CYCLIN D1 (G870A) POLYMORPHISM AND BREAST CANCER RISK IN GUILAN PROVINCE POPULATION OF IRAN

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Abstract:

Background and Objective: Cyclins are the key regulator of the cell cycle, and their over-expression has been seen in many cancers, including breast cancer. Cyclin D1 is an oncoprotein encoded by the CCND1 gene located on chromosome 11 (11q) that regulates the cell cycle in shifting from the G1 to the S phase. It's the main target for steroids and mitogenic growth hormones in breast epithelial cells. This study aimed to evaluate the relationship between Cyclin D1 G870A polymorphism and breast cancer risk in a population of Guilan province in the north of Iran.
Methods: Whole blood samples (CBC Tube) were collected from 82 patients with breast cancer and 66 healthy women. DNA extraction and genotyping were performed by Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP) technique.
Results: Genotypic prevalence of AA, AG, and GG genotypes among patients were 40.2%, 35.3%, and 24.4%, and in controls were 30%, 47%, and 23%, respectively. There was no significant difference in CCND1 G870A genotype polymorphism between patients and the control group (p=0.32). Also, the allelic prevalence of A and G alleles in breast cancer patients was 58% and 42%, in controls were 54% and 46%, respectively. The present study showed that there is no significant association between CCND1 G870A polymorphism and the risk of breast cancer.
Conclusion: The results of this study revealed that there is no significant association between CCND1 G870A genetic polymorphism and the risk of breast cancer in the population of Guilan province of Iran. More studies with larger samples of cases and controls would be beneficial.
Keywords: Breast cancer; ccnd1; polymorphism

Introduction

Cell division is very critical for organism life. It's divided into four steps, including G1, S, G2, and M phases. There are Regulatory proteins that control cell cycle phase transitions. At the G1, D-type Cyclins bind to their Cyclin-Dependent Kinases (CDKs), resulting in the phosphorylation of retinoblastoma protein (Rb). At the end of G1, Activated Rb will release

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E2F (family of genes that encoded transcription factors) that regulates gene transcriptions necessary for the S phase. Cyclin D1 is an oncoprotein that regulates the cell cycle G1 phase (John et al., 2017).

Cancer development needs oncogene activation through gene amplification. Some genomic regions tend to be more amplified. Amplification in locus 13 of the long arm of chromosome 11 (11q13) has proven to be associated with many malignancies, including breast cancer (Pabalan et al.,2008; Lundgren et al., 2008; Ramos-Garcia et al., 2017; Pandey et al., 2018; Yang et al., 2017; Salimi et al., 2017; Wen et al., 2014). CCND1 (Cyclin D1) is an oncogene mapped on 11q13, amplified in most breast cancer cases (Ormandt et al., 2003). CCND1 encodes a protein, Cyclin D1, a member of the cyclin-dependent kinase family and the main regulator of the cell cycle G1/S phase transition (Sherr, 1996). Amplification of CCND1 leads to overexpression of Cyclin D1, which has an essential role in breast cancer progression (Buckley et al., 1993). Approximately 35-50 % of breast cancer cases are associated with Cyclin D1 overexpression (Soleimani et al., 2017). However, some studies reported no significant association between CCND1 and breast cancer risk (Grieu et al., 2003).

CCND1 gene consists of 5 exons and 4 introns. It has 2 transcripts: transcript a (G allele), which is normal, and a mutant product named transcript b (A allele) (Gupta et al., 2008). G to A transition leads to skipping exon 5 and instead translating some parts of intron 4; this Results in an alternate transcript (Transcript b) with a lack of proline (P), glutamic acid (E), serine (S), and threonine (T) peptides (PEST) motif. PEST consists of a phosphorylated threonine residue and contributes to transcript-product transformation and Ubiquitination, so it is essential in the degradation of the Cyclin D1 protein (Inoue and Fry, 2015). Therefore, transcript b (A allele) has a longer abnormal half-life than the G allele. Cells with CCND1 G870A transition tend to bypass the G1/S checkpoint and become cancerous (Betticher et al., 1995). So, this project aimed to evaluate the association of CCND1 G870A Polymorphism with the risk of breast cancer in the Guilan province population of Iran.

