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Differential expression of alternative transcripts of soluble guanylyl cyclase, GYCY1a3 and GUCY1b3 genes, in the malignant and benign breast tumors

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ABSTRACT

Extensive alterations in splicing is one of the molecular indicator for human cancers. Soluble guanylyl cyclase (sGC), an obligatory heterodimer, is composed of α1 and β1 subunits. Each subunit is encoded by a separate gene, GUCY1a3 and GUCY1b3, correspondingly. sGC activity has been regulated by an alternative splicing and it has an important effect on the breast cancer. sGC alternative splicing has been evaluated in the 55 malignant, 25 benign and 30 normal breast tissues using qRT-PCR and RT-PCR. The differences between groups were analyzed by Mann-Whitney U. The expression of six different splice forms have been detected, three for α1 and three for β1 sGC. Expressions of Tr1, Tr2 β1 sGC and Tr7, Tr6 α1 sGC mRNA in the malignant breast tumors were significantly lower than those of benign and normal breast tissues. However, the expression of Tr3 α1 sGC mRNA was significantly higher than that of benign and normal tissues.

Present data have provided some evidences for an alteration in the expression of α1 and β1 sGC alternative splicing forms which may contribute to the loss of sGC functions in the breast cancer. The observed information might be discussed by the cGMP status.

1. Introduction

Separation of short exons by long non-coding introns is the description of eukaryotic genome. Removal of introns and joining exons together are occurred after splicing [1]. Following alternative splicing, approximately 95% of human genes transcribe more than one transcript and produce variant protein isoforms with similar or entirely different functions, [2–4]. RNA splicing might be tissue dependent [5]. Different molecular mechanisms are involved in the regulation of gene expression including epigenetic modulation, microRNAs and alternative splicing, [6]. Normal cell differentiation and growth, migration, cell-to-cell communication and cell death are regulated by an alternative splicing [7]. Therefore, abnormal alternative splicing can disturb normal cellular function and thus cell growth [7]. So extensive alterations in splicing is one of the molecular indicator for human cancers [8]. Transcriptome analysis of different types of cancer revealed an abnormal splicing patterns in the malignant tissues [9–11]. An alternative splicing has substantial effect on the cell genesis and development of breast cancer such as tumor progression or suppression, some spliceosomal proteins or some other RNA-binding proteins [12]. Recent studies have shown that Soluble guanylyl cyclase, sGC (EC 4.6.1.2), has an important effect on the progression of breast cancer [13–17]. It has been shown that sGC activity has been regulated by an alternative splicing [18]. Mammalian sGC is a heterodimeric enzyme, comprising α_1 and β_1 subunits; although, α_2 and β_2 subunits have also been recognized. It is activated by binding of NO to heme moiety [19,20]. The C-terminal domain of α and β subunits is necessary to form a functional catalytic site with low cyclic guanosine monophosphate (cGMP)-forming activity, although NO receptor portion of sGC, located in the N-terminal domain of β subunit, is essential for the enzyme activity, NO binds to heme moiety with high affinity [21]. NCBI (National Center for Biotechnology Information) nucleotide database identifies seven alternatively spliced transcripts for α1 subunit (_1-Tr1 to 1-Tr7) and six for β1 subunit (_1-Tr1 to _1-Tr6) [18]. Expression of several sGC splice forms modulate sGC function and it's enzymatic properties [22–24]. Alteration in sGC splicing may be occur in some illnesses such as cancer and heart disease [18,25]. Alternative splicing in sGC and its biological role have not been previously studied in the breast cancer, although

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Table 1
Clinicopathological characteristics of patients.

