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Sperm-Associated Antigen 9 as a Candidate Diagnostic and Prognostic Biomarker in Breast Cancer

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Abstract

Background: Cancer/testis antigens are a unique class of tumor antigens with normal expressions restricted to the testis and various cancers, but not in adult somatic tissues. *Sperm-associated antigen 9* (*SPAG9*) has been introduced as a new member of Cancer Testis Antigens family involved in c-Jun-NH2-kinase signaling module.

The objective of this research was to investigate the potential of *SPAG9* as a diagnostic and prognostic biomarker in breast cancer. We further aimed to find any significant association between *SPAG9* expression and clinicopathologic features of the cancer.

Methods: In this retrospective study, 35 breast cancer tissues and 35 adjacent non-cancerous tissues were collected and examined using RT-PCR to explore *SPAG9* mRNA expression. Statistical analysis was done utilizing SPSS 22.0 software.

Results: Unexpectedly, we detected *SPAG9* expression in 54% of adjacent non-cancerous tissues. Moreover, *SPAG9* mRNA was expressed in 57% of cancerous tissues. Statistical analysis showed a significant association between *SPAG9* expression and tumor size, lymph node metastasis, and cancer stage.

Conclusion: The association between the gene expression and tumor size, lymph, node and metastasis, and cancer stage suggests that *SPAG9* can potentially be considered as a prognostic biomarker in breast cancer. However, it may not be a candidate diagnostic biomarker.

Keywords: *SPAG9*, Cancer/testis antigens, Breast cancer, Biomarker, RT-PCR

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Introduction

Breast cancer is the second most prevalent cancer worldwide, and by far, the most frequent cancer among women, with an estimated 1.7 million new cancer cases diagnosed in 2012. Breast cancer is the fifth cause of overall cancer-related mortality.[1] Statistics of breast cancer incidence among Iranian women report about 10,000 new cases yearly.[2] In addition, annually, there are 1063 cases of breast cancer death in Iran.[3] In addition to the high prevalence of

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breast cancer in Iran, the fact that the average age of breast cancer among Iranian women is at least a decade earlier than reported in the developed countries, makes it a particularly more important concern.[4]

A biomarker is a molecule, gene, or characteristic that can be measured in the body to predict the incidence of outcome or disease. Prognostic biomarkers are a type of cancer biomarkers which enable the monitoring of the advances in anticancer therapy, the assessment of the tumor stage, and its potential malignancy. Molecular prognostic factors are more important nowadays; however, traditional markers such as the number of regional metastatic lymph nodes, tumor size, and tumor grade are still considered.[5]

Cancer/testis antigens (CTAs) are a group of genes normally expressed in germ-cells and trophoblasts and abnormally activated in up to 40% of various types of cancers.[6] So far, no defined biological function of CTAs has become known; however, it has been proposed that these molecules are involved in signaling, transcription, translation, and chromosomal recombination.[7] It is further suggested that the aberrant expression of CTAs in the tumor may contribute to different malignant properties such as immortality, migration, invasion, and metastatic capacity.

Sperm-associated antigen 9 (*SPAG9*) is a member of the CTAs family expressed from a single copy gene located on human chromosome 17q2. *SPAG9* functions as a scaffolding protein in c-Jun-NH2-kinase (JNK) signaling module. This suggests its involvement in physiological processes, including apoptosis, survival, proliferation, and tumorigenesis.[8] Scaffold proteins act by modulating the signaling strength of their cognate mitogen-activated protein kinase (MAPK) module through regulating the signal amplitude and duration. *SPAG9* has been suggested as a novel biomarker for early diagnosis of ovarian cancer, chronic myeloid leukemia, and bladder transitional cell carcinoma.[9]

The purpose of this study was to investigate *SPAG9* expression as a probable diagnostic and prognostic biomarker in Iranian breast cancer patients and find any significant association between *SPAG9* expression and the clinicopathologic features of cancer.