Materials and Methods

Collected Blood Specimens were from Razi Hospital and Jam Clinic, Guilan province of Iran. All patients were diagnosed based on mammography and biopsy. The age distribution of patients was between 32 and 78 years (50.05±11.78). Controls were also selected in the same area age range (52.82 ± 12.38) (P=0.81). After interviewing and considering the characteristics of participants, 66 healthy women as the control group and 82 patients with diagnosed breast cancer were included in this study.

This project was accepted by the University of Guilan Ethics Committee and has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Collected Whole blood specimens were in EDTA-containing tubes. DNA extracted from samples according to the GPP solution kit procedure made by Gene Pajhouhan Pooya factory-Iran. The extraction procedure was based SDS-proteinase K method (Protein Precipitation Solution, chloroform, Nacl, and Ethanol). Each DNA sample was incubated in Tris_EDTA (TE) Buffer and stored at -20°C. DNA extraction validity was checked by agarose gel 1% electrophoresis.

Samples ran on polymerase chain reaction (PCR). Used Primers for amplifying the gene sequence were as follows. The reverse primer sequence was; CCCAGCCCCAACCTTGTCA and the Forward primer sequence was; GCCTCAGATACCGAGTGCTT. Primers obtained from Generay Biotech factory-Iran.

Initial denaturation was done at 94°C for 5 minutes, followed by 35 cycles at 94°C for 1 minute, 65°C for 45 seconds for annealing, 72°C for 1 minute to primer aid extension, and final extension at 72°C for 7 minutes.

The restriction Fragment Length Polymorphism (RFLP) technique was used to digest PCR products enzymatically. BSRI enzyme (a restriction endonuclease from Bacillus stearothermophilus that recognizes 5' ACTGG3') obtained from Thermo-Scientific factory-US. According to the site of BsrI enzyme cleavage and investigation of point mutation in nucleotide 870 and substitution of G (guanine) with A (adenine), genotypic expectation in individuals homozygous for G was one band fragment(399bp), homozygous individuals for allele A was two fragments (279 and 120 bp) and individuals Heterozygous AG was three pieces of band(399,279 and 120 bp).

The frequency of genotype in both the patient and control groups was analyzed by conducting a $\chi 2$ (Chi-Square) test. The Hardy-Weinberg equilibrium assumption was assessed by comparing the genotype frequencies with those expected based on the observed frequencies. The logistic regression approach was used to obtain an adjusted odds ratio (OR) and 95% confidence interval (CI) for genetic polymorphisms.

Results

This study included a total number of 82 patients with breast cancer and 66 healthy controls.

We used the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method to analyze CCND1 SNP (Single Nucleotide Polymorphism) in patients and the control group.

The main PCR product was 399bp and detected by 2% agarose gel electrophoresis, and a 50bp marker was used as a ladder (loading dye).

To distinguish between product bands of A and G alleles, RFLP- PCR products were electrophoresed using 2% agarose gel and visualized by ethidium bromide staining.

No significant association in genotypic (p=0.32) or allelic frequency was observed between patients and controls. PCR products of CCND1 detected by 1% agarose gel electrophoresis shown in Figure 1. PCR-RFLP result is shown in Figure 2. Both groups were in the same age range. The main characteristic features of patients are shown in Figure 3. Genotyping frequency among cases was AA, 40.2% (n=33), AG, 35.3% (n=29), and GG, 24.4% (n=20), and in the control group were AA, 30% (n=20), AG, 47% (n=31) and GG, 23% (n=15) (figure 4). Moreover, A and G allelic frequencies in the breast cancer group were 58% and 42%, and in controls were 54% and 46% respectively (figure 5) (figure 6).

