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ORIGINAL ARTICLE

β-Secretase 1 and its Naturally Occurring Anti-Sense RNA are Down-Regulated in Gastric Cancer

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Abstract

β-secretase (BACE1) and its naturally occurring anti-sense RNA (*BACE1-AS*) have established role in the pathologic process leading to Alzheimer's disease. Their possible implication in the neoangiogenesis suggests that they might be involved in the tumorigenesis events as well. In the present study, we compared transcript levels of these genes in 30 gastric cancer samples and their adjacent non-cancerous tissues (ANCTs) to find whether their altered expression might facilitate discrimination of these two sets of samples. Expressions of both genes were associated with site of primary tumor. Both genes were significantly down-regulated in tumoral tissues compared with ANCTs. Significant correlations were detected between transcript levels of these genes in both sets of samples. Transcript levels of *BACE1* and *BACE1-AS* had the diagnostic power of 75% based on Receiver operating characteristic curve analysis. The current study provides evidences for contribution of *BACE1* and *BACE1-AS* in gastric cancer evolution and suggests their potential as diagnostic markers.

Keywords BACE1 · BACE1-AS · Gastric cancer

Introduction

Beta-secretase 1 (BACE1) is an aspartic-acid protease which participates in construction of myelin sheaths in peripheral nerve cells [1]. Apart from this physiologic function, this enzyme catalyzes consecutive breakage of the amyloid precursor protein (APP) and production of amyloid-β peptides that amassed in the brain of Alzheimer's patients [2]. A long non-coding RNA (lncRNA) has been demonstrated to be transcribed from the opposite strand of *BACE1* and promptly increase *BACE1* expression following exposure to [3]. The observed over-expression of APP is in the endothelium of neoformed blood vessels has provided primary evidences for its contribution in angiogenesis. Notably, beta-secretase inhibitors have decreased endothelial cell proliferation and suppressed development of microvessels and tumor growth in xenograft animal models [4].

Gastric cancer (GC) is one of the most aggressive human malignancies with high metastatic potential. Based on the established role of neoangiogenesis in evolution of tumor metastases, several anti-angiogenic treatment strategies have been developed and tested in GC [5]. Considering the initial reports regarding the safety and efficacy of beta-secretase inhibitors [4], this kind of anti-angiogenic agents might be used for GC patients as well. We designed the current study to assess expression level of *BACE1* and its regulatory lncRNA in GC samples to explore whether expression of these genes are elevated in GC samples.

Material and Methods

Patients

Sixty gastric samples including tumoral ($n = 30$) and paired adjacent non-cancerous tissues (ANCTs) ($n = 30$) were obtained from patients during gastric surgery. Patients with prior history of chemo/radiotherapy have been excluded from the study. All tissue samples were examined by pathologists to

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assess the presence of tumoral cells. The study protocol was approved by the ethical committee of Shahid Beheshti University of Medical Sciences. All patients have signed written informed consent forms. Informed consent form has also obtained from parents of patients under age 18 years.

Expression Study

Total RNA was extracted from all tissue samples using TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA). About 50–100 ng of RNA samples was used for cDNA synthesis using Applied Biosystems High-Capacity cDNA Reverse Transcription Kit. Expressions of BACE1 and BACE1-AS were measured in the Rotor Gene 6000 Real-Time PCR Machine using TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Expression levels of genes were normalized to transcript levels of *HPRT1*. The sequences of primers and probes and PCR product length are shown in Table 1.

Detection of *Helicobacter Pylori* (*H. pylori*) Infection

Extracted RNA was used to synthesize cDNA using 25 pmol of random hexamer primers. cDNA synthesis was performed using Geneall Hyperscript cDNA synthesis Kit according to manufacturer's instruction. No DNAase I treatment was performed. Real-time PCR was performed using RealQ Plus 2x Master Mix Green from Ampliqon and primers against *H. pylori* 16 s rRNA (F: AGCGTTACTCGGAATCACTG; R: CACATACCTCTCACACACTC) at final concentration of 0.2 pmol/μl and 100 ng of synthesized cDNA. Reaction samples were incubated at 95 °C for 15 min and then 95 °C for 15 s and 60 °C for 1 min for 40 cycles followed by melt curve analysis.

Statistical Analysis

Fold changes of expression levels in tumoral tissues vs. ANCTs were measured using REST 2009 software. The significance of difference in expression of mentioned genes between paired GC samples and ANCTs was evaluated using the Student's paired t-test. The association between tumor features and relative expression of genes was assessed using Chi-square test. The correlation between relative expressions of *BACE1* and *BACE1-AS* was measured using the regression model. For all statistical tests, the level of significance was set at $P < 0.05$. The suitability of transcript levels of these genes in differentiation of tumoral from non-tumoral tissues was assessed by plotting the receiver operating characteristic (ROC) curve.