	Normal (n = 30)	Benign (n = 25)	Malignant (n = 55)
Age (year)			
< 40	6 (20%)	20 (80%)	10 (18%)
40–60	18 (60%)	5 (20%)	31 (56%)
> 60	6 (20%)	0 (0%)	14 (26%)
Tumor size			
< 2.5		23 (95%)	43 (78%)
> 2.5		2 (5%)	12 (22%)
Grade			
1			6 (11%)
2			37 (67%)
3			12 (22%)
Stage			
I			11 (20%)
II			25 (45%)
III			19 (35%)
ER/PR			
Positive			36 (65%)
Negative			19 (35%)
c-erbB2			
Positive			19 (35%)
Negative			36 (65%)
Ki67			
< 20%			30 (54%)
20–40%			8 (15%)
> 40%			17 (31%)

ER estrogen receptor, PR progesterone receptor.

alteration in sGC splicing affected by hydrogen Peroxide, has been shown in MDA-MD-468 cancer cell line [25]. Therefore, the present study was designed to investigate the pattern of expression in the alternative splicing of α1 and β1 sGC in the malignant, benign and normal breast tissues.

2. Material and method

2.1. Tissues collection

With local ethical approval and informed consent, 55 patients with breast ductal cell carcinoma and 25 with benign, fibroadenoma, breast tumors referred to Bahman and Day Hospital of Tehran, Iran, were included in this investigation. Tumor tissues were collected during surgery from December 2015 to December 2017. Tumor samples were taken away from non-necrotic proliferative regions after their surgical removal and 30 normal adjacent tissues were sampled away. The histopathology of all specimens were confirmed by a pathologist as soon as they were harvested. Patients received no therapy prior to the surgery. Fresh tissues were carefully trimmed of necrotic and adipose tissue, wrapped in aluminum foil and frozen in liquid nitrogen, immediately. Tissues were stored at −80 °C until assayed. The correlation of alternative splicing with Patients' age, menopausal status, tumor grade, tumor size, estrogen and progesterone receptor status, P53, c-erbB2, and Ki67 have been studied. Prognostic biomarkers, evaluated by immunohistochemistry, has been mentioned in pathology reports. The clinicopathological characteristics are summarized in Table 1.

2.2. Total RNA extraction, cDNA synthesis

Frozen tissues were pulverized to a fine powder using a Mikro-Dismembrator (Mikro-Dismembrator, Braun, Germany). Total RNA was isolated by RiboEx reagent, according to the manufacturer's protocol (GeneAll, South Korea) and stored at −80 °C. RNA concentration was quantified using UV spectrophotometer at 260 nm, the purity and integrity were determined by A260/A280 ratio. Total RNA was treated with DNase (Thermo fisher, USA) and then reversely transcribed into first-strand cDNA in a 20-μl reaction volume using cDNA kit, according to the manufacturer's protocol (GeneAll, South Korea).

2.3. Transcripts variants analysis

α1 and β1 splice variants were detected using quantitative and semi-quantitative reverse transcriptase polymerase chain reaction (qRT-PCR and RT-PCR), as described by Martin et al. [18]. RT-PCR products were separated by electrophoresis on a 2.5% agarose gel and visualized by DNA Green Viewer (Pars tous Iran) staining. Electrophoretograms were quantified by densitometry using image j software. GAPDH levels were used as internal controls for normalization in quantitation of RT-PCR results. The fold difference in the expression was calculated as $2^{\Delta\Delta CT}$ for qRT-PCR.

2.4. Western blot analysis

Western blot analysis was performed as described previously [26]. Frozen tissue samples were homogenized in equal amounts of digestion buffer [150 mM NaCl, 25 mM Tris–HCl (pH 7.4), 2 mM EDTA, 1.0% NP40, 1.0% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 200 mM PMSF] containing a cocktail of complete protease inhibitors for 1 h on ice. The homogenate was centrifuged at 14,000 × *g* for 20 min at 4 °C. The clear supernatant fluid, cytosol, was collected and protein concentration was determined using Bradford assay. Total of 30 μg protein in 62.5 mM Tris loading buffer (pH 6.8, containing 25% glycerol, 2% SDS, 0.01% bromophenol blue, and 5% β-mercaptoethanol) was boiled for 5 min and loaded into each lane of 10% SDS-PAGE gel. The polypeptides were electrotransferred to PVDF. Non-specific binding was blocked with TBST (10 mM Tris (pH 8.0), 150 mM NaCl, and 0.05% Tween 20) containing 5% skimmed milk. After blocking, membranes were incubated with either α1 sGC (ab50358) or β1 sGC (ab154841) and β-actin (sc-130301), as primary antibodies, that have been diluted in 0.5% skimmed milk in TBST for overnight at 4 °C and then washed with TBST solution. After washing, the secondary antibodies diluted in 0.5% skimmed milk in TBST were added to the membrane and incubated for 1.5 h at room temperature. They were then washed with TBST solution. The antigen-antibody complexes were detected by enhanced chemiluminescence and exposed to X-ray film. Western blot bands were measured with Image j software to analyze the integrated density value (IDV). The average IDV values for α1 sGC and β1 sGC were compared with β-actin, and the average relative values were obtained.

