The Angiotensin II/Angiotensin II Receptor System Correlates with Nodal Spread in Intestinal Type Gastric Cancer

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Abstract

We aimed to substantiate the putative significance of angiotensin II receptor type 1 (AT1R) and type 2 (AT2R) for gastric cancer biology by investigating the correlation of their expression with various clinicopathologic variables and patient survival. Local expression of AT1R, AT2R, and angiotensin-converting enzyme (ACE) was investigated by immunohistochemistry in tumor and corresponding non-tumor specimens obtained from 100 patients with gastric cancer, and compared with the ACE insertion/deletion gene polymorphism. AT1R and AT2R were found in the tumor epithelial cells of 26 (26%) and 95 (95%) patients, respectively. AT1R was significantly more prevalent ($P < 0.001$) in intestinal type gastric cancer than in diffuse type gastric cancer. In intestinal type gastric cancer, its expression correlated with the N category ($P = 0.009$) and the International Union Against Cancer tumor stage ($P = 0.024$). $AT1R^+$ intestinal type gastric cancers had a larger number of lymph node metastases ($P = 0.026$), a higher International Union Against Cancer tumor stage ($P = 0.032$), and a shorter survival time ($P = 0.009$) than $AT1R^-$ tumors. Multivariate analysis with lymph nodes as a dependent variable showed that AT1R status and ACE-I/D gene polymorphism are independent risk factors. Irrespective of the genotype, $AT1R^+$ gastric cancers had a relative risk of lymph node metastases of 4.40 (95% confidence interval, 1.30-14.86). When the ACE genotype was included, the relative risk of having lymph node metastases increased considerably in $AT1R^+$ tumors being heterozygous or homozygous for the ACE D allele (odds ratio, 19.00; 95% confidence interval, 1.45-248.24). Our study shows that AT1R and AT2R are expressed locally in gastric cancer and that the combination of AT1R expression and ACE I/D gene polymorphism correlates with nodal spread in intestinal type gastric cancer. (Cancer Epidemiol Biomarkers Prev 2007;16(6):1206–12)

Introduction

Gastric cancer is among the leading causes of cancer-related deaths worldwide (1). Its prognosis is poor, as it is usually first diagnosed in advanced stages, when curative treatment is impossible. At the time of diagnosis, most gastric cancers have already spread to the lymph nodes, which is the most significant independent prognostic factor for this disease: the 5-year survival rate for patients with lymph node metastases is $9.8 \pm 4\%$ compared with $58 \pm 11\%$ for patients without lymph node metastases (2, 3). Identifying patients with an increased risk of developing lymph node metastasis in gastric cancer is of high importance because this would influence both the extent of surgical resection (D1 or D2 resection), and/or the indication for neoadjuvant or adjuvant therapy.

Recently, we have shown that the angiotensin-converting enzyme (ACE) is expressed locally in gastric cancer (4), and that the insertion/deletion gene polymorphism correlates with the metastatic spread (5). Patients with the DD genotype were significantly more commonly associated with a greater number of lymph node metastases and advanced International Union Against Cancer (UICC) tumor stage, than patients with the ID or II genotype (5). ACE is a type I cell surface zinc metallopeptidase that generates angiotensin II, the major effector of the angiotensin II/angiotensin II receptor system. Angiotensin II binds with high affinities to the two G protein–coupled cell surface receptors, angiotensin II receptor type 1 (AT1R) and type 2 (AT2R), which often mediate different effects. Binding to AT1R has proliferative, proinflammatory, proangiogenic, and antiapoptotic effects (6), whereas binding to AT2R inhibits mitogen-activated protein kinase activity by the induction of phosphatases, and has proapoptotic and antiproliferative effects (6). However, angiogenesis also seems to be regulated by AT2R working in concert with AT1R (7).

Intrigued by our previous observations, we aimed to further substantiate the putative significance of the angiotensin II/angiotensin II receptor system in gastric cancer biology by investigating the correlation of AT1R and AT2R expression with various clinicopathologic variables and patient survival.