Results

General Demographic and Clinical Data of Patients

Table 2 shows the tumor features and demographic data of study participants which were obtained from assessment of patient' records and questionairs.

Relative Expression of *BACE1* and *BACE1-AS* in Tumoral Tissues Compared with ANCTs

Expressions of both *BACE1* and *BACE1-AS* were significantly lower in GC samples compared with ANCTs (Fold change values = 0.35 and 0.24, *P* values = 0.03 and 0.002 respectively). Figure 1 shows the –delta CT values (CT *HPRT1*- CT target gene) in GC tissues and ANCTs.

Table 1 The primers and probes sequences and PCR product length

Gene name	Primer and probe sequence	Primer and probe length	Product length
HPRT1	F: AGCCTAAGATGAGAGTTC	18	88
	R: CACAGAACTAGAACATTGATA	21	
	FAM -CATCTGGAGTCCTATTGACATCGC- TAMRA	24	
BACE1	F: CCAAGACGACTGTTACAA	18	79
	R: GAAGCCCTCCATGATAAC	18	
	FAM-TTGCCATCTCACAGTCATCCAC-TAMRA	22	
BACE1-AS	F: GACACTGTACCATCTCTTTTACCC	24	113
	R: CACCACCAACCTTCGTTTGC	20	
	FAM - AGTCCACTCACGGAGGAGGTCGCC -TAMRA	24	

Table 2 General demographic and clinical data of patients

Variables		Values
Age (mean ± SD (range))		42.53 ± 10.1 (14–55)
Gender	Male	78.6%
	Female	21.4%
Site of primary tumor	Cardia	41.4%
	Antrum	31%
	Body	27.6%
Histologic grade	2	37.5%
	3	58.3%
	4	4.2%
Lymphatic invasion	Yes	82.8%
	No	17.2%
Vascular invasion	Yes	82.8%
	No	17.2%
Peritoneal invasion	Yes	62.1%
	No	37.9%
TNM stage	I	3.4%
	II	31%
	III	44.8%
	IV	20.8%
Histological form	Intestinal	46.7%
	Diffuse	53.3%
H. pylori infection	Positive	50%
	Negative	50%
Smoking	Never Smoker	50%
	Current Smoker	13.6%
	Ex-Smoker	36.4%

Association Between Expression of *BACE1*/ *BACE1-AS* and Tumor Features

Relative expressions of both genes were significantly associated with site of primary tumor in a way that both genes were down-regulated in all tumor samples originated from cardia. Other clinicopathological features were not associated with expression levels of either gene. Table 3 shows the results of association analysis between relative expressions of genes in GC samples compared with ANCTs and tumor features.

Pairwise Correlation Between Expressions of *BACE1* and *BACE1-AS*

Based on the Spearman correlation coefficients, significant pairwise correlations were detected between transcript levels of these genes in both GC tissues and ANCTs (Fig. 2a and b respectively).

ROC Curve Analysis

Based on area under curve (AUC) values, the diagnostic power values of *BACE1* and *BACE1-AS* in GC were estimated to be 0.67 and 0.74 respectively. Combination of transcript levels of both genes slightly increased the diagnostic power (0.75) and significance ($P < 0.001$). Table 4 shows the detailed data of ROC curve analysis.

Finally, we assessed the diagnostic power of genes in relation to tumor localization (Table 5). As expected from the results of expression analysis, *BACE1* and