2.5. In silico analysis

In silico analysis was performed to evaluate potential biological functions of different mRNA splicing. These splicing were analyzed by https://asia.ensembl.org and https://genome.ucsc.edu online database.

2.6. Data analysis

The statistical analysis was performed using the Statistical Package for Social Science, SPSS 18 (SPSS, Chicago, IL). Mann-Whitney *U* Test was used to analyze the difference between groups. Correlation analysis between variables was performed by Graphpad PrismV.5.0 software (GraphPad Software, San Diego CA, USA). Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. In silico analysis result

In silico analysis showed that alternative splicing of α1 and β1 sGC can potentially effect on the arrangement and function of sGC isoforms (Fig. 1).

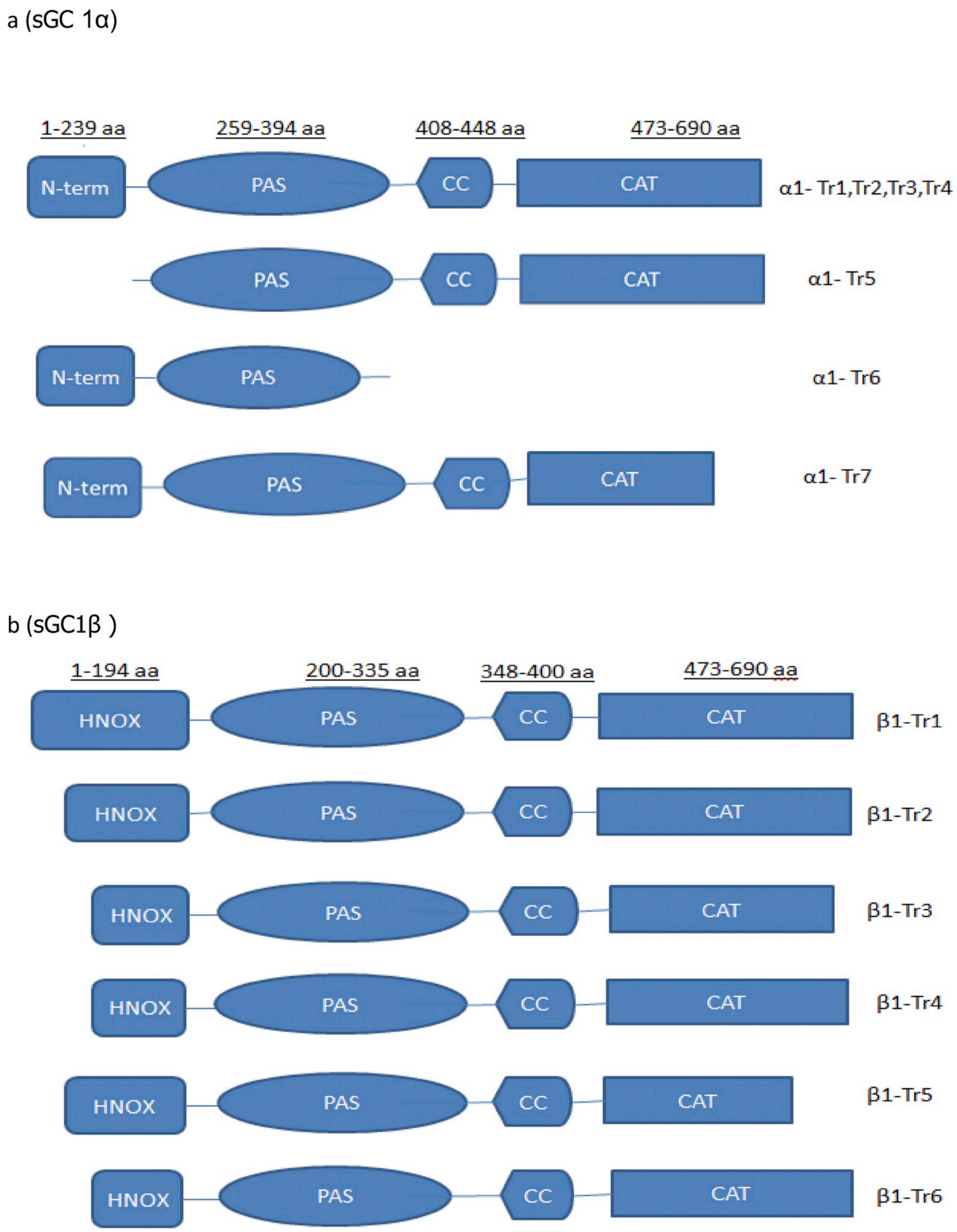

Fig. 1. Domain organization of sGC α1 (a) and β1(b) [18]. The underlined numbers above bars are domain boundaries. The numbering is in respect to canonical variants of human α1 and β1 sGC. The names of splice form transcripts and proteins are in accordance with the updated NCBI nomenclature. HNOX bindingheme/NO/oxygen domain; PAS, Per/Arnt/Sim domain; CC, coiled coil domain; CAT, catalytic domain.

NCBI nucleotide database classifies 7 alternatively spliced transcripts for human GUCY1A3 gene of α1 subunit (α1- Tr1 to α1-Tr7) and 6 alternatively spliced transcripts for human GUCY1B3 gene of β1 subunit (β1-Tr1 to β1-Tr6). Tr1-Tr4 encoded the same, 690 amino acids, protein isoform A α1 subunit. Tr5 encoded 432 amino acid protein isoform B, Tr6 encoded 366 amino acid protein isoform C and Tr7 encoded 624 amino acid protein isoform D α1 subunit. For β1 subunit, we found 6 different isoforms that were encoded by 6 different transcripts (isoform 1 to 6) [21].

