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Expression of gamma-aminobutyric acid receptors on neoplastic growth and prediction of prognosis in non-small cell lung cancer

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Abstract

Background: Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the adult mammalian brain, but exerts physiologic effects other than that on neurotransmitter in non-neuronal peripheral tissues and organs. GABA may affect cancer growth through activation GABA receptors. We investigated the gene expression of GABA receptors in tissue of non-small cell lung cancers (NSCLC) and non-cancerous tissues, and found that the gene expression of GABA receptor phenotypes was correlated with tumorigenesis and clinical prognosis.

Methods: Sixty-one snap-frozen human samples of NSCLC tissues and paired non-cancerous tissues (5cm away from tumor) were analyzed. Gene expression of GABA receptors was detected by Real-time quantitative PCR (RT-qPCR). Survival times in relation to the expression of GABA receptor phenotypes were analyzed. Human NSCLC cell lines H1299, A549, H520, H460 and human bronchial epithelial cell line BEAS-2B were used to determine the phenotypes of GABA inhibitory effects on cancer cell growth. The effects of exogenous administration of GABA on H1299 cell growth were examined.

Results: The gene expressions were significantly higher in NSCLC tissues than in the paired non-cancerous tissues for GABA_A receptor subunit $\alpha 3$ (GABR_{A3}, $P = 0.030$); for GABA_A receptor subunit epsilon (GABR_E, $P = 0.036$); and GABA_B receptor subunit 2 (GABBR₂, $P = 0.005$). Kaplan-Meier curves showed that patients with high expression of GABBR₂ gene and low expression of GABR_{A3} gene had a better prognosis ($P < 0.05$). The administration of GABA resulted in suppressed proliferation of NSCLC cell lines in a dose- and time-dependent manner. The use of the GABA receptor antagonist CGP35348 could reverse the inhibitory effect.

Conclusions: The pattern of GABA receptor gene phenotype expression may be involved in the regulation of tumorigenesis. A high expression of GABBR₂ with a low expression of GABR_{A3} may predict a better outcome. The treatment with GABA attenuates cancer cell growth in vitro. The expression of GABA receptor may be not only promising genetic therapeutic targets but may also serve as valuable prognostic markers for NSCLC.

Keywords: Gamma aminobutyric acid receptor, Survival, Biomarker, Prognosis

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Background

Cancer is a major global public health problem. One in 4 deaths in the United States is due to cancer [1]. Lung cancer is comprising 17% of the total new cancer cases and 23% of the total cancer deaths [2]. Non-small cell lung cancer (NSCLC) accounts for about 80% of all lung cancer cases where adenocarcinoma is dominantly presented [3]. Conventional treatment of NSCLC has improved survival, but the 5-year survival rate is approximately 16% over the past 30 years [1]. Novel and effective methods are urgently required for lung cancer therapy.

Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the adult mammalian brain via activation of specific GABA receptors highly expressed in the central nervous system (CNS) [4,5]. GABA receptors are composed of GABA_A and GABA_B receptors. GABA_A receptors are ligand-gated chloride channels composed of five subunits. These subunits are encoded by 19 different genes that have been grouped into eight subclasses based on sequence homology (α 1–6, β 1–3, γ 1–3, δ , ϵ , θ , π , ρ 1–3). GABA_B receptors are heterodimeric G-protein-coupled receptors (GPCRs) composed of GABBR₁ and GABBR₂ subunits which are both required for normal receptor functioning [6].

It has recently revealed that GABA and its receptors also exist in non-neuronal peripheral tissues and organs, indicating that GABA exerts physiologic effects other than the inhibitory neurotransmitter property. In fact, GABA has been shown to be involved in the development of many tissues and organs, including the peripheral nervous system [7], the development of the palate [8], lung [9], pancreas [10], digestive tract [11], liver [12], chondrocytes [13], testicular cells [14] and even stem cells [15].

Given that GABA participates in the proliferation of various normal cell types and tissues, it is intriguing to consider the potential function of GABA in cancer cells. Recent studies gave the evidences that GABA and its receptors seemed to play critically regulative effects on many kinds of cancers [12,16-31]. In most cases, the levels of GABA receptors accompanying other growth signaling components had significant changes in cancer cells. This raised the possibility that manipulating GABA receptor activity might inhibit tumor growth [25].

In this study, we tested the hypothesis that GABA receptor profiles modulate cancer survival. We thus investigated the gene expression of GABA receptor phenotypes in NSCLC tissues and paired non-cancerous tissues obtained from surgical patients to correlate the GABA receptor gene profiles with clinical outcome. To examine the specific effects of GABA receptor on lung cancer cell growth, we investigated the GABA receptor profiles in cancer cell lines and in normal human epithelial cell line in the presence and absence of exogenous administration of GABA.