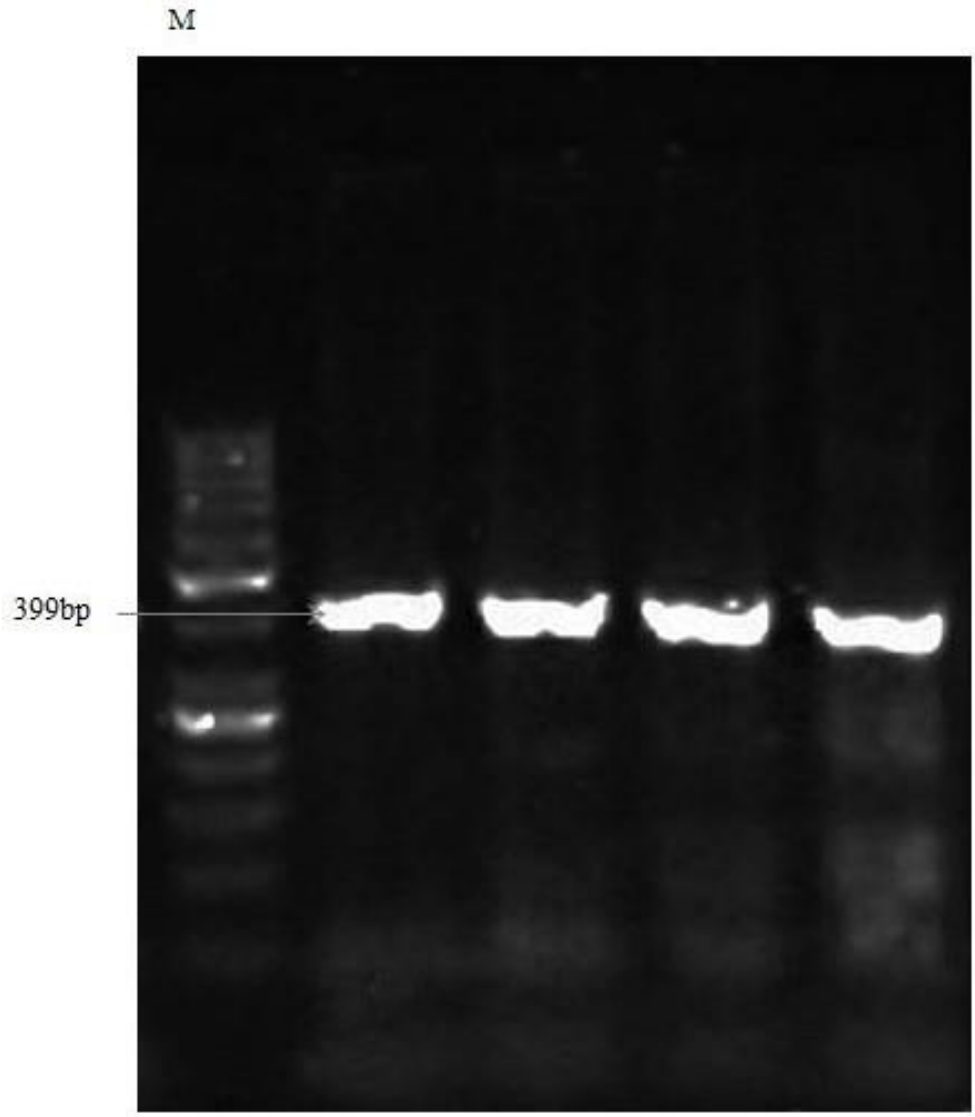

Fig 1. 1%Agarose gel electrophoresis after PCR amplification of CCND1. The size of the PCR product was 399 bp. M represents a 50bp marker.