Materials and Methods

Patients and tissue samples

The Ethics Committee of the National Institute of Genetic Engineering and Biotechnology approved this retrospective study (Ethics Code No: IR.NIGEB.EC.1395.5.6). After obtaining written informed consent from all patients, we collected 35 breast cancer tissues as an

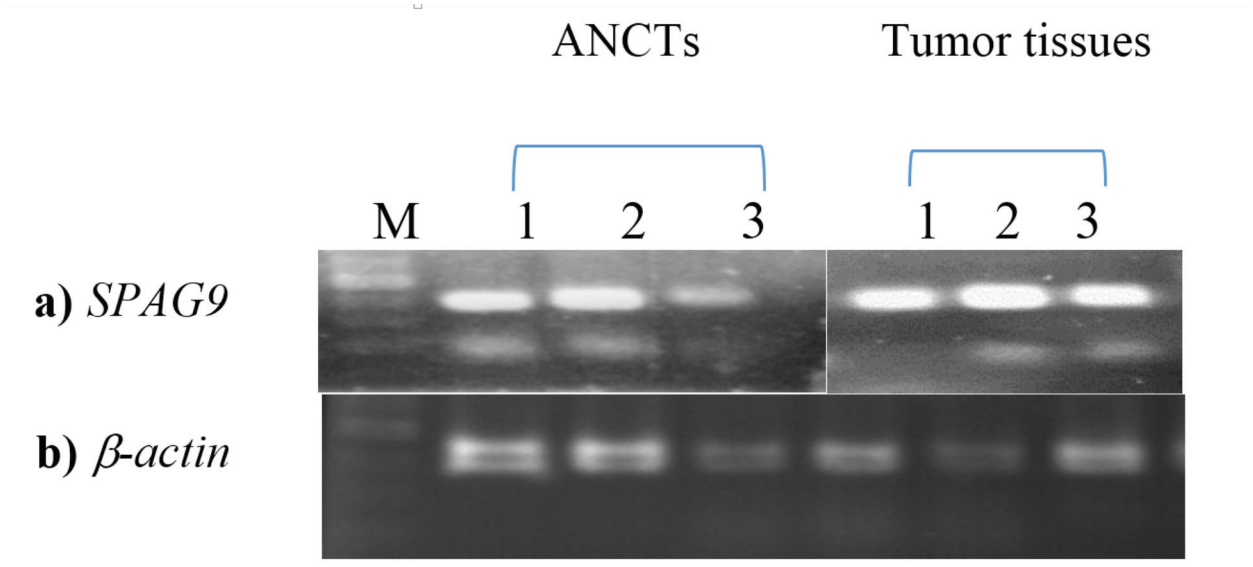

Figure 1. a) RT-PCR analysis of SPAG9 mRNA expression showing specific 141bp products in breast cancer and adjacent non-cancerous tissues. M, molecular size marker b) β-actin expression as an internal control.

experimental group and 35 adjacent non-cancerous tissues (ANCTs) as a control group. The obtained tissue samples belonged to women admitted to the Khatam Al-Anbia semi-private Hospital in Tehran and surgically treated without chemotherapy. Tissue specimens were immediately snap-frozen in liquid nitrogen and archived at -70°C until use. Pathology reports provided the patients' demographics and clinicopathologic variables. Sample size calculation was done using the following formula:[10]

$$n \geq \frac{Z^2_{\alpha}/2*pq}{d^2}$$

n – Sample size

Zα/2 – Critical value for the desired confidence degree, usually: 1.96 (95%)

d–Standard error, usually: ±5% of the proportion of cases (absolute precision)

p – Proportion of cancerous samples in the population

q – Proportion of adjacent non-cancerous samples in the population (q=1-p).

Table 1. Patients' demographic data*

Clinicopathologic features	Frequency N (%)
Age (years)	
<50	19 (54.3)
≥50	16 (45.7)
Tumor size	
T1(<2 cm)	9 (25.7)
T2(2-5 cm)	26 (74.3)
TNM Stage	
I	12 (34.3)
II	17 (45.7)
III	6 (17.1)
Histological type	
IDC	24 (68.6)
ILC	4 (11.4)
others	7 (20)
Histological Grade	
G1	2 (5.9)
G2	23 (67.6)
G3	9 (26.5)
Lymph node metastasis	
No	16 (47.1)
Yes	18 (52.9)
ER status	
Negative	8 (25)
Positive	24 (75)
PR status	
Negative	7 (21.9)
Positive	25 (78.1)
Her-2 status	
Negative	24 (75)
Positive	8 (25)
Necrosis	
No	10 (37)
Yes	17 (63)

*TNM, tumor, node, and metastasis; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; others, invasive ductal and lobular carcinoma, ductal carcinoma in situ and medullary carcinoma; G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated.