Methods

Cancer cell lines

Human NSCLC cell lines H1299(adenocarcinoma), A549 (adenocarcinoma), H520(squamous cell carcinoma), H460 (large cell carcinoma) and normal human bronchial epithelial cell line BEAS-2B (ATCC, Rockville, Maryland) were cultured at 37°C with 5% CO₂ in DMEM (Gibco, Beijing, China) supplemented with 10% FBS (Gibco, Beijing, China) without antibiotics.

MTT assays for cell proliferation

To measure the proliferation of cells, the colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assays (Sigma) were used in vitro. In the MTT assays, cells were seeded into 96-well plates (2×10^3 cells per well), grown overnight, washed in PBS, and incubated with GABA (Sigma-Aldrich) at 0.1 μ mol/L to 500 μ mol/L in the presence or absence of 100 μ mol/L picrotoxin (PTX, GABA_A receptor antagonist) or CGP35348 (CGP, GABA_B receptor antagonist), respectively. MTT was then added (10 μ g/well) for 4h. Formazan products were solubilized with DMSO, and the optical density was measured at 490 nm.

Tissues and subjects

Sixty-one samples of NSCLC tissues and paired non-cancerous tissues (5cm away from tumor) were collected from the Thoracic Cardio Surgery Department of the First Affiliated Hospital of Guangzhou Medical College under full ethical clearance by the Guangzhou Medical College Ethics Committee for experimentation on human subjects. Informed written consent was obtained from the participants. All subjects were Chinese. The collected samples were immediately cut into small pieces and snap-frozen in liquid nitrogen until further use. All tumor tissue and paired non-cancerous tissue samples were pathologically confirmed.

RNA extraction and cDNA synthesis

Total RNA from cell lines and liquid-nitrogen-frozen NSCLC tissue samples were extracted using Trizol reagent (Invitrogen). First-strand cDNAs were synthesized using primerscript RT reagent kit (Takara). Briefly, a mix of 2 μ g RNA, 2 μ l of 5 \times gDNA eraser buffer and 1 μ l of gDNA eraser in a final volume of 10 μ l with RNase free DH₂O, was incubated at 42°C for 2 min to get rid of gDNA, and placed on ice for at least 1 min. Then 4 μ L 5 \times Primerscript buffer, 1 μ L Primerscript RT enzyme mix I, 1 μ L RT primer mix, and 3 μ L RNase free DH₂O were added, and incubated at 37°C for 15 min followed by 85°C for 5 sec. The cDNA samples were stored at -20°C until use.

Primer design

Primers were designed using Primer Premier 5.0 and oligo 7 Software. Each primer pair was verified for gene specificity using Nucleotide Basic Local Alignment Search Tool from the GenBank non-redundant nucleotide sequence database (National Centre for Biotechnology Information, 2009, <http://www.ncbi.nlm.nih.gov/>). The sequence of each primer pair and the expected amplicon size were summarised in Table 1.

PCR

PCR was performed to pick out the genes expression in NSCLC cancer cell lines using Premix Taq[®] version 2.0 kit (Takara). The reaction condition was followed: initial denaturation at 95°C for 1 min; 35 cycles of 30 sec at 95°C; 30 sec at 58°C; and 30 sec at 72°C; followed by a final 3 min extension at 72°C. 3% Agarose gel was used in electrophoresis to separate the reaction products at 80V, 40min. After dyeing with ethidium bromide, the Gel Doc[™] EZ Imager (BIO-RAD) was used for imaging.

RT-qPCR

Real-time quantitative PCR was carried out in tissues cDNA samples using the SYBR[®] Premix Ex Taq[™] reagent kit (Takara) through ABI PRISM[™]7900 HT Fast Real-Time PCR system (Applied Biosystems). Briefly, 2 µl of each cDNA product was amplified in a mixture containing 12.5 µl of 2 × SYBR[®] Premix Ex Taq[™], 0.5 µl of 10 µmol/L PCR sense primer and the same quantitative antisense primer with dH₂O in a final volume of 25 µL. The RT-qPCR was performed using the following parameters: initial denaturation at 95°C for 30 sec, 40 cycles of 5 s at 95°C, 30 s at 60°C. The dissolved curve was added to verify the specificity of amplified products. Wells with no template were

included for each primer set as a negative control. In each experiment, samples were amplified in duplicate for each of the genes of interest and the reference gene. Only average CT values with a standard deviation <0.5 were accepted.

Statistic analysis

The expression of each gene of interest was determined in relation to the reference gene 18S rRNA. The difference in the mean CT values of the duplicate samples against the reference gene was calculated to give the ΔCT . The relative quantitation value was then expressed as two times $-\Delta CT$ ($2^{-\Delta CT}$). Analysis of the $2^{-\Delta CT}$ values data was performed using SPSS 13.0 followed by Paired-Samples *t* Test and Independent-Samples *t* Test where appropriate. Correlation analysis was carried out for GABA concentration and cell growth over time. Survival times between groups were displayed by Kaplan-Meier curves after a log-rank test. $P < 0.05$ was considered statistical significance.