3.2. The expression of α1 and β1 sGC

α1 or β1 sGC transcripts were analyzed by RT-PCR. We identified six different splice forms expressed in the breast tissues (Transcript 3,6 and 7 for α1; Transcript 1,2 and 4 for β1) (Table 2). These data are demonstrating an alternative splicing in α1 and β1 sGC genes.

Significant differences were observed in Tr3, Tr6 and Tr7, α1 sGC mRNA expressions between normal and malignant tissue. In the malignant breast tumors, expressions of Tr1, Tr2 β1 sGC and, Tr7, Tr6 α1 sGC mRNA were significantly lower and the expression of Tr3 α1 sGC mRNA was significantly higher than those of benign and normal tissues. However, no significant differences were detected for Tr4 (Fig. 2). No significant association was observed between alternative splicing distributions and Clinicopathological manifestations.

3.3. Expression of α1 and β1 sGC protein

In the malignant tumors, the expression of full-length β1 sGC protein was lower than those of benign and normal breast tissues; albeit, these differences were not statistically significant ($P > 0.05$).

Table 2
Detection of human α1 sGC (GUCY1a3) and β1 (GUCY1b3) sGC transcripts.

α1	Detect in breast	isoform
*Transcript*1	–	A
*Transcript*2	–	A
*Transcript*3	+	A
*Transcript*4	–	A
*Transcript*5	–	B
*Transcript*6	+	C
*Transcript*7	+	D
β1		
*Transcript*1	+	1
*Transcript*2	+	2
*Transcript*3	-	3
*Transcript*4	+	4
*Transcript*5	-	5
*Transcript*6	-	6

However, the level of full-length α1 sGC protein was higher in the malignant and benign breast tumors than that of normal tissues ($P < 0.001$), (Fig. 3 and Fig. 4).

