therapy including ACE inhibitors may prove useful in cancer treatment (7). A large number of ACE inhibitors are available, which are inexpensive, well tolerated, and seem to reduce the risk of side effects of other chemotherapeutics (32). The poor prognosis of gastric cancer is often related to the presentation in an advanced tumor stage and is most strongly influenced by lymph node involvement (odds ratio, 4.6; ref. 36). Thus, ACE inhibitors might prove useful for the treatment of gastric cancer, particularly by preventing or reducing nodal spread. The simple and rapid procedure of determining the *ACE* genotype could assist in tailoring the administration of ACE inhibitors by directing the treatment towards the higher risk patients (i.e., those with the *DD* or *ID* genotypes).

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