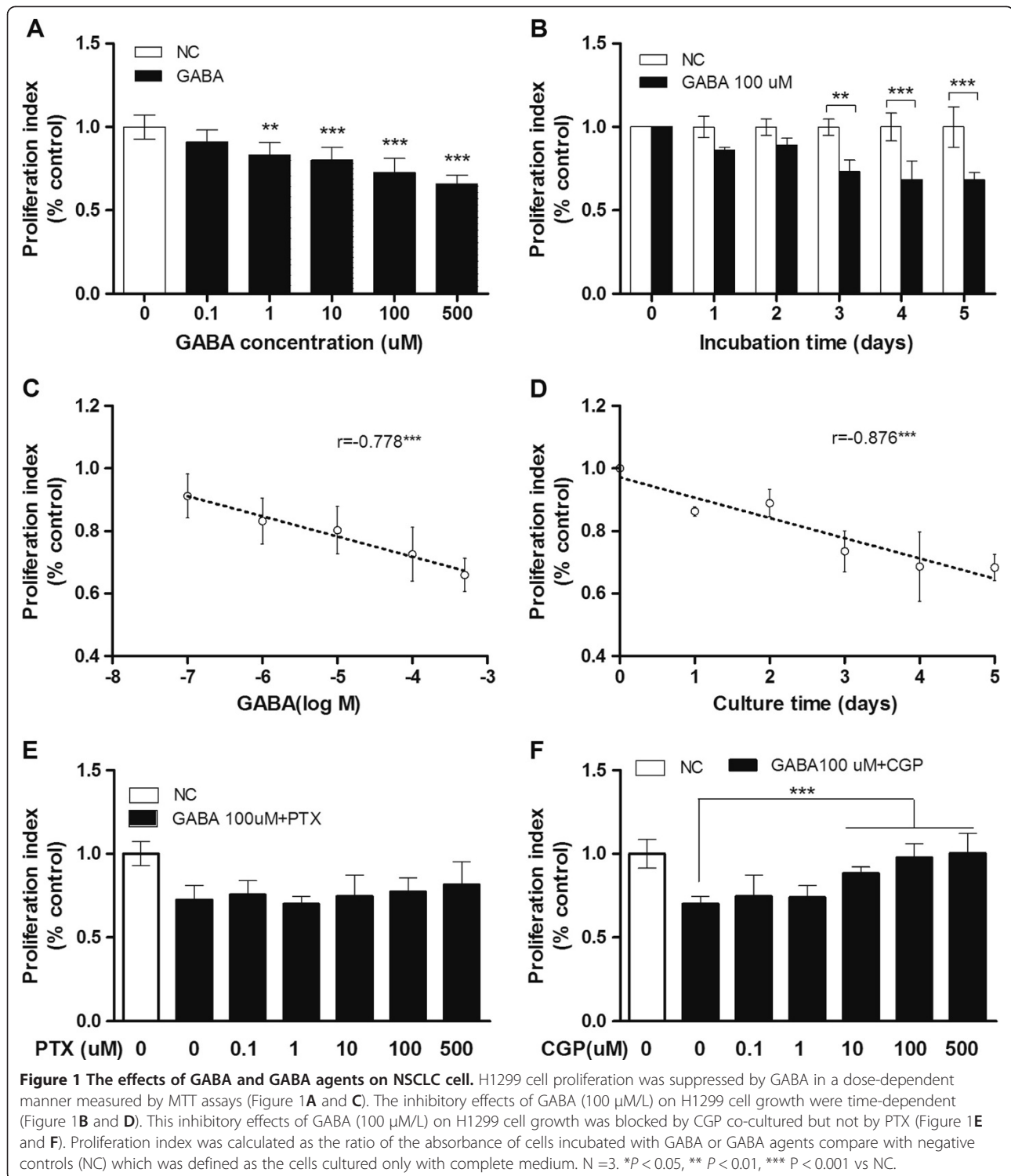
Results

Effect of GABA on cancer cell proliferation

To examine the effects of administration of exogenous GABA on cancer cells proliferation in vitro, MTT assays were performed on the H1299 cells at broad range of concentrations of GABA (0.1-500 µmol/L). There was a dose- and time-dependent inhibition of cell proliferation with GABA (Figure 1A, B). The GABA concentration used was negatively correlated with the proliferation index (Figure 1C, $r = -0.778$, $p < 0.001$; Figure 1D, $r = -0.876$, $p < 0.001$). This inhibitory effects of GABA on cell proliferation was blocked by the use of CGP, a GABA_B receptor antagonist, while PTX, a GABA_A receptor antagonist, had no effect (Figure 1E, F). Similar results were observed in