4. Discussion

There are Growing evidences to suggest that NO/cGMP signaling pathway is related to the development of different cancers, such as breast cancer [15,17,27–29]. Soluble guanylate cyclase (sGC) is encoded by separate genes and can be regulated independently, although heterodimeric form of sGC is necessary for the enzyme activity [13]. Therefore, an alternative splicing could generate different isoforms. Alternative splicing regulates mRNA expressions and several changes in alternative splicing have been known as a hallmark of cancer [30]. Different studies have revealed that an alternative splicing has a key role in the regulation of cGMP and NO/cGMP signaling pathways, such as cGMP-dependent protein kinase I [31] and cGMP-dependant phosphodiesterase (PDE) [32,33]. It has been shown that α1sGC splicing regulates human sGC activity and higher expression of various splice forms have been described in some tissues [24]. Expression and regulation of sGC subunit can be affected by different situations in the cancer cells such as epigenetic [13] and alternative splicing (present findings).

Our findings demonstrated that the expression level of full-length α1 sGC protein was higher in the malignant and benign breast tumors than that of normal tissues. Similarly, an increase in the expression of α1 sGC subunit has been detected in glioma cell lines [34]. Higher expressions of sGCα1 and sGCβ1 have also been shown in the breast cancer cell line, MDA-MB-468 [35].

In the present study, the expression of different alternative splicing forms have been evaluated in the malignant, benign and corresponding normal breast tissues. The expression of six, out of 13, different splice forms have been detected, three for α1 and three for β1 sGC. The presence of three alternative α1 sGC splice transcripts have been reported in human heart, brain, artery, and Bcells [36]. Our results revealed that sGC mRNA expressions of Tr7 and Tr6 for α1 subunits in the malignant breast tumor were significantly lower than those of normal and benign tissues, no significant differences were observed between benign and normal tissues. In the present investigation, mRNA expressions of Tr3 in the malignant tumor was found to be higher than those of benign and normal breast tissues which could be explained by the elevation of cGMP in the breast cancer [37,38]. No significant changes were found in the benign tumors when compared to those of normal tissues. On the contrary in an investigation in aortas with aneurysm disease, using the same methods as ours (RT-PCR), a much higher level of transcripts coding for α1-IsoD (Tr7) and α1-IsoC (Tr6) splice variants have been obtained [18]which results in the reduction of cGMP in this disorder.

Transcript 6, isoform C (N1-α1 variant) encodes α1 sGC protein with extensive deletion in the catalytic domain and inhibits the activity of α1/β1 sGC [24]. Transcript 7, isoform D, is also produced by an alternative splicing in polyadenylation site on exon 10 [39]. This mRNA isoform encodes an aberrant polypeptide that missing 66 amino acid residues at the C-terminal (39). The deletion which is located in close proximity to the catalytic domain, might affect the catalytic properties of sGC's α1 subunit [39].

Concerning Transcript 5, sGC's α1 subunit in the present investigation, no expression has been detected. However, in an in vitro study using cancer cell lines, high expression of Tr5 of α1 has been found by Cote et al. They showed that an alternative splicing could be changed by antioxidant balance in the presence of H_2O_2, [25].

The results of present study revealed that Tr1 and Tr2 expressions, related to β1 sGC, in the malignant tumors were lower than those of benign and normal breast tissues, no significance differences were observed for Tr4, sGC's β1. Similarly, no expressions were detected for Tr3, Tr5 and Tr6 sGC's β1. Our results are validated by the facts that proteins encoded by alternative transcripts β1-Tr 3, 5, and 6 are expected to be dysfunctional since, they have missing regions in the catalytic domain or deletions/insertions in the heme-binding region [18], although Tr1 and Tr2 expressions observed in the present study, are essential for the enzyme activity.