Materials and Methods

Patient Populations and Samples. Samples from 100 patients with gastric cancer operated on between 1995 and 2002 were retrieved from the archives of the Department of Pathology, Otto-von-Guericke University, Magdeburg, Germany (Table 1). Tissue samples used in the present study were obtained from patients who had undergone either complete (75 of 100 cases) or partial (25 of 100 cases) gastrectomies. Data were encoded to ensure patient protection. This study population was part of a previous investigation, in which ACE insertion/deletion (I/D) gene polymorphisms and ACE expression were studied in detail (5). No patient had received any adjuvant therapy (chemotherapy and/or radiation). This

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Table 1. Characteristics of entire patient population (n = 100)

Patients with gastric cancer	Total	AT1R$^-$	AT1R$^+$	P	AT2R$^-$	AT2R$^+$	P
Patients, n (%)	100	74 (74)	26 (26)		5 (5)	95 (95)	
Age (mean ± SD)	63.8 ± 12.5	63.3 ± 13.2	65.2 ± 10.2	0.475	59.6 ± 9.9	64.0 ± 12.6	0.383
Gender							
Men, n (%)	74 (74)	53 (72)	21 (28)	0.443	5 (7)	69 (93)	0.323
Women, n (%)	26 (26)	21 (81)	5 (19)		0	26 (100)	
Tumor type							
Intestinal, n (%)	59 (59)	39 (66)	20 (34)	<0.001*	4 (7)	55 (93)	1.0*
Diffuse, n (%)	29 (29)	29 (100)	0		1 (3)	28 (76)	
Mixed, n (%)	8 (8)	3 (38)	5 (62)		0	8 (100)	
Indeterminate, n (%)	4 (4)	3 (75)	1 (25)		0	4 (100)	
Localization							
Gastroesophageal junction, n (%)	14 (14)	10 (71)	4 (29)	0.739	2 (14)	12 (86)	0.130
Corpus/fundus, n (%)	67 (67)	51 (76)	16 (24)		2 (3)	65 (97)	
Antrum/pylorus, n (%)	19 (19)	13 (68)	6 (32)		1 (5)	18 (95)	
T category							
pT_1, n (%)	13 (13)	12 (92)	1 (8)	0.337	1 (7)	12 (93)	0.073
pT_{2a}, n (%)	17 (17)	14 (82)	3 (18)		0	17 (100)	
pT_{2b}, n (%)	34 (34)	22 (65)	12 (35)		2 (6)	32 (94)	
pT_3, n (%)	27 (27)	20 (74)	7 (26)		0	27 (100)	
pT_4, n (%)	9 (9)	6 (67)	3 (33)		2 (22)	7 (78)	
Lymph nodes							
Patients with metastases, n (%)	68 (68)	46 (62)	22 (84)	0.033	3 (60)	65 (69)	0.501
Studied (mean ± SD)	26.3 ± 12.3	25.7 ± 11.2	27.8 ± 14.9	0.521	32.0 ± 10.4	26.0 ± 12.3	0.340
No. with metastases (mean ± SD)	7.1 ± 8.9	5.7 ± 8.1	10.7 ± 10.1	0.029	1.2 ± 1.0	7.3 ± 9.0	<0.001
Mean with metastases, mean ± SD (%)	29.5 ± 31.8	26.4 ± 32.2	37.5 ± 30.0	0.122	4.8 ± 3.9	30.6 ± 32.1	<0.001
N category							
pN_0, n (%)	28 (28)	25 (89)	3 (11)	0.077	1 (4)	27 (96)	0.608
pN_1, n (%)	36 (36)	27 (75)	9 (25)		3 (8)	33 (92)	
pN_2, n (%)	21 (21)	13 (62)	8 (38)		0	21 (100)	
pN_3, n (%)	15 (15)	9 (60)	6 (40)		1 (7)	14 (93)	
M category							
pM_0, n (%)	74 (74)	55 (74)	19 (26)	1.0	4 (5)	70 (95)	1.0
pM_1, n (%)	26 (26)	19 (73)	7 (27)		1 (4)	25 (96)	
UICC tumor stage							
Stage IA, n (%)	9 (10)	8 (89)	1 (11)	0.104	0	9 (100)	0.908
Stage IB, n (%)	20 (20)	18 (88)	2 (10)		2 (10)	18 (90)	
Stage II, n (%)	16 (16)	12 (80)	4 (20)		1 (6)	15 (94)	
Stage IIIA, n (%)	15 (15)	12 (80)	3 (20)		0	15 (100)	
Stage IIIB, n (%)	5 (5)	2 (40)	3 (60)		0	5 (100)	
Stage IV, n (%)	35 (32)	22 (63)	13 (37)		2 (6)	33 (94)	
ACE genotype							
II, n (%)	21 (21)	15 (71)	6 (29)	0.830	0	21 (100)	0.703
ID, n (%)	51 (51)	37 (73)	14 (28)		3 (6)	48 (94)	
DD, n (%)	28 (28)	22 (79)	6 (21)		2 (7)	26 (93)	
ACE immunohistochemistry							
ACE-positive tumor cells, n (%)	56 (56)	37 (66)	19 (34)	0.065	2 (4)	54 (96)	0.652
ACE-negative tumor cells, n (%)	44 (44)	37 (84)	7 (16)		3 (7)	41 (93)	
AT1R/AT2R immunohistochemistry							
Tumor cells		74 (74)	26 (26)	<0.001	5 (5)	95 (95)	<0.001
Nonneoplastic foveolar epithelium		98 (98)	2 (2)		100 (100)	0	

*Comparing only intestinal with diffuse type gastric cancer.

study is in accordance with the guidelines of the Ethics Committee of the University of Magdeburg and all patients gave written informed consent for molecular analyses.