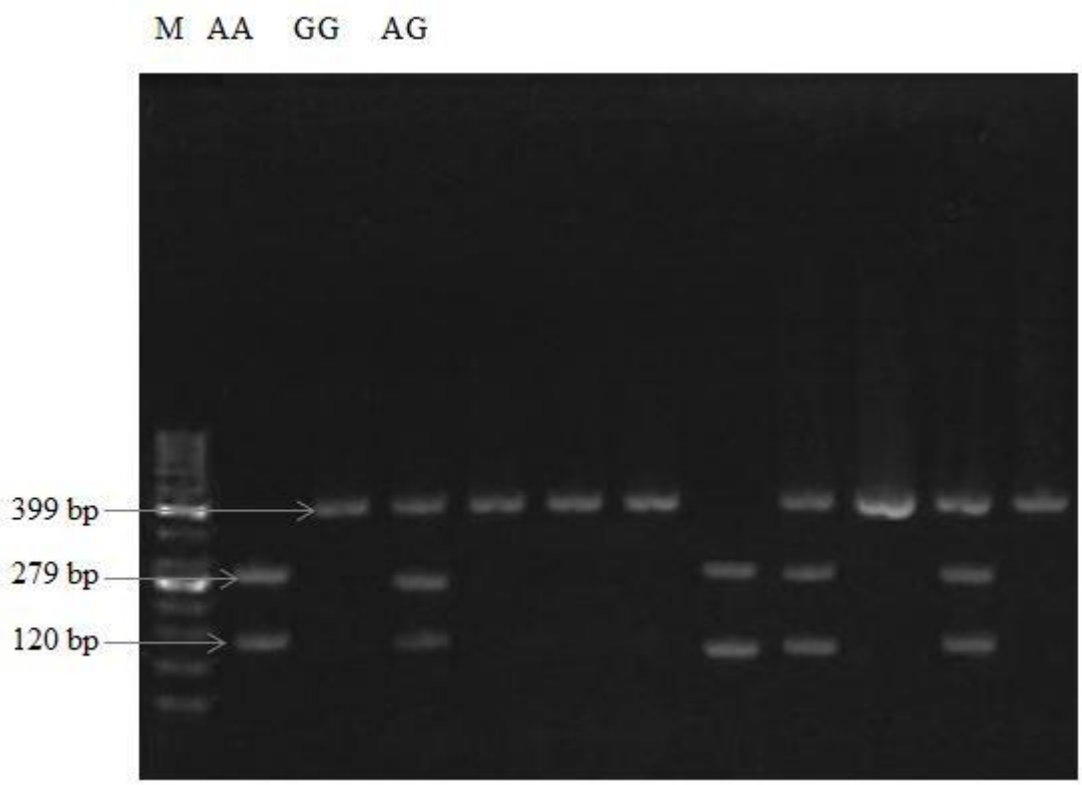

Fig 2. 2%Agarose gel electrophoresis of the CCND1 gene PCR-RFLP amplification products. M=Molecular marker. AG generated three bands of 399, 279, and 120 bp (lane 1), GG generated a single band of 399 bp (lane 2), and AA had two bands of 279 and 120 bp (lane 3).

Controls No.		Age Range (year old)
19		28-42
38		51-60
9		60-79
Patients NO.		Age Range (year old)
20		32-50
51		51-59
11		60-72
Patients Parameters	**No.**	**%**
Stage		
I	12	15
II	42	51
III	18	22
IIIc/IV	10	12
Metastasis		
Metastatic	3	4
Non metastatic	79	96
Pathology		
IDC (invasive ductal carcinoma)	57	69.5
ILC (invasive lobular carcinoma)	25	30.5
Menstrual status		
Premenopausal	29	35
Postmenopausal	53	65

Fig 3. Age distribution and Clinical and Biological parameters among breast cancer patients and healthy controls.