In an initial study, two different mRNA for β1 sGC expression have been detected in the human lung tissue [40]. An increase in α1 and a decrease in β1 expressions have also been shown in the present study. These results are validated by Cabilla eat al findings [41], since, they have demonstrated that estrogen can regulate sGC/cGMP pathway by a decrease in β1 and an increase in α1 expressions in some tissues.

In conclusion, our data have provided some evidences for α1 and β1 sGC alternative splicing forms that may contribute to the loss of sGC functions in the breast cancer. Accurate understanding of sGC splicing regulation may develop a new approach for the treatment of breast cancer and optimizing sGC-targeting therapy.

Declarations of interest

The authors declare that they have no conflict of interest.

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Ethics approval

All procedures performed in this study were approved by the Tarbiat Modares University Ethics Committee and in accordance with the ethical standards of 1964 Helsinki declaration and its later amendments or comparable ethical standards.

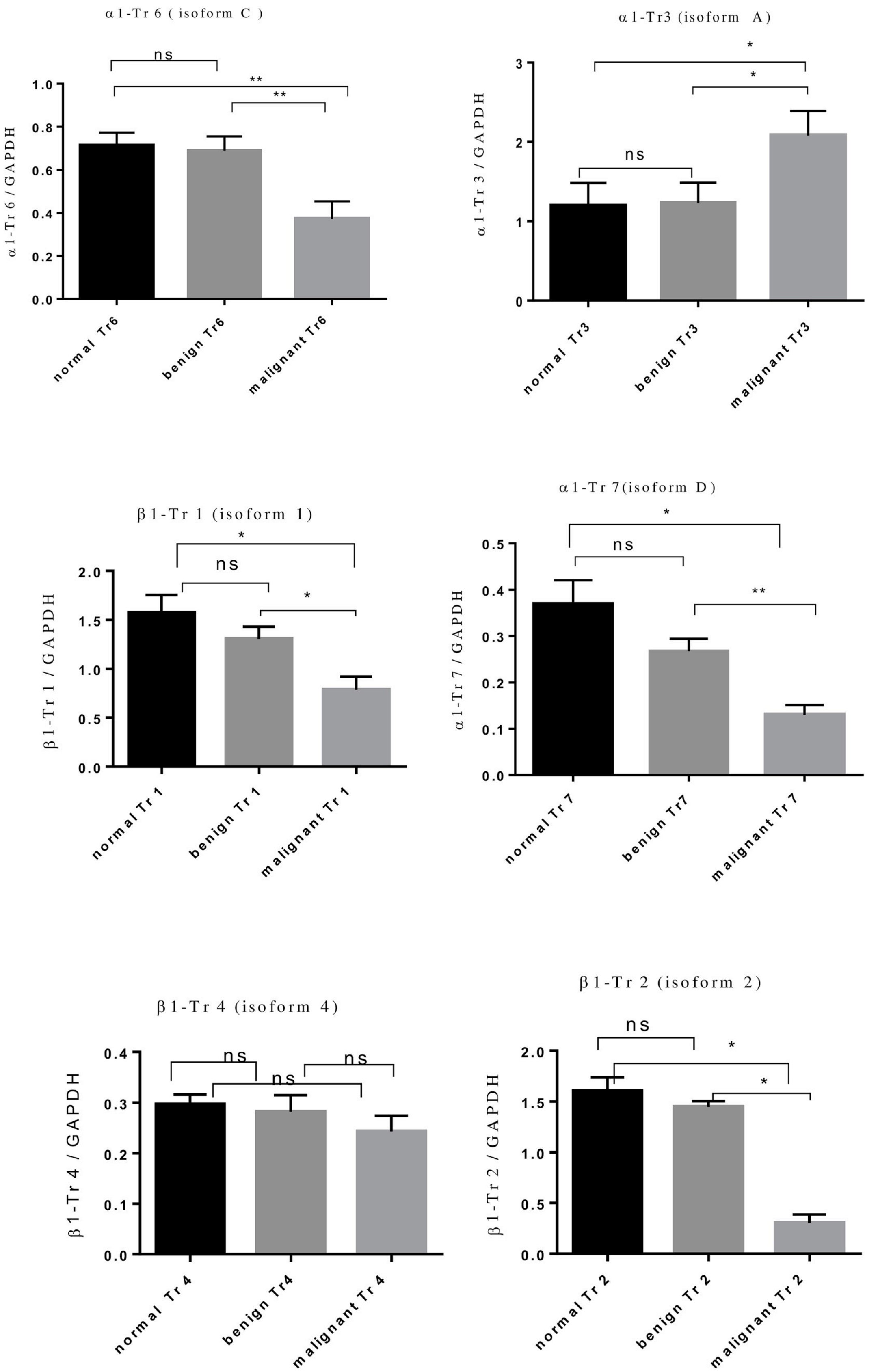

Fig. 2. mRNA expressions of α1 and β1 transcript variants in the malignant, benign and normal breast tissues (*$P < 0.001$; **$P < 0.05$; ns not significant).

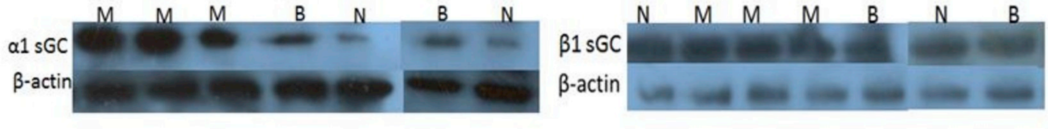

Fig. 3. α1 and β1 sGC expressions in the normal, benign and malignant breast tissues. α1 and β1 sGC expressions were detected by western blot. β-actin was used to normalize (N, normal breast tissue; B, benign breast tumor; M, malignant breast tumor).

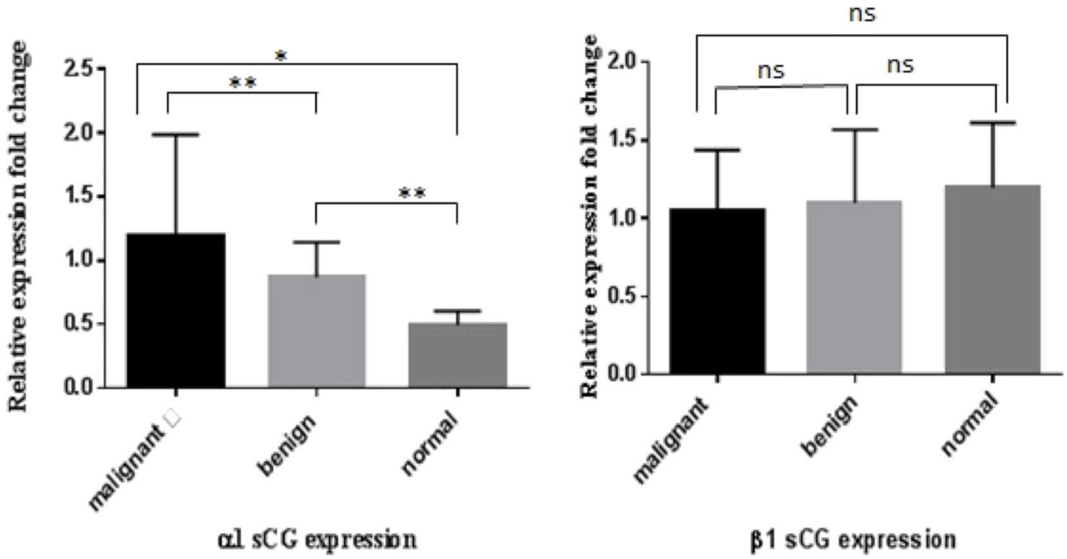

Fig. 4. Expression of sGC subunits (α1, β1) in the malignant, benign, and normal breast tissues (*P < 0.001; **P < 0.05; ns, not significant).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.niox.2018.12.009.

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