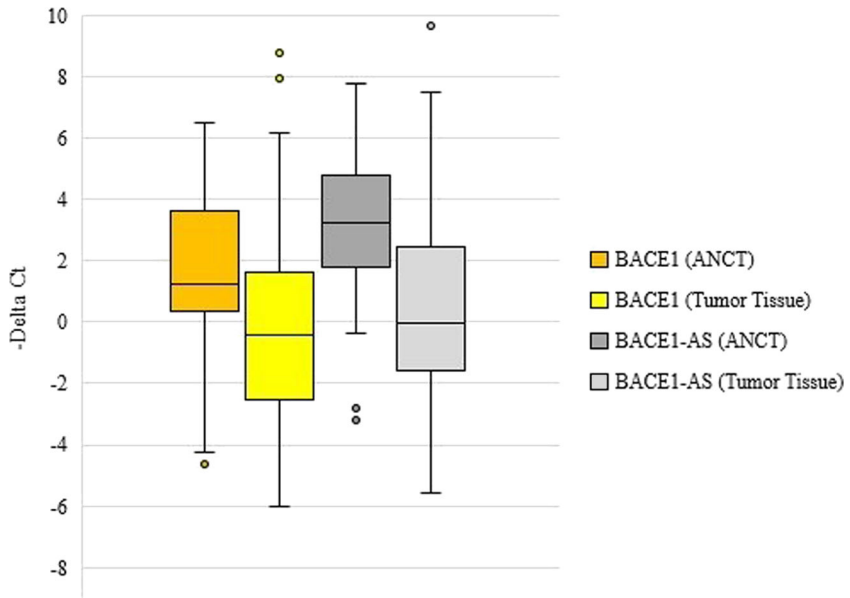
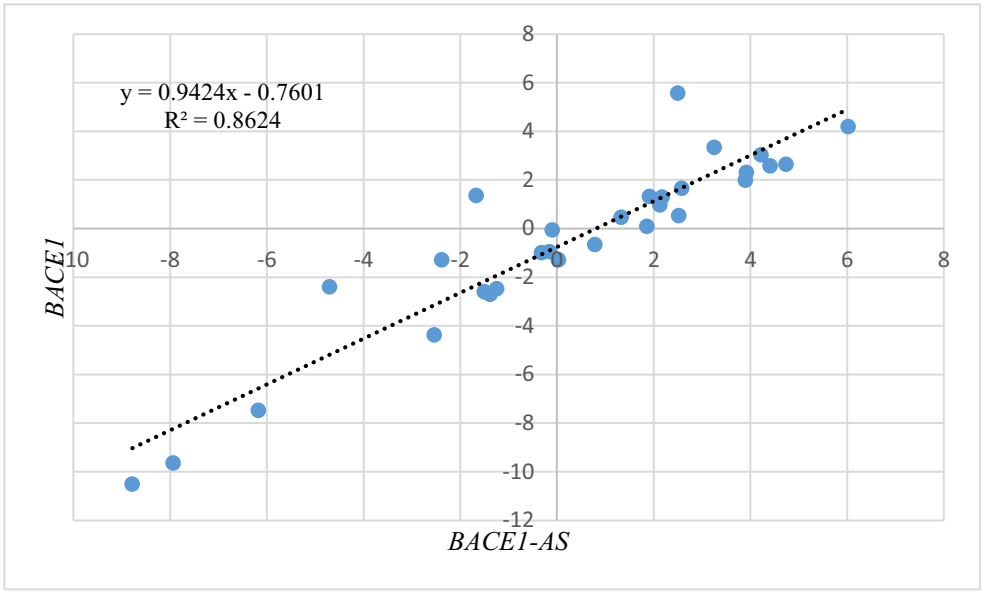


Fig. 1 Relative expression of *BACE1* and *BACE1-AS* in GC samples ($n = 30$) and ANCTs ($n = 30$) as described by –delta CT values (CT *HPRT1*- CT target gene)

Table 3 The results of association analysis between relative expressions of *BACE1* and *BACE1-AS* in GC tissues compared with ANCTs and tumor features (Up/down regulation of genes was defined on the basis of relative expression of each gene in tumoral tissue compared with the paired ANCT)