RNA extraction and cDNA synthesis

We carried out the total RNA extraction using the TriPure Isolation Reagent (Roche) according to the manufacturer's protocol. After determining the extracted RNA concentration, 5.5µl RNA of each sample was utilized for complementary DNA (cDNA) synthesis using Thermo Scientific Revert Aid First Strand cDNA Synthesis kit.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

To examine *SPAG9* mRNA expression, we designed specific primers with the amplicon size of 141bp using Oligo7; NCBI/blast was then employed to confirm the specificity. Avoiding any genomic DNA contamination, we designed the primers from exon-exon junction regions of the target gene.

SPAG9 Forward Primer: 5'-GCAGTAAACAGC-

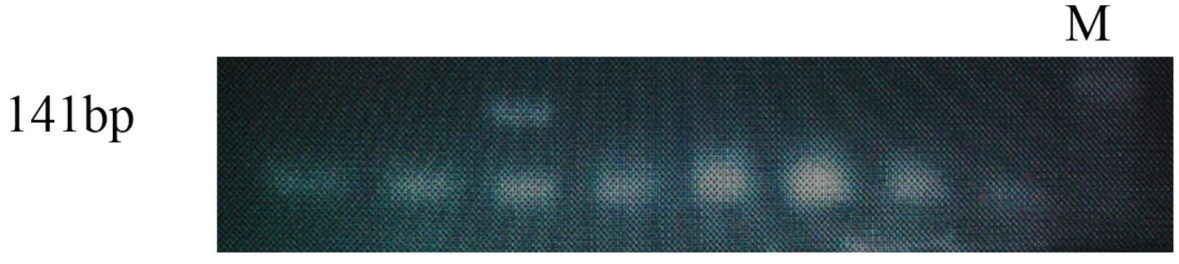

Figure 2. SPAG9 mRNA expression in normal breast tissue. M, molecular size marker.

GAAGTG-3'
SPAG9 Reverse Primer: 5'-CTTTTGTAGCC-GAATGAGT-3'

We first performed gradient PCR to find the most optimal annealing temperature. RT-PCR was then employed using Ampliqon Taq 2x master mix (Red,1.5 mM mgcl2) in a volume of 10µL containing 5µL of Master, 0.5µL of each primer (10 µM) and 0.5µL of (diluted 1:10) cDNA. Afterwards, we carried out 40 cycles of amplification. Ensuring the accuracy of the results, all experiments were repeated three times. PCR products were then analyzed on 1.5% agarose gel and photographed under UV light (Figure 1).

Statistical analysis

Statistical analysis was performed using SPSS 22.0 software. We assessed the association between *SPAG9* expression and clinicopathologic variables using Pearson's χ^2 and Mann-Whitney U tests. *P* values of 0.05 or less were statistically significant.

Results

Patients' demographic data

In total, 35 patients and 11 healthy women, undergone mammoplasty surgery, enrolled in this study. Table 1 summarizes the patients' demographic data.

SPAG9 expression in cancerous and adjacent non-cancerous tissues

Unexpectedly, this gene was expressed in 54% (19 of 35) of adjacent non-cancerous tissues (Figure 1) and 57% (20 of 35) of cancerous tissues. As mentioned, *SPAG9* is a CTA which, in theory, is not expected to be expressed in normal tissues, except testis and certain cancer cells.

According to the results, we decided to test *SPAG9* mRNA expression in normal breast tissues. Therefore, we collected normal breast tissues from 11 healthy women, undergone mammoplasty surgery, with a mean age of 37. Interestingly, *SPAG9* expression was once again observed in one of the samples (Figure 2).

SPAG9 expression in cancerous tissues and clinicopathologic variables

SPAG9 expression and patients age

The estimated mean age of patients was 52.2±11.7 years. *SPAG9* was expressed in the cancerous samples of 57% (11 of 19) of patients younger than 50 years old and 56% (9 of 16) older than 50 years. *SPAG9* expression was independent of age (*P*=0.93).