Table 1 Primer sequences, NCBI gene ID and amplicon size

Gene	NCBI gene ID		Sequence(5'—3')	Amplicon size (bp)
GABR _{A3}	2556	Sense	CCGTCTGTTATGCCITTTGTATT	165
		Antisense	TGTTGAAGGTAGTGCTGTTTT	
GABR _{B3}	2562	Sense	GGAGATACCCCTGGACGAGCA	196
		Antisense	GGATAGGCACCTGTGGCGAAGA	
GABR _E	2564	Sense	ATGCTTCTCTAAACTCCGCC	248
		Antisense	CTTAAAACGCTTGCACCACTC	
GABR _p	2568	Sense	CGACCGTGTATCAATGACC	120
		Antisense	CCCCAAACACAAAGCTAAAGCA	
GABBR ₁	2550	Sense	CAGATAAATGGATTGGAGGGT	101
		Antisense	GAGAACTGAGACGGAGATAAAGAG	
GABBR ₂	9568	Sense	GAGTCCACGCCATCTTCAAAAAT	108
		Antisense	TCAGGATACACAGGTCGATCAGC	
18S rRNA	100008588	Sense	GCAATTATCCCCATGAACG	123
		Antisense	GGCCTCACTAAACCATCCAA	

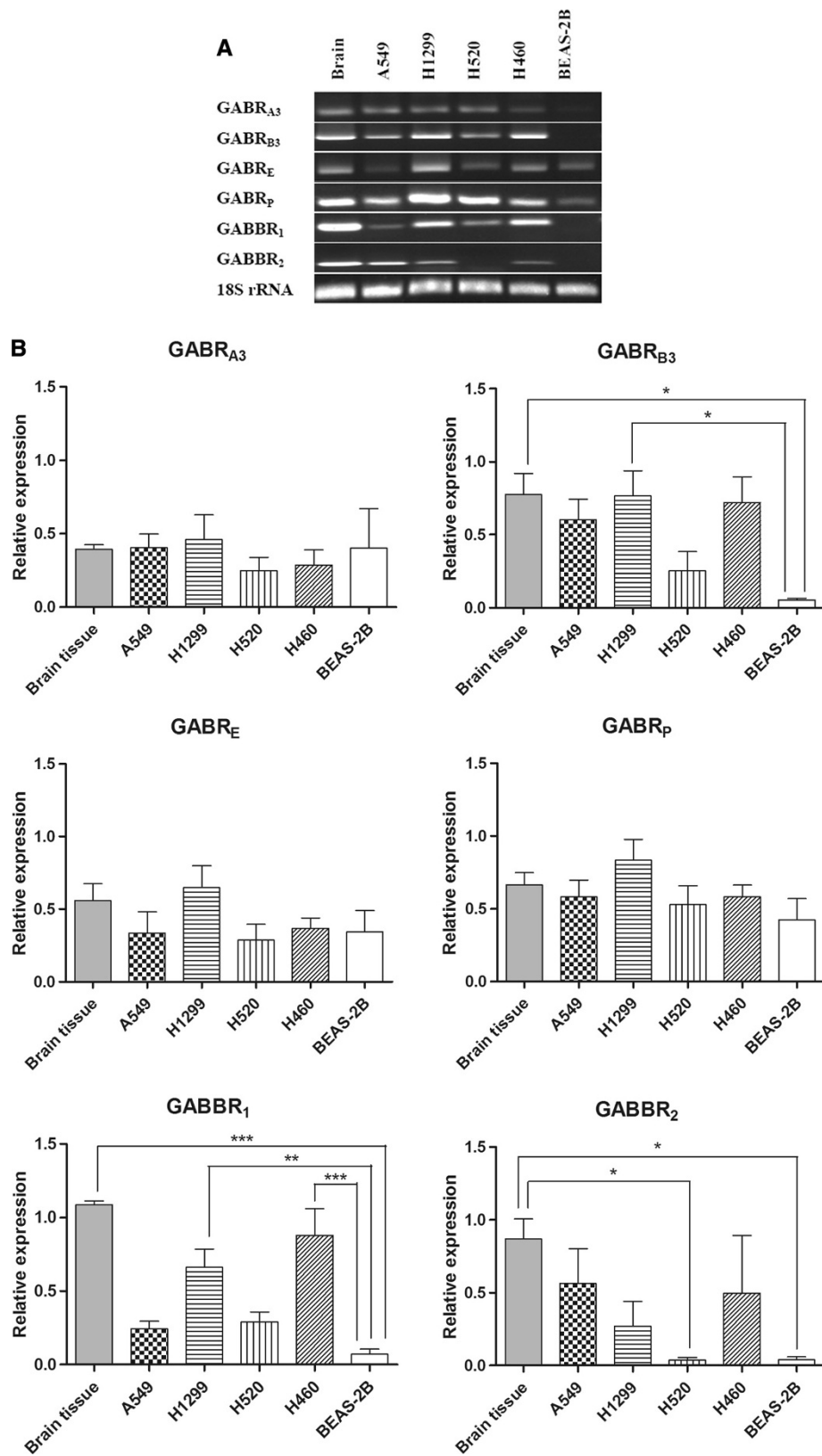


other NSCLC cell lines, such as A549, H520, H460 (data not show).