Histology. 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 (8) into diffuse, intestinal, and mixed tumor types (Table 1). The tumor-node-metastasis stage was determined according to the UICC guidelines and was based on histologic confirmation.

Immunohistochemistry. Immunostaining was done with affinity-purified polyclonal antibodies directed against AT1R (dilution, 1:20) or AT2R (1:50; both from BioTrend). Following antigen retrieval (AT1R: Na citrate, 3 × 10 min, 600 W; AT2R: EDTA, 8 min, 12 min, 450 W), incubation with the primary antibodies was done in a moist chamber at 37°C for 1 h. Goat anti-rabbit IgG (30 min, room temperature; Immunotech) served as a secondary antibody. Slides were washed between steps with TBS. Immunoreactions were visualized via an avidin-biotin complex, using the Vectastain ABC alkaline phosphatase kit (distributed by CAMON), with Fast Red/Naphthol Mx (Immunotech) as chromogen. The specimens were counterstained with hematoxylin. Immunostaining for ACE was done as described elsewhere (4). Omission of primary antibodies served as negative controls.

For the quantification of the immunohistochemical results, a numerical scoring system was applied. The observed expression of AT1R and AT2R in epithelial cells was assessed using two categories. Category A documented the number of immunoreactive epithelial cells as 0 (no immunoreactive cells), 1 (<10%), 2 (11-50%), and 3 (>50%). A positive case was defined as having a category A value of 1. Category B documented the intensity of the immunostaining as 0 (no immunostaining), 1 (weak), 2 (moderate), and 3 (strong). A case was considered positive when the addition of values for category A and B gave a minimum ''immunoreactivity score'' of 2. The maximum available score was 6. Lack of immunostaining of any tumor cells was categorized as negative.

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). DNA was dissolved at 100 ng/μL in 10 mmol/L of Tris-HCl and 1 mmol/L of EDTA (pH 8.0). The ACE genotype of patients and healthy controls was determined by PCR according to Yoshida et al. (9). 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′-CTggAgACCACTCCCATCCTTTCT; ACE-DS: 5′-gATgTggC-CATCAC-ATTCgTCAgAT), and 5% (v/v) DMSO. An initial 15 min denaturation at 95°C was followed by 40 cycles of 1 min at 64°C, 1 min at 72°C, and 0.6 min 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. An independent PCR analysis was carried out for each sample.

Statistical Analysis. Univariate tests were used to search for correlations between AT1R or AT2R expression and the various clinicopathologic variables, e.g., age, gender, tumor type, tumor, node, and metastasis category, and UICC stage (Table 1). The characteristics of two groups were compared using the *t* test (Satterthwaite's approximation of variance) and the Fisher exact test for continuous and categorical variables, respectively (10). The correlation between the number of lymph nodes and AT1R expression and ACE genotype was also evaluated using Fisher exact test. The immunohistochemical results, i.e., expression of AT1R and AT2R in neoplastic and nonneoplastic cells, were analyzed using the sign test modified by McNemar (11). A subsequent subgroup analysis was applied in a hierarchical manner to estimate the effects of AT1R and ACE genotype. The odds ratios were estimated from contingency tables. The dependency of patient outcome on AT1R expression and ACE genotype was evaluated using the Kaplan-Meier method and compared using the log-rank test (12). The interactions between the main factors were analyzed using multivariate analyses. A logistic regression was carried out to estimate the influence on tumor progression, i.e., lymph node metastases, and subsequently, an ANOVA was used to test whether the number of lymph node metastases provides further information. Finally, a Cox regression was used to evaluate the outcome with regard to survival time (13). All statistical decisions were made two-tailed with a critical probability of $\alpha = 5\%$ without α-adjustment, and P values supported the interpretation. For that reason, the results should be interpreted in an exploratory manner. All statistical analyses were carried out using the SPSS program version 13.