SPAG9 expression and tumor size

The average assessed tumor size of samples was 2.5 ± 1.0. We found the mRNA expression of *SPAG9* in 88% (8 of 9) of tumors smaller than

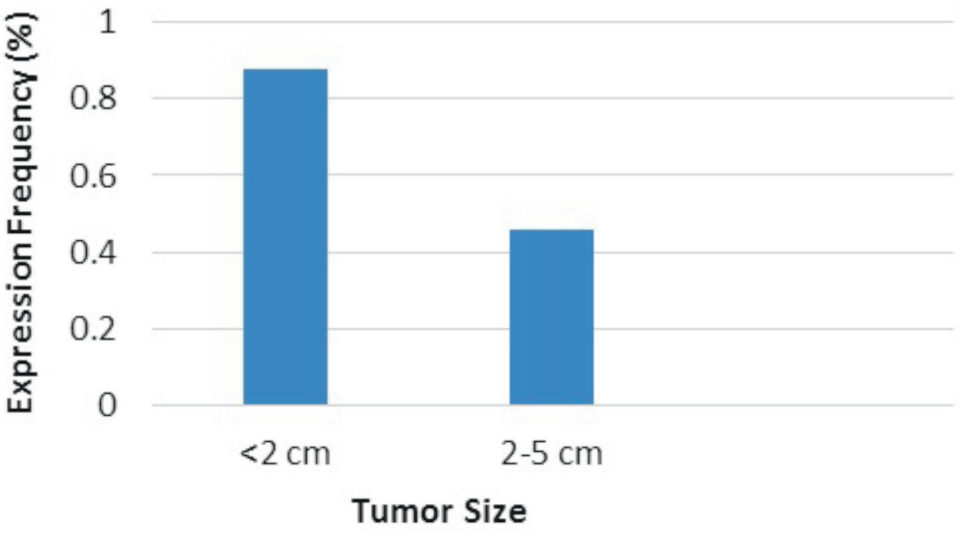

Figure 3. SPAG9 expression and tumor size.

2cm and 46% (12 of 26) of tumors with 2-5cm size. There was a significant association between *SPAG9* expression and tumor size (*P*=0.05) (Figure 3).

SPAG9 expression and cancer stage

SPAG9 expression was observed in 83% (10 of 12) of stage I, 47% (8 of 17) of stage II, and 33% (2 of 6) of stage III. A significant association existed between *SPAG9* expression and TNM stage (*P*=0.05) (Figure 4).

SPAG9 expression and histological type

SPAG9 was expressed in 58% (14 of 24) of invasive ductal carcinoma (IDC), 75% (3 of 4) of invasive lobular carcinoma (ILC), and 42% (3 of 7) of other histological types. We further observed that *SPAG9* expression was not associated with the histological type of samples (*P*=0.61).

SPAG9 expression and histological grade

SPAG9 was expressed in 50% (1 of 2) of grade 1, 60% (14 of 23) of grade 2, and 44% of (4 of 9) grade 3; however, there was no association between expression and histological grade (*P*=0.69).

SPAG9 expression and lymph node metastasis

We identified *SPAG9* mRNA in 81% (13 of 16) of breast cancer tissues with negative lymph node involvement compared to 33% (6 of 18) tissues with lymph node involvement. There was a significant association between *SPAG9* expression and lymph, node, and metastasis (*P*=0.01) (Figure 5).

SPAG9 expression and ER status

SPAG9 expression was observed in 37% (3 of 8) of ER- and 62% (15 of 24) of ER+ specimens without any significant association between these two factors (*P*=0.20).

SPAG9 expression and PR status

SPAG9 was expressed in 60% (15 of 25) of PR- and 42% (3 of 7) of PR+ tissues. We found no association between *SPAG9* expression and PR status (*P*=0.35).

SPAG9 expression and HER-2 status

We detected *SPAG9* expression in 62% (5 of 8) of HER-2- together with 54% (13 of 24) of HER-2+ samples; however, no association existed between HER-2 status and *SPAG9* expression (*P*=0.50).