GABA receptor profiles in NSCLC cell lines

The mRNA profile of GABA receptors was determined in NSCLC cell lines including H1299, A549, H520,

H460 and BEAS-2B. The mRNA of human brain (a kind gift from Dr. He Junde, Department of neurosurgery in the same affiliation as ZX) was used as positive control and the normal lung bronchiolial epithelial cells BEAS-2B served as negative control. We found that six GABA receptor subunits were expressed in most NSCLC cell lines



(See figure on previous page.)

Figure 2 The mRNA profile of GABA receptors in NSCLC cell lines. Figure 2A show that GABRA₃, GABRB₃, GABRE, GABRP, GABBR₁ and GABBR₂ express in NSCLC cell lines. Brain expression acts as a positive control and BEAS-2B as a negative control. Figure 2B show three times of the PCR for gene relative expression. * $P < 0.05$, ** $P < 0.01$.

including GABA_A receptor subunits (GABRA₃, GABRB₃, GABRE, GABRP) and GABA_B receptor subunits (GABBR₁ and GABBR₂) (Figure 2A and B).

GABA receptor profiles in NSCLC tissues and paired non-cancerous tissues

The gene expression of GABRA₃, GABRE and GABBR₂ was significantly higher in primary NSCLC tissues than in the paired non-cancerous tissues (GABRA₃, $t = 2.227$, $P = 0.030$; GABRE, $t = 2.148$, $P = 0.036$; GABBR₂, $t = 2.928$, $P = 0.005$; Figure 3A, B and C). There was no significant difference observed in the expression of GABRB₃ ($t = -0.330$, $P = 0.742$), GABRP ($t = 1.072$, $P = 0.288$) or GABBR₁ ($t = -0.326$, $P = 0.746$, Figure 3D, E and F) in the same tissues examined (Table 2).

Survival analysis in relation to GABA receptor gene profiles

Using the median of $2^{-\Delta CT}$ value as cutoff point, the data was divided into high and low expression for analysis. A

high expression of GABBR₂ in the lung tissue was associated with better survival as compared to those where low expression was detected (Figure 4B). However, a higher expression of GABRA₃ was associated with a lower survival rate (Figure 4A). Smoking and TNM stage were also the important influential factors of decreased survival (Figure 4E, F). Expression of GABRE and pathological grade did not appear to influence on survival time (Figure 4C, D).

GABA receptors profiles and selected clinical characteristics

The gene expression of GABRA₃ was significantly higher in the NSCLC tissues in male patients, while the expression of GABBR₂ was significantly higher than paired controls in the patients of female (Table 3). It appeared that the gene expression of both GABRA₃ and GABBR₂ was higher in the patients with early stage of pathology, but not in the advanced stages.