Results

AT1R and AT2R in Gastric Cancer. Table 1 summarizes the clinicopathologic characteristics of the patients. The mean age of the patients was 63.8 ± 12.5 years, and they consisted of

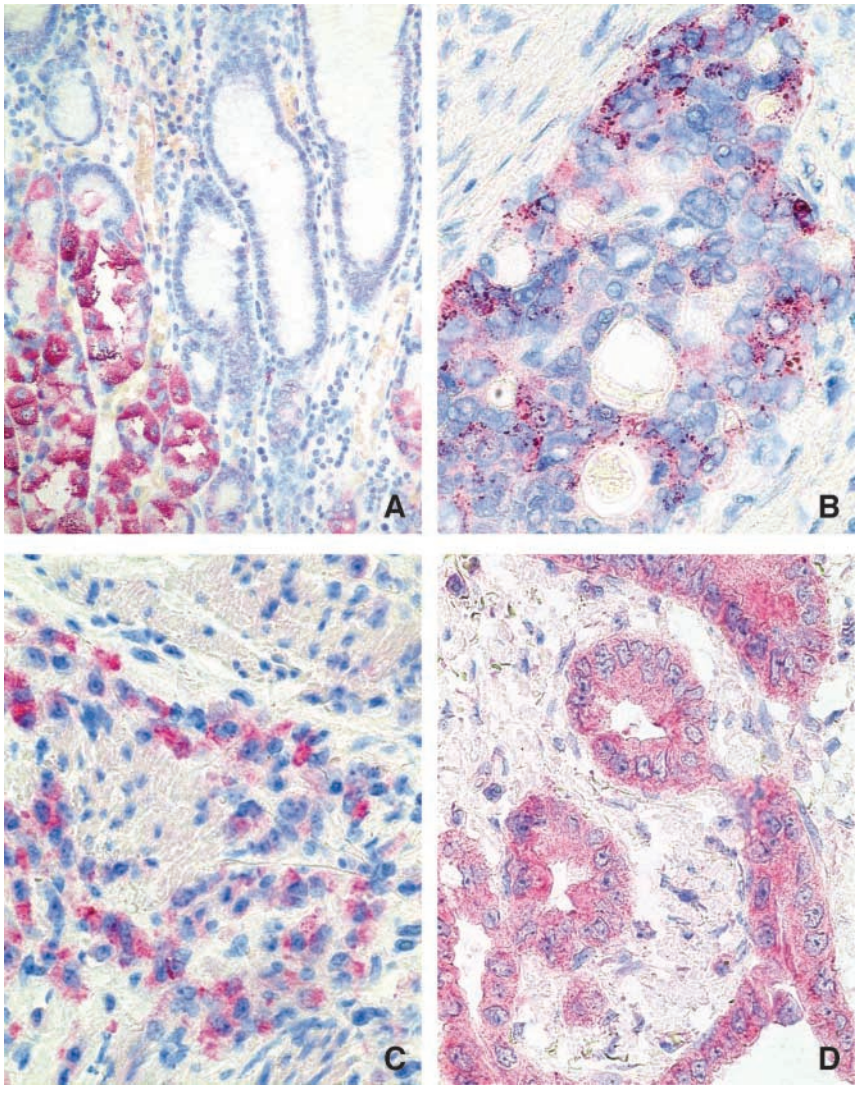

Figure 1. Expression of AT1R and AT2R in gastric cancer. The distribution and expression pattern of AT1R and AT2R in gastric carcinomas was investigated by immunohistochemistry. AT1R was found in oxyntic mucosa (**A**) and tumor cells of intestinal type gastric cancer (**B**). Note immunonegative foveolar epithelium in **A**. AT2R was found in tumor cells of diffuse (**C**) and intestinal (**D**) type gastric cancers. Polyclonal anti-AT1R antibody (**A** and **B**); polyclonal anti–AT2R antibody (**C** and **D**); hematoxylin counterstain (original magnifications, ×400).

74 men and 26 women (59 intestinal, 29 diffuse, 8 mixed, and 4 indeterminate type). We examined the expression of AT1R, AT2R, and ACE in gastric cancer and corresponding non-tumorous gastric tissue.

AT1R was found in the cytoplasm of tumor cells in 26 (26%) patients, and in the corresponding nonneoplastic and non-metaplastic foveolar and surface epithelium of 2 (2%) patients (Fig. 1). Intestinal metaplasia was found in 34 (34%) patients and showed immunoreactivity in 21 (62%) cases. AT1R was also detected in glandular epithelia of oxyntic mucosa from 62 specimens (Fig. 1).