SPAG9 expression and necrosis

SPAG9 was expressed in 58% (10 of 17) of breast tissues without necrosis and 60% (6 of 10) of tissues with necrosis. There was no association between these two parameters (*P*=0.63).

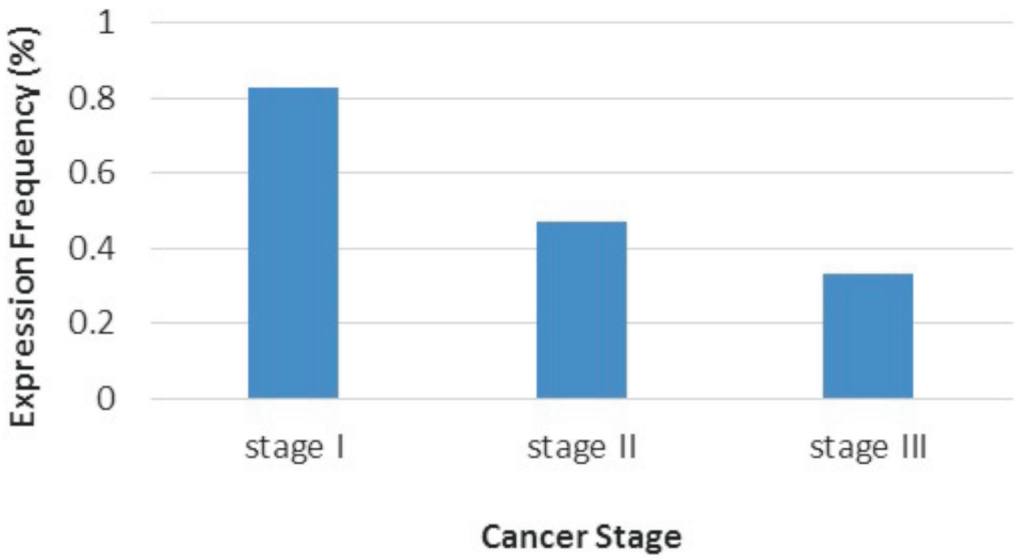

Figure 4. SPAG9 expression and cancer stage.

SPAG9 expression and patients' age in adjacent non-cancerous tissues

In adjacent non-cancerous samples, *SPAG9* was expressed in 50% (9 of 18) of patients younger than 50 years old and 58% (10 of 17) of those older than 50 years. *SPAG9* expression was independent of age (P=0.44).

Discussion

Despite the data from previous studies,[16,17] our results confirmed that *SPAG9* was indeed expressed in adjacent non-cancerous tissues and even the normal breast tissues. This makes the value of this gene as CTA doubtful.

SPAG9 encodes a protein which functions as a scaffolding protein and interacts with c-Jun NH2-terminal kinase subgroup of mitogen-activated protein kinases. Recently, *SPAG9* has been reported as a candidate cancer-associated marker in various cancers including colorectal cancer,[11] endometrial cancer,[12] lung cancer,[13] osteosarcoma,[14] cervical cancer,[15] and renal cell carcinoma.[7]

To the best of our knowledge, there are only two published papers regarding *SPAG9* mRNA expression in breast cancer. The first study was performed by Kanojia et al. in 2009. They reported *SPAG9* expression in cancerous tissues, but not in adjacent non-cancerous tissues. Furthermore, *SPAG9* expression was independent of tumor stage, yet significantly associated with the grades.[16]

The second study was performed in 2013 by Sinha et al. on four breast cancer cell lines, namely MCF-7, BT-474, SK-BR3, and MDA-MB-231 together with normal mammary epithelial cells. Their results confirmed *SPAG9* mRNA expression in all examined samples except normal mammary epithelial cells. This obviously shows *SPAG9* exclusive expression in cancerous cells.[17]

Our study showed *SPAG9* expression in 54% (19 of 35) of adjacent non-cancerous tissues, 57% (20 of 35) of cancerous tissues, and one of the normal breast tissues. Possible explanations for the attained results are:

As stated earlier, any gene that exhibits an mRNA expression restricted to the testis and neoplastic cells can be called a CT gene. In the literature, the existing definitions of CT genes vary from "genes expressed exclusively in adult testis germ cells and malignant tumors" to "dominant testicular expression, possible additional presence in placenta, ovary and epigenetic regulation, and membership of a gene family and localization on the X chromosome".[6] Therefore, due to the paucity of a distinct definition for CTAs, a growing number of CT candidates have appeared in the literature.