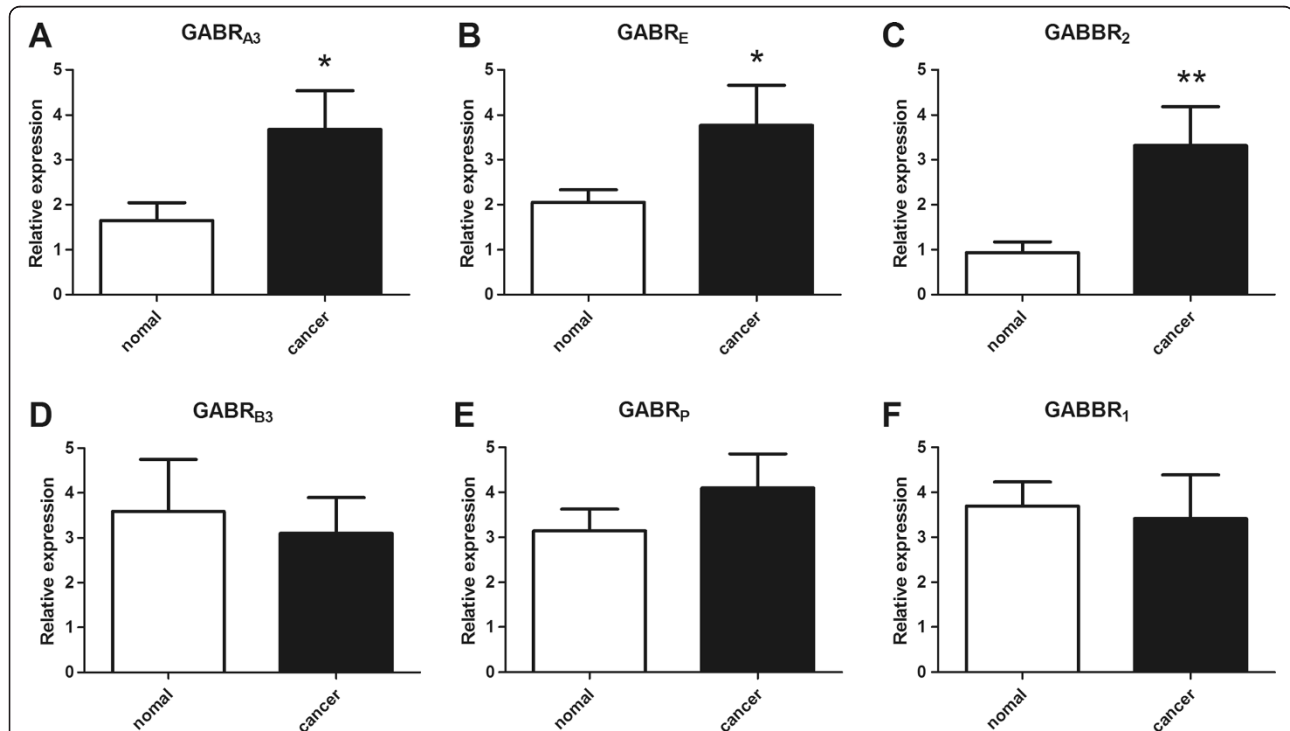


Figure 3 Gene expression levels in all NSCLC tissues and paired non-cancerous tissues. Relative expression is presented as $2^{-\Delta CT} \pm S.E$. The expression of 18S rRNA acts as reference gene. There is a significant increase in gene expression for GABRA₃ (* $P = 0.030$, Figure 3A), GABRE (* $P = 0.036$, Figure 3B) and GABBR₂ (** $P = 0.005$, Figure 3C) in NSCLC tissues, with no difference in expression for GABRB₃ ($P = 0.742$, Figure 3D), GABRP ($P = 0.288$, Figure 3E) and GABBR₁ ($P = 0.746$, Figure 3F). * $P < 0.05$, ** $P < 0.01$.

Table 2 Comparison of GABA receptor gene relative expression between cancer and paired non-cancerous tissue

	Normal(n = 61)	Cancer(n = 61)	P value
GABRA ₃	1.647 ± 0.394	3.681 ± 0.853	0.030*
GABRE	2.054 ± 0.276	3.767 ± 0.883	0.036*
GABBR ₂	0.940 ± 0.236	3.315 ± 0.868	0.005**
GABRB ₃	3.585 ± 1.163	3.103 ± 0.792	0.742
GABRP	3.147 ± 0.475	4.095 ± 0.760	0.288
GABBR ₁	3.693 ± 0.538	3.413 ± 0.976	0.746

The data of GABA receptor gene relative expression were expressed as mean ± SE. Normal: Paired non-cancerous tissue. * $P < 0.05$, ** $P < 0.01$.

Discussion

GABA and GABA receptors act as an inhibitory neurotransmitter in the mature CNS, but their functions in non-neuronal cells or tumor cells are not well addressed. Previous investigation has reported that GABA is significantly decreased in NSCLC tissues [26]. The previous observation maybe explained by our current findings demonstrating that GABA exerts inhibitory effects on human NSCLC cell. Our results further demonstrate that GABA_B receptors play an important role in mediating the GABA-inhibitory effects on NSCLC cells since the effects were blocked by using the antagonist CGP35348 specific for GABA_B receptor. Decreased GABA level might have resulted in impaired inhibitory effects on cancer cell proliferation as seen clinically [26]. A compensatory mechanism may be required by enhancing the expression of GABA_B receptors in order to redeem the impaired inhibitory effects.