AT2R was found in the cytoplasm of tumor cells from 95 (95%) patients. It was not expressed by the corresponding nonneoplastic and nonmetaplastic gastric foveolar and surface epithelium. Intestinal metaplasia showed immunoreactivity for AT2R in 31 of 34 (91%) cases. AT2R was also detected in glandular epithelia of oxyntic mucosa in 52 specimens (Fig. 1).

The study population investigated here was part of a previous investigation, in which the ACE insertion/deletion (I/D) gene polymorphism and ACE expression were studied in detail (5). ACE was expressed in tumor epithelial cells of 56 (56%), and in endothelial cells of all patients (100%), as described elsewhere (5). ACE was not expressed by nonneoplastic and nonmetaplastic gastric foveolar and surface epithelium. Twenty-one (21%) of the 100 patients with gastric cancer had the II genotype, 51 (51%) had the ID genotype, and 28 (28%) had the DD genotype. The distribution of the ACE I/D gene polymorphism in this population does not differ from the series studied previously (5).

Statistical Analyses. The univariate analyses showed that both AT1R and AT2R were significantly more commonly found in tumor cells than in nonneoplastic foveolar and surface epithelium ($P < 0.001$; McNemar; ref. 11). Furthermore, immunoreactive ($AT1R^+$) tumor cells were more commonly of the intestinal type than the diffuse type of gastric cancer ($P < 0.001$, Fisher exact test). In contrast, the expression of AT2R was not influenced by the tumor type.

We then studied the correlation between AT1R and AT2R expression and various other clinicopathologic variables (Table 1). The expression of either AT1R or AT2R did not correlate with age (*t* test), gender, tumor localization (gastroesophageal junction, corpus/fundus, antrum/pylorus), local tumor growth (T category), nodal status (N category), distant metastases (M category), UICC tumor stage, ACE genotype, or expression of ACE by tumor cells (Fisher exact test). However, a correlation was found between AT1R expression and the number of metastases, especially to the number of patients with metastases ($P = 0.033$; Fisher exact test) and to

Table 2. Characteristics of patients with intestinal, mixed, and indeterminate type gastric cancer (*n* = 71)

Patients with gastric cancer	Total	$AT1R^-$	$AT1R^+$	*P*
Patients, *n* (%)	71	45	26	
Age, mean ± SD	65.5 ± 10.7	65.7 ± 11.1	65.2 ± 10.2	0.824
Gender				
Men, *n* (%)	58 (81.7)	37 (63.8)	21 (36.2)	1.000
Women, *n* (%)	13 (18.3)	8 (61.5)	5 (38.5)	
Tumor type				
Intestinal, *n* (%)	59 (83)	39 (66)	20 (34)	0.301
Mixed, *n* (%)	8 (11)	3 (38)	5 (62)	
Indeterminate, *n* (%)	4 (6)	3 (75)	1 (25)	
Localization				
Gastroesophageal junction, *n* (%)	10 (14.1)	6 (60.0)	4 (40.0)	0.762
Corpus/fundus, *n* (%)	47 (66.2)	31 (66.0)	16 (34.0)	
Antrum/pylorus, *n* (%)	14 (19.7)	8 (57.1)	6 (42.9)	
T category				
pT_1, *n* (%)	12 (17)	11 (92)	1 (8)	0.072
pT_{2a}, *n* (%)	13 (18)	10 (77)	3 (23)	
pT_{2b}, *n* (%)	23 (32)	11 (48)	12 (52)	
pT_3, *n* (%)	17 (24)	10 (59)	7 (41)	
pT_4, *n* (%)	6 (8)	3 (50)	3 (50)	
Lymph nodes				
Patients with metastases, *n* (%)	47 (66)	25 (56)	22 (85)	0.011
Studied (mean ± SD)	26.8 ± 13.3	26.2 ± 12.3	27.8 ± 14.9	0.652
No. with metastases (mean ± SD)	7.3 ± 9.8	5.2 ± 9.1	10.7 ± 10.1	0.026
Mean with metastases, mean ± SD (%)	29.0 ± 32.6	23.6 ± 33.4	37.5 ± 30.0	0.081
N category				
pN_0, *n* (%)	22 (31)	19 (86)	3 (14)	0.023
pN_1, *n* (%)	24 (34)	15 (63)	9 (37)	
pN_2, *n* (%)	14 (20)	6 (43)	8 (57)	
pN_3, *n* (%)	11 (15)	5 (45)	6 (55)	
M category				
pM_0, *n* (%)	56 (79)	37 (66)	19 (27)	0.382
pM_1, *n* (%)	15 (21)	8 (53)	7 (47)	
UICC tumor stage				
Stage IA, *n* (%)	8 (10)	7 (88)	1 (12)	0.032
Stage IB, *n* (%)	16 (20)	14 (88)	2 (12)	
Stage II, *n* (%)	10 (16)	6 (60)	4 (40)	
Stage IIIA, *n* (%)	9 (15)	6 (67)	3 (33)	
Stage IIIB, *n* (%)	4 (5)	1 (25)	3 (75)	
Stage IV, *n* (%)	24 (32)	11 (46)	13 (54)	
ACE genotype				
II, *n* (%)	17 (23.9)	11 (64.7)	6 (35.3)	1.000
ID, *n* (%)	37 (52.1)	23 (62.2)	14 (37.8)	
DD, *n* (%)	17 (23.9)	11 (64.7)	6 (35.3)	
ACE immunohistochemistry				
ACE-positive tumor cells, *n* (%)	45 (63.4)	26 (57.8)	19 (42.2)	0.215
ACE-negative tumor cells, *n* (%)	26 (36.6)	19 (73.1)	7 (26.9)	