Another classification system for CTAs is based on their expression profile, dividing them

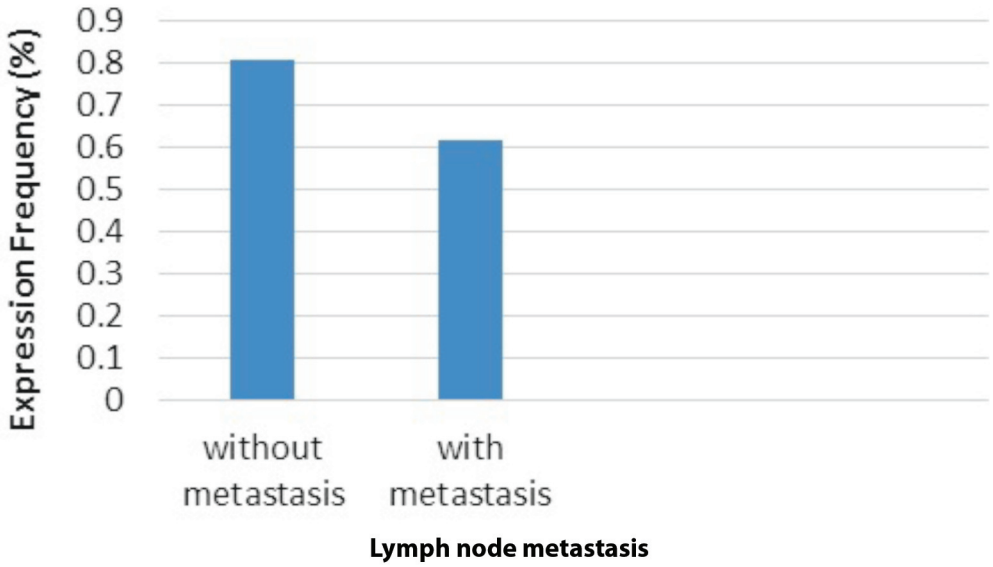

Figure 5. SPAG9 expression and lymph node metastasis.

into three subclasses: (a) testis restricted (found only in the testis), (b) testis-brain restricted (expressed in the testis and central nervous system), (c) testis selective (expressed in the testis and no more than two additional tissues at lower levels than in the testes).[18]

Based on The Human Protein Atlas (https://www.proteinatlas.org/), *SPAG9* RNA and protein expression has been reported in numerous tissues including breast tissue.

Transcriptome analysis of healthy, adjacent non-cancerous, and tumor tissues in 6506 samples from eight tissues including breast tissue showed that adjacent non-cancerous tissues presented a unique intermediate state between healthy and tumor. Also, differential gene expression and protein-protein interaction analyses revealed altered pathways among adjacent non-cancerous tissues across tissue types. A set of 18 genes specifically expressed in adjacent non-cancerous tissues were ultimately characterized.[19]

Regarding the relationship between the expression and clinicopathologic factors, we found no association between the expression of this gene and the patients' age, grade, ER, PR, Her-2, and necrosis; however, there existed a significant association between *SPAG9* expression and tumor size, cancer stage, and lymph node involvement. Lymph node involvement is particularly related to the risk of metastasis; therefore, it can be proposed that *SPAG9*-positive expression may be considered as a poor prognostic marker in breast cancer.

This study had certain limitations such as the relatively small number of the patients and the bias towards the middle or high-income patients (often low-income patients refer to the governmental hospitals); Therefore, more investigations are required to obtain more robust results.

Conclusion

Due to the expression of *SPAG9* mRNA in adjacent non-cancerous tissues and normal breast tissues, *SPAG9* may not be used as a diagnostic biomarker. However, the association of *SPAG9* expression with tumor size, lymph node metastasis, and cancer stage suggests the use of this gene as a possible prognostic biomarker in patients diagnosed with breast cancer.

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Conflict of Interest

None declared.

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