	BACE1 up-regulation	*BACE1* down-regulation	*P* value	*BACE1-AS* up-regulation	*BACE1-AS* down-regulation	*P* value
Age			1			0.59
>50	6 (28.6%)	15 (71.4%)		5 (23.8%)	16 (76.2%)	
≤50	2 (28.6%)	5 (71.4%)		1 (14.3%)	6 (85.7%)	
Gender			0.96			0.55
Female	1 (16.7%)	5 (83.3%)		0 (0%)	6 (100%)	
Male	6 (27.3%)	16 (72.7%)		5 (22.7%)	17 (77.3%)	
Site of primary tumor			0.002			0.007
Cardia	0 (0%)	12 (100%)		0 (0%)	12 (100%)	
Antrum	6 (66.7%)	3 (33.3%)		5 (55.6%)	4 (44.4%)	
Body	2 (25%)	6 (75%)		1 (12.5%)	7 (87.5%)	
Histological grade			0.75			1
2	2 (22.2%)	7 (77.8%)		2 (22.2%)	7 (77.8%)	
2	5 (35.7%)	9 (64.3%)		3 (21.4%)	11 (78.6%)	
3	0 (0%)	1 (100%)		0 (0%)	1 (100%)	
Lymphatic invasion			0.59			0.26
Yes	6 (25%)	18 (75%)		4 (16.7%)	20 (83.3%)	
No	2 (40%)	3 (60%)		2 (40%)	3 (60%)	
Vascular invasion			0.59			0.26
Yes	6 (25%)	18 (75%)		4 (16.7%)	20 (83.3%)	
No	2 (40%)	3 (60%)		2 (40%)	3 (60%)	
Peritoneal invasion			1			0.64
Yes	5 (27.8%)	13 (72.2%)		3 (16.7%)	15 (83.3%)	
No	3 (27.8%)	8 (72.2%)		3 (27.8%)	8 (72.2%)	
Tumor size			0.83			
T2b	1 (25%)	3 (75%)		0 (0%)	4 (100%)	
T3	3 (17.6%)	14 (82.4%)		3 (17.6%)	14 (82.4%)	
T4	2 (33.3%)	4 (66.7%)		1 (16.7%)	5 (83.3%)	
Lymph node status			0.1			0.55
N0	2 (22.2%)	7 (77.8%)		2 (22.2%)	7 (77.8%)	
N1	1 (11.1%)	8 (88.9%)		1 (11.1%)	8 (88.9%)	
N2	5 (62.5%)	3 (37.5%)		3 (37.5%)	5 (62.5%)	
N3	0 (0%)	3 (100%)		0 (0%)	3 (100%)	
TNM staging			1			0.8
I	0 (0%)	1 (100%)		0 (0%)	1 (100%)	
II	2 (22.2%)	7 (77.8%)		2 (22.2%)	7 (77.8%)	
III	4 (30.85%)	9 (69.2%)		3 (23.1%)	10 (76.9%)	
IV	2 (33.3%)	4 (66.7%)		1 (16.7%)	5 (83.3%)	
Histological form			1			0.37
Intestinal	4 (28.6%)	10 (71.4%)		4 (28.6%)	10 (71.4%)	
Diffuse	4 (25%)	12 (75%)		2 (12.5%)	14 (87.5%)	
H. pylori infection			0.68			0.16
Positive	3 (20%)	12 (80%)		1 (6.7%)	14 (93.3%)	
Negative	5 (33.3%)	10 (66.7%)		5 (33.3%)	10 (66.7%)	
Smoking			0.64			0.64
Never smoker	3 (27.3%)	8 (72.7%)		3 (27.3%)	8 (72.7%)	
Current smoker	1 (33.3%)	2 (66.7%)		1 (33.3%)	2 (66.7%)	
Ex- smoker	1 (12.5%)	7 (87.5%)		1 (12.5%)	7 (87.5%)	



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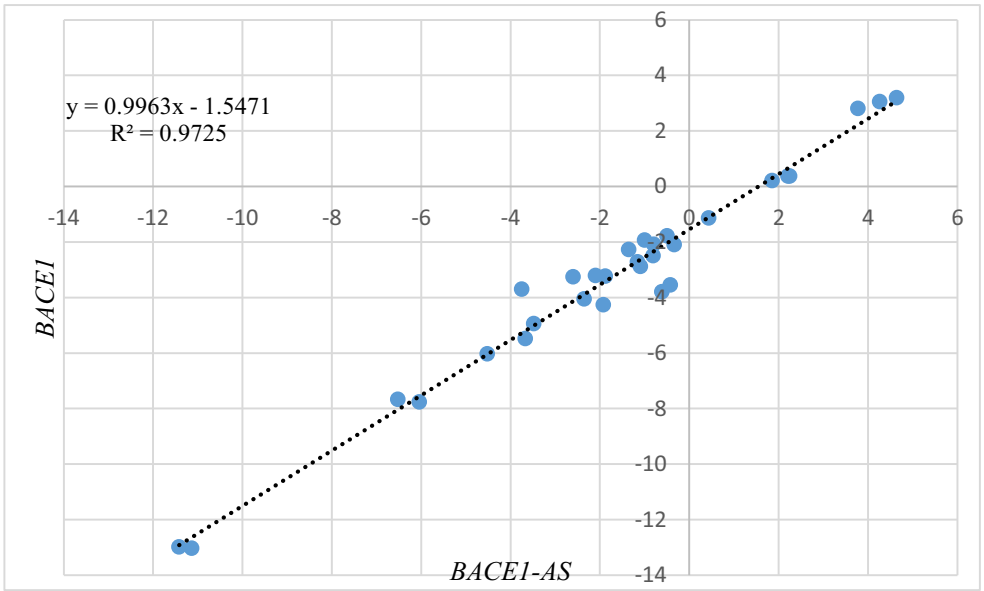

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Fig. 2 Pairwise correlation between expression levels of *BACE1* and *BACE1-AS* in GC samples (**a**) and ANCTs (**b**)