Table 3. Influence of ACE phenotype, AT1R phenotype, and ACE I/D genotype on nodal spread in the patient population, comprised of intestinal, mixed, and indeterminate type gastric cancer

Phenotype/genotype	No. of patients with lymph node metastases		Mean no. of lymph node metastases		Mean no. of lymph node metastases in nodal-positive cases		Odds ratios (95% CI)
	n (%)	95% CI	Mean / max	95% CI	Mean	95% CI	
$AT1R^-$ tumor cells + any ACE genotype (n = 45)	25 (56)	40.00-70.36	4.82 / 49	2.16-7.49	8.68	4.37-12.99	
$AT1R^+$ tumor cells + any ACE genotype (n = 26)	22 (85)	65.13-95.64	10.73 / 34	6.66-14.80	12.68	8.36-17.01	4.40 (1.30-14.86)
$AT1R^+$ tumor cells + ACE II genotype (n = 6)	3 (50)	11.81-88.19	5.00 / 22	−4.00-14.00	10.00	−15.94-35.94	
$AT1R^+$ tumor cells + ACE ID genotype (n = 14)	13 (93)	66.13-99.82	11.14 / 34	4.90-17.39	12.00	5.51-18.49	13.00 (0.98-172.95)
$AT1R^+$ tumor cells + ACE DD genotype (n = 6)	6 (100)	54.07-100.00	15.50 / 26	7.11-23.89	15.50	7.11-23.89	19.00* (1.45-248.24)

*ACE ID and ACE DD genotypes in relation to ACE II genotype.

the number of lymph nodes with metastases (P = 0.029; t test), showing that gastric cancer with $AT1R^+$ and $AT2R^+$ tumor cells had a higher number of lymph node metastases ($P < 0.001$ for $AT2R^+$) than gastric cancers with receptor-negative tumor cells. Because 26.3 ± 11.2 lymph nodes were studied on average per patient, with no differences in the number of lymph nodes investigated between receptor-positive and -negative gastric cancers, the total number of lymph nodes investigated did not influence the number of lymph node metastases observed (Table 1). The Fisher test for nodal status (N category; P = 0.077) and the expression of ACE by tumor cells gave P values near the significance level.

Because AT1R (but not AT2R) was most commonly expressed by intestinal type gastric cancers, we next studied the significance of AT1R in only intestinal type gastric cancers, by excluding diffuse type gastric cancers (Table 2). Table 2 shows the results from univariate analyses. AT1R expression now correlated with the mean number of lymph node metastases (P = 0.026), N category (P = 0.023), and UICC tumor stage (P = 0.032). Note that the UICC tumor stage is also dependent on the N category. Thus, the lack of correlation between $AT1R^+$, N category and UICC tumor stage in the analysis of the entire study population was largely influenced by the presence of the $AT1R^-$ diffuse type gastric cancers. Interestingly, after exclusion of diffuse type gastric cancers, $AT1R^+$ gastric cancers were also more commonly associated with advanced local tumor growth. However, this did not reach statistical significance (Table 2).

Similar to AT1R, ACE is also more frequently expressed in intestinal type than in diffuse type gastric carcinomas (5), and in the subgroups consisting mainly of intestinal type gastric cancers, $AT1R^+$ is no longer correlated with ACE immunoreactivity, indicating that the correlation observed for the entire study population is due to their common expression in intestinal type gastric cancers.