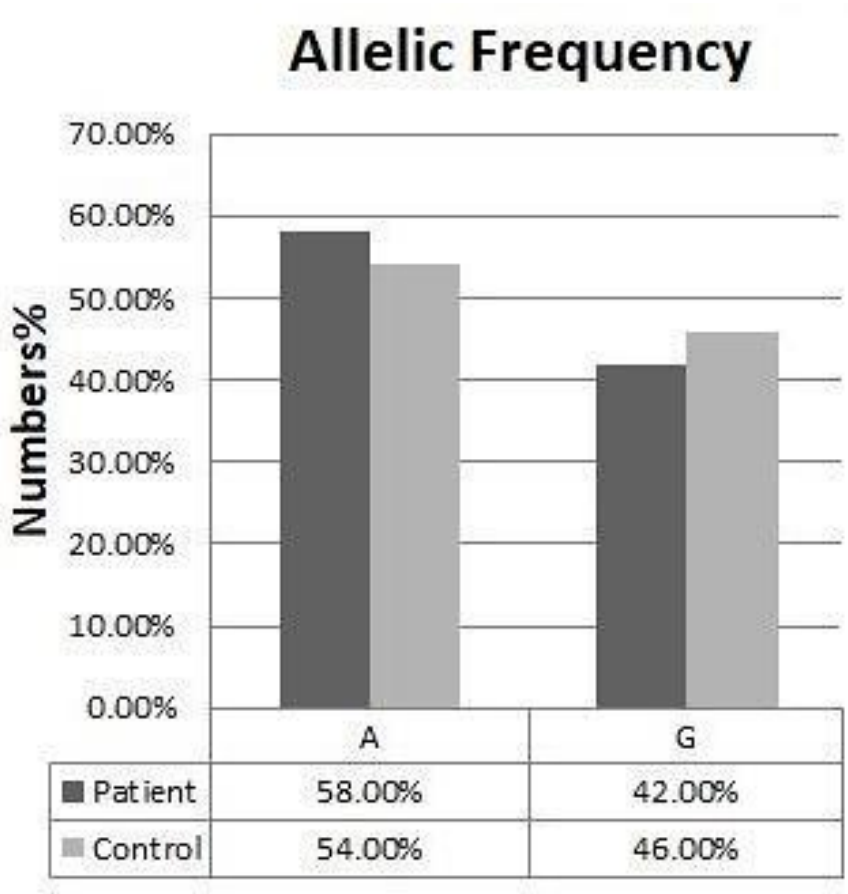

Fig 4. Genotypic frequency chart. AA genotype in patients and AG genotype in controls are more frequent.