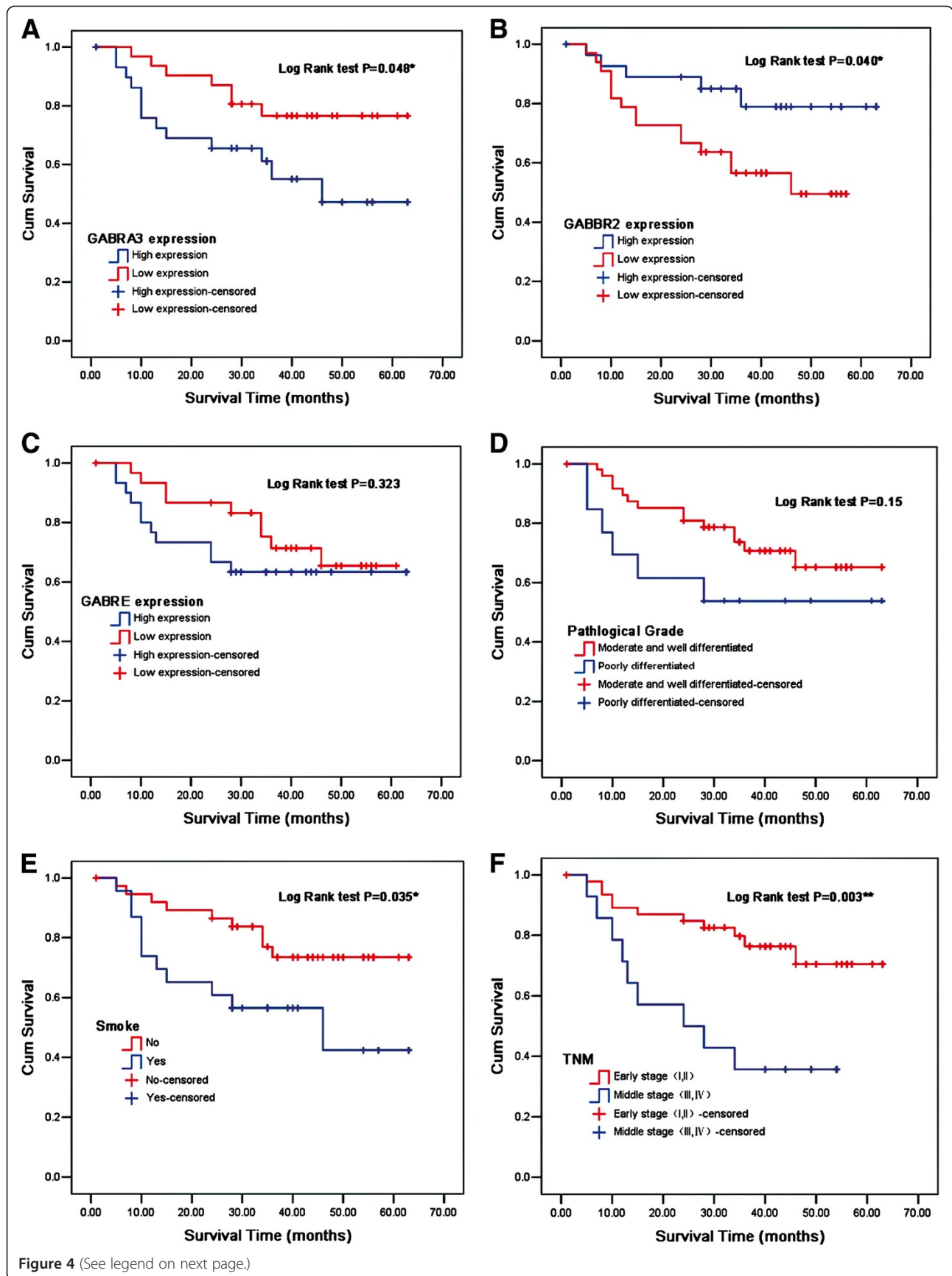
The etiological factors of lung cancer are still not clear, but the tumor progression is associated with genetic changes and is reflected in phenotypic changes such as altered gene expression profiles. In this study, we found that the six genes of GABA receptors are expressed in most of NSCLC cells and tissues, including GABRA₃, GABRB₃, GABRE, GABRP, GABBR₁ and GABBR₂. These subunits might compose functional GABA_A and GABA_B receptor. These genetic changes suggest that GABA receptors have close relationship with NSCLC progression.

As for GABA_B receptors, it is now well accepted that GABA_B receptors assemble into heteromers composed of one GABBR₁ and one GABBR₂ subunit, which are both required for normal receptor function [6,32]. Some recent reports have suggested that GABA inhibits neoplastic proliferation via GABA_B receptor [23,26]. Since GABA_B receptor could strongly inhibit base level and isoproterenol-induced cAMP, p-CREB, cyclic adenosine monophosphate response element-luciferase activity and p-extracellular regulated kinase-1 (ERK1)/2 and effectively blocked DNA synthesis and cell migration. The inhibitory cancer cells arrest in G(0)/G(1) phase which is

associated with down-regulation of intracellular cAMP level [33]. In our study, GABA inhibited proliferation of NSCLC cells in a dose-dependent and a time-dependent manner. This inhibitory effect could be blocked in the presence of GABA_B receptor inhibitor CGP35348. However, co-cultured with GABA_A receptor inhibitor picrotoxin, there were no significant proliferative effects on cancer cells versus control. These results imply that GABA inhibition of NSCLC cell proliferation was associated with GABA_B receptor which coincides with the report by Schuller and Al-Wadei [23,26]. The high level expression of GABA_B receptor gene in NSCLC tissues compared with the adjacent non-tumor lung tissues, implicated that GABA and GABA_B receptor pathways could be a critical factor in regulation of NSCLC cells proliferation. This pathway might be a promising molecular target for the development of new therapeutic strategies for antineoplaston.

As for GABA_A receptors, it has been demonstrated that GABA_A receptors are usually composed of two α subunits, two β subunits, and one γ subunit, and sometime the γ subunit is replaced by other subunits, such as δ , ϵ , π and θ [34]. Different GABA_A receptor subunits have been detected in many cancer cell lines and tissues. Li et al. [12] detected overexpression of GABR_Q in hepatocellular carcinoma cell line HepG2, and half of the tested hepatocellular carcinoma tissues. Takehara [35] identified the overexpression of GABA receptor pi subunit (GABRP) in PDAC cells. In gastric cancer, more than five GABA_A receptor subunits were associated with stimulating KATO III cells [28]. The similar results that GABA_A receptors are related to cancer cell proliferation were reported in prostate cancer [29], breast carcinoma [36], even in normal human small airway epithelial cells [37]. These findings imply that the inward Cl⁻ ionic current transport and the activated mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/Erk) cascade via GABA_A receptors positively promotes cell proliferation. Our study revealed that the GABRA₃ receptor gene was overexpression in NSCLC tissues compare with paired non-cancerous tissues, this was consistent with the study reported by Liu et al. [38]. In addition, beside overexpression of GABRA₃ and GABRE genes, the other GABA_A receptor genes expression in our study including GABRB₃ and GABRP were also detected in lung cancers, although there was no significant difference between NSCLC tissues and paired non-cancerous tissues. These GABA_A receptor subunits may form a functional pentameric chloride channel. We observe that the overexpression GABRA₃ and GABRE genes were not associated with proliferative effects on cancer cells. This indecipherable phenomenon will lend us to take more investigations of their roles in lung cancers.