Our present and the previous studies (5) provided evidence that two components of the angiotensin system, i.e., the patients' ACE genotype and the AT1R status of the tumor cells are both related to the presence and the number of lymph node metastases in intestinal type gastric cancer. Multivariate analyses without diffuse type gastric cancer now aimed to show whether these were independent factors. First, we looked for lymph node metastases as an expression of progression. In the logistic regression analysis with AT1R status, ACE genotype and ACE immunohistochemistry as independent variables, AT1R had the lowest P value (P = 0.017). Using the three-factor ANOVA, including only patients with lymph node metastases (number of lymph nodes with metastases as a dependent variable), we found neither significant main factors nor any interactions between the different independent variables. These results support the contention that AT1R status and ACE indicate the presence of lymph node metastases, but not the number of lymph node metastases. A stepwise Cox regression (Wald, forward), using AT1R immunoreactivity, ACE genotype and ACE immunoreactivity as factors, showed the same results for patient outcome. AT1R is the most important factor (P = 0.011). After the first step, no further effects were found.

Because AT1R and ACE genotypes were found to be independent factors without interactions, we assessed the association of various combinations of AT1R phenotypes ($AT1R^+$ and $AT1R^-$) and ACE I/D genotypes (II, ID, and DD) with nodal spread in the patient population comprising intestinal, mixed, and indeterminate type gastric cancer (excluding diffuse type gastric cancers). As shown in Table 3, the mean number of lymph node metastases varied remarkably, according to the phenotype/genotype combination observed. Irrespective of the genotype, $AT1R^+$-gastric cancers had a relative risk of lymph node metastases of 4.40 [95% confidence interval (CI), 1.30-14.86]. When the ACE genotype was included, the relative risk of having lymph node metastases increased considerably in $AT1R^+$ tumors with an ACE-ID genotype only (odds ratio, 13.00; 95% CI, 0.98-172.95), or either an ACE-ID or ACE-DD genotype (odds ratio, 19.00; 95% CI, 1.45-248.24) compared with the $AT1R^+$ tumors with an

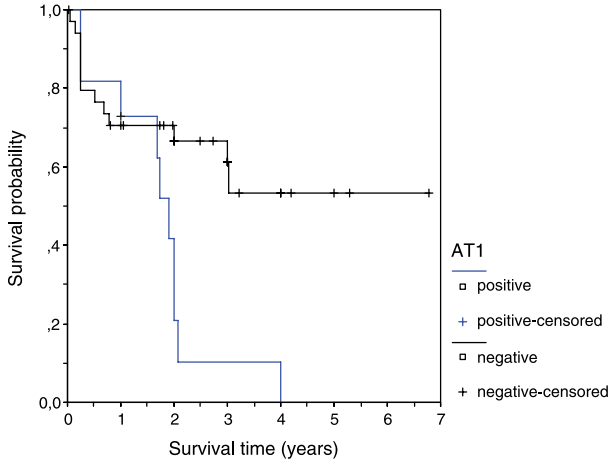

Figure 2. Kaplan-Meier survival curves for the presence (positive) or absence (negative) of AT1R in gastric cancer cells. Patients with $AT1R^+$ gastric cancer cells had significantly shorter survival times than patients with $AT1R^-$ tumor cells.

ACE-II genotype (Table 3). This showed that the phenotype/genotype combination correlates with nodal spread.

Finally, we studied the influence of AT1R status on patient survival in the entire study population, i.e., including diffuse and intestinal type gastric cancers. Overall, patients with $AT1R^+$ tumors had a significantly shorter survival time than patients with $AT1R^-$ gastric cancers ($P = 0.009$; Fig. 2).

Discussion

There is now abundant evidence that the angiotensin II/angiotensin II receptor system is involved in tumor biology (see Fig. 3; for a review, see ref. 7). Members of the angiotensin II/angiotensin II receptor system, such as ACE, are regularly found in tumors (7, 14). AT1R was detected in carcinomas of the larynx, lung, liver, pancreas, kidney, breast, ovary, cervix, in malignant melanoma, and in sarcomas (15-23). Epidemiologic studies found a significant inverse correlation between cancer mortality and the treatment with and the duration of administration of ACE inhibitors (24). Experimental studies have shown that the angiotensin II/angiotensin II receptor system can influence tumor biology by promoting neoangiogenesis and by enhancing microvessel density in solid tumors (17, 23, 25), by promoting tumor cell proliferation (26, 27), by promoting the remodeling of the interstitial matrix (28), and by modulating the local, peritumorous inflammatory reaction (29).