Table 4 The results of ROC curve analysis

	Estimate criterion	AUC	J[a]	Sensitivity	Specificity	*P*-value[b]
BACE1	> −0.33	0.67	0.43	66.7	76.7	0.01
BACE1-AS	> −1.77	0.74	0.5	73.3	76.7	0.0004
Combination of *BACE1* and *BACE1-AS*	> 0.47	0.75	0.46	76.7	70	<0.0001

[a] Youden index, [b] Significance level P (Area = 0.5), Estimate criterion: optimal cut-off point for gene expression

Table 5 The results of ROC curve analysis in relation with site of primary tumor

		Estimate criterion	AUC	J[a]	Sensitivity	Specificity	*P*-value[b]
Cardia	*BACE1*	> −0.43	0.79	0.58	75	83.3	0.002
	BACE1-AS	> 0.38	0.86	0.66	66.7	100	<0.0001
	Combination of two genes	> 0.51	0.89	0.66	83.3	83.3	<0.0001
Antrum	*BACE1*	≤ −4.7	0.58	0.22	22.2	100	0.58
	BACE1-AS	≤ 2	0.51	0.33	100	33.3	0.96
Body	*BACE1*	> −1	0.73	0.5	62.5	87.5	0.08
	BACE1-AS	> − 1.93	0.76	0.62	75	87.5	0.05

[a] Youden index, [b] Significance level P (Area = 0.5), Estimate criterion: optimal cut-off point for gene expression

BACE1-AS could differentiate disease status in cardia region with acceptable diagnostic power values (AUC values of 0.79 and 0.86, P values of 0.002 and < 0.0001 respectively).

Discussion

In the present study, we detected significant down-regulation of *BACE1* and *BACE1-AS* in GC samples compared with ANCTs. *BACE1* expression and beta-secretase function has been shown to be elevated by hypoxia through participation of inducible factor 1a (HIF1a) [6, 7]. HIF1a has an established role in the pathogenesis of gastric cancer [8]. Although we did not assess expression of HIF1a in our cohort of patients, based on the results of previous studies we anticipated elevated levels of this transcription factor in GC samples [9] and subsequent over-expression of *BACE1*. However, we detected the opposite. The first implication of our results might be unsuitability of beta-secretase inhibitors for GC patients. To find possible explanations for the observed down-regulation of *BACE1* in GC we searched for regulatory mechanisms of its expression. The *BACE1* promoter has binding sites for various transcription factors such as Sp1, NF-κB, YY1, MZF1, HNF-3β and GATA [10]. Luciferase reporter assays have indicated the role of NF-κB site as a repressor of *BACE1* transcription, while a GATA containing site possibly activate *BACE1* expression [11]. There are some reports of elevated expression of NF-κB in GC samples and cell lines [12]. Meanwhile, *H. pylori* strains carrying the *cag*PAI have up-regulated expression of NF-κB [13]. However, we could find detect any associations between expression of genes and *H. pylori* infection which might indicate an alternative mechanism for down-regulation of *BACE1* in GC samples which should be explored in future studies. Other studies have reported epigenetically silencing of GATA-4 and GATA-5 transcription factor genes in GC [14]. *BACE1* expression has also been shown to be controlled by Nuclear Factor of Activated T-cells (NFAT) [15]. Different members this family of transcription factors have dissimilar role in the regulation of cell proliferation, apoptosis, cell cycle and tumor cell proliferation [16]. Consequently, the observed down-regulation of *BACE1* in the current study might be attributed to dys-regulation of expression of diverse transcription factors in the context of GC. Functional studies and simultaneous assessment of expression of these transcription factors and BACE1 in GC samples are needed to explore the underlying mechanism of BACE1 down-regulation in GC.

We also detected significant associations between expression levels of both genes and site of primary tumor in a way that both genes were down-regulated in all tumor samples originated from cardia. Previous biologic, epidemiologic and clinicopathological studies have revealed that cardia tumors are more closely related to esophageal tumors than non-cardia GC [17].

Although the overall performance of transcript levels of these genes as diagnostic markers for GC was not ideal, the detected down-regulation of *BACE1* and *BACE1-AS* in all cardia tumors compared with paired ANCTs indicates the suitability of these genes as biomarkers for detection of cancer status in cardia region. Such speculation was supported by the calculated acceptable AUC values in cardia tumors. Future studies are needed to verify our suggestion in a larger cohort of cardia tumors.

Finally, the significant correlations between transcript levels of *BACE1* and *BACE1-AS* in both GC tissues and ANCTs provide additional support for the previously reported role of BACE1-AS in increasing the stability of BACE1 [3].

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Compliance with Ethical Standards

Conflict of Interest The authors declare they have no conflict of interest.

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