Furthermore, our clinical data analysis showed that both GABA_A receptors (GABRA₃) and GABA_B receptor (GABBR₂) genes were significantly expressed in the early



(See figure on previous page.)

Figure 4 Kaplan-Meier survival curves of NSCLC patients based on various influential factors. Figure 4A, Overall survival rate in patients with high expression of GABRA₃ was significantly lower than that in patients with low expression. Figure 4B, Overall survival rate in patients with high expression of GABBR₂ was significantly higher than that in patients with low expression. Figure 4E, F, the smokers and the patients in advanced stage had a shorter survival time. Figure 4C, D, In spite of curve separated, GABRE and pathological grade seemed to have no influence on survival time. * $P < 0.05$, ** $P < 0.01$.

pathological stage (stage I and II) of the lung cancer patients, and the expression was gone in stage III and IV. This suggests that high level of gene expression of these GABA receptors may be critical in inhibition of early stage of cancer cells and this regulatory effect got impaired in advanced stage. We speculate that therapeutic intervention approaches that enhance GABA receptors maybe beneficial in late stages of NSCLC patients.

We observe a correlation between the high expression of GABBR₂ gene and the greater survival rate in patients with NSCLC. The overexpression of GABBR₂ gene was mostly seen in female patients who had better outcome. This suggests that patients with higher level GABBR₂ might have better outcome. This observation is consistent

with previous in vitro data showing that high level GABBR₂ gene expression is associated with inhibition of cancer cell proliferation [26,39,40].

In contrast, high level GABRA₃ gene expression is correlated with cancer cell development [12,35,41], and thus these patients had worse outcome. We indeed showed that the higher gene expression of GABRA₃ was mostly detected in those male patients who had a worse prognosis.

Our data was based on a relatively small sample size of 61 patients with NSCLC who were followed for 3–5 years. Our data suggests that the gene expression of certain GABA receptor subunits may be useful for prediction of NSCLC prognosis. We believe that a longer term follow-up study with larger sample size would be required to confirm our current findings.

Table 3 GABRA3, GABRE and GABBR2 gene expression in NSCLC tissues compare with paired non-cancerous tissues

Variable	N(%)	P value		
		GABRA ₃	GABRE	GABBR ₂
Total	61(100)	0.030*	0.036*	0.005**
Gender				
Male	40(66)	0.040*	NS	NS
Female	21(34)	NS	NS	0.008**
Age				
<60	36(59)	NS	NS	0.046*
>60	25(41)	NS	NS	0.014*
Smoking history				
Yes	23(38)	NS	NS	NS
No	38(62)	NS	NS	0.014*
Histotype				
Adenocarcinoma	43(70)	NS	0.049*	NS
Squamous cell carcinoma	18(30)	NS	NS	0.020*
Pathological Grade				
I, II	48(79)	NS	NS	0.008**
III	13(21)	NS	NS	NS
Pathological Stage				
I, II	47(77)	0.050*	NS	0.008**
III	9(15)	NS	NS	NS
IV	5(8)	NS	NS	NS

Pathological grade I: well differentiation, grade II: moderately differentiation, grade III: poorly differentiation. Pathological stage refers to the 7th Edition of TNM Classification of Malignant Tumors form UICC. P-value represents the gene expression of GABA receptor in NSCLC tissues compared with that in paired non-cancerous controls. NS: no significant difference. * $P < 0.05$, ** $P < 0.01$.

Conclusion

The present study has identified significant gene profiles of GABA receptors in NSCLC and the gene profiles are correlated with patients' survival. Exogenous administration of GABA can inhibit NSCLC growth by activation on GABA receptors. Our data suggests that GABA receptors can modulate cancer cell proliferation and their gene profiles may be able to help predict prognosis in patients with NSCLC.

Competing interests

The authors declare they have no competing interests of this article.

Authors' contributions

ZXX participated in all experiments and drafted the manuscript; ZR participated in the RT-qPCR assays; ZYJ, SJF, LJ, SXS, HLY, THL, and LJ carried out collection of the NSCLC tissue, RNA extraction and clinical data analysis; XDK took part in the statistical analysis; HJX participated in the study design; ZHB initiated the study design, participated in data analysis and manuscript writing. All authors read and approved the final manuscript.

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