Recently, we have shown that ACE is expressed locally in gastric cancer (4), and that the insertion/deletion gene polymorphism of the ACE gene influences tumor development (30), as well as metastatic behavior (5). ACE generates the octapeptide angiotensin II, which then binds to AT1R and AT2R (Fig. 3). Based on our previous observation, we hypothesized that the biological effect of the ACE insertion/deletion gene polymorphism might be mediated through the activation of AT1R and/or AT2R. However, until now, AT1R and AT2R had not been studied in human gastric cancers. In searching for further evidence to support our hypothesis, we are the first to show the local expression of AT1R and AT2R in human gastric cancers on the protein level. In addition, we provide evidence for the biological significance of AT1R in gastric cancer biology: the expression of AT1R in gastric cancers, particularly those with intestinal type differentiation, correlated with the presence and mean number of lymph node metastases, the N category, as well as the UICC tumor stage. Previously, we made a similar observation regarding the ACE gene polymorphism in patients with gastric cancer (5). However, although the expression of AT1R and ACE coincide in intestinal type gastric cancers, the expression level of ACE in the tumor cells does not correlate with nodal spread (data not shown; ref. 5). The pathophysiologic effect of the ACE gene polymorphism may also be mediated through heterotypic signaling.

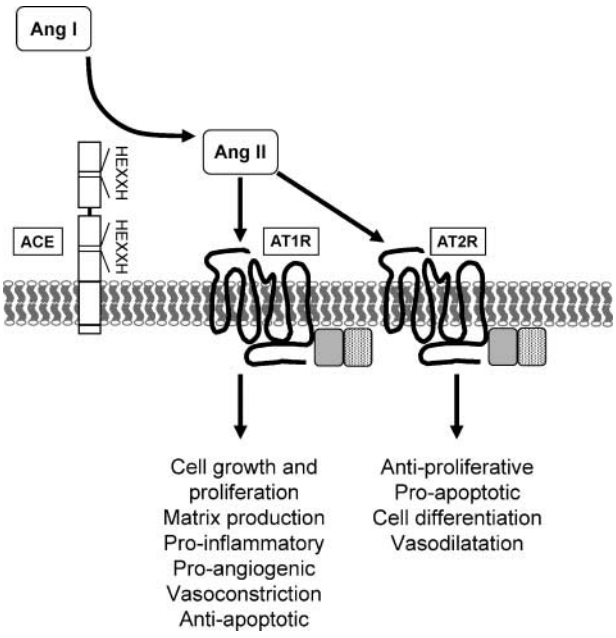

Figure 3. Schematic outline of the local angiotensin system. Angiotensin I (*Ang I*) is cleaved by ACE into angiotensin II (*Ang II*), which then binds to angiotensin receptor type 1 (*AT1R*) and type 2 (*AT2R*). This system plays an important role in tumor biology, influencing tumor cell proliferation, remodeling of the interstitial matrix, neoangiogenesis, and metastatic behavior.

Extending our previous studies, we are now able to show that the combination of the $AT1R^+$ phenotype and the ACE ID and DD genotypes define a high-risk patient group. Comparison of node-positive patients shows that patients with $AT1R^-$ tumor cells bear a lower risk of developing lymph node metastases, whereas patients with $AT1R^+$ tumor cells and an ACE ID or DD genotype carry a much higher risk for nodal spread (odds ratios, 13.00 and 19.00, respectively). However, because several analytical steps were required to show this correlation, our observations should be validated by an independent study series. Furthermore, we were unable to test whether the expression of AT1R per se contributes directly to tumor progression. AT2R was also differentially expressed in gastric cancer cells. However, the number of patients with $AT2R^-$ tumors was too low to be informative with regard to its putative biological significance. Further studies into this topic are warranted.

In summary, we provide evidence that the angiotensin II/angiotensin II receptor system might influence tumor progression and the risk of developing lymph node metastases through the ACE gene polymorphism and the local expression of angiotensin type II receptors. Patients with $AT1R^+$ gastric cancer and an ACE DD genotype had all developed lymph node metastases, leading to the conjecture that this is a specific high-risk patient population. The poor prognosis for gastric cancer is often related to presentation at an advanced tumor stage, and is most strongly influenced by lymph node involvement (3). Early identification of a high-risk gastric cancer group might allow the identification of patients who should undergo extensive lymph adenectomy (D2 versus D1) in gastric cancer surgery, as well as enabling the therapeutic approach to be tailored towards a more aggressive regimen in these patients. Furthermore, ACE inhibitors have already been considered for ''novel'' antineoplastic treatment and cancer prevention strategies (24, 31). Based on our observations, we propose that the combination of AT1R expression and ACE I/D gene polymorphisms allow the risk assessment of nodal spread in gastric cancer, possibly before metastases develop and that AT1R antagonists might prove to be useful for the treatment of gastric cancer, particularly by preventing or reducing nodal spread in high-risk patient groups.

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