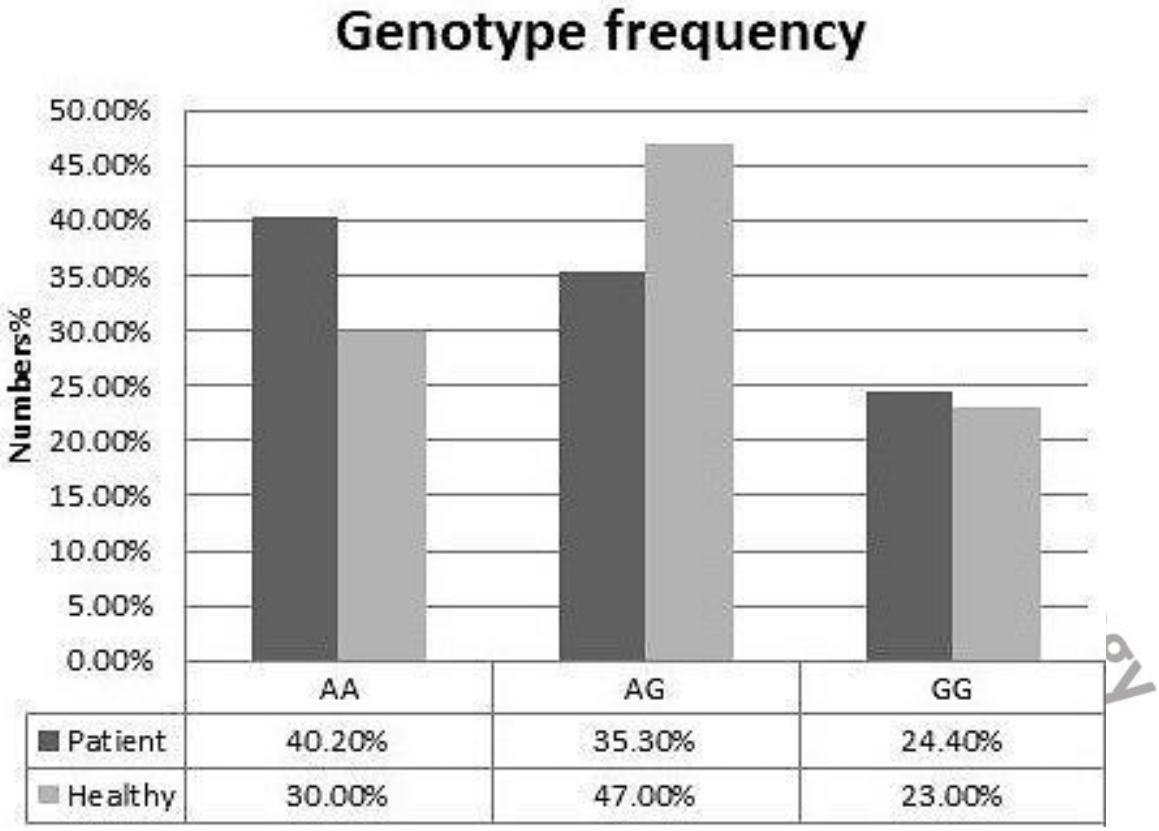

Fig 5. Phenotypic frequency chart. Allelic frequency in patients and controls is similar.

Genotypic frequency	Control %	NO.	Patient %	NO.	P-Value
AA	30	20	40.2	33	0.32
AG	47	31	35.3	29	
GG	23	15	24.4	20	
Allelic frequency					
A	54	71	58	95	0.22
G	46	61	42	69	0.19

Fig 6. Genotypic and Allelic frequencies of CCND1 G870A polymorphism among patients and controls and association with risk of breast cancer

Discussion

Cancer etiology consists of both genetics and environmental factors (Anand et al., 2008). Cyclin D1 is a proto-oncogene manufactured by the CCND1 gene and regulates G1 to S phase transition of the cell cycle (Knudsen et al., 2006). Cyclin D1 is activated after binding to CDK4, and increased activation of this complex is often observed in human cancers (Palmero and Peters, 1996). Cyclin D1 protein is unstable due to a C-terminal sequence (PEST Motif) (Dragnev et al., 2001). Cyclin D1 splicing in 870th nucleotide results in an alternative transcript of cyclin D1 protein called transcript b (D1b). The D1b transcript encodes a protein with an altered C-terminal domain that lacks PEST Motif, So this protein overexpresses during the cell cycle and results in malignancy (Gautschi et al., 2007). Cyclin D1 is associated with cell cycle regulation and has an important, oncopathogenic role in breast cancer (Mohammedi et al., 2019).

The association of TP53, FGFR2, ApE1, SEPP1, and AEP15 gene polymorphisms with the risk of breast cancer in the Iranian population has been studied (Kazemi et al., 2009; Salehi et al., 2015; Mashayekhi et al., 2015; Mohammaddoust et al., 2018). It has been suggested that there is a significant association between Cyclin D1 overexpression and CCND1 amplification. Overexpression of Cyclin D1 was observed in a high proportion of breast cancer patients and should be considered for routine diagnosis (Mohammedi et al., 2019).

In this study, we aimed to evaluate the association of cyclin D1 (CCND1) G870A polymorphism with the risk of breast cancer in the Guilan province of Iran. There was no significant association between genotype distribution in breast cancer cases and controls in our studied population. Moreover, no significant association was observed in allele frequencies

between the two groups. CCND1 G870A polymorphism is not associated with breast cancer risk in Guilan province.

A meta-analysis study by Zhang and colleagues showed an association of CCND1 G870A polymorphism with gastric cancer (Zhang et al., 2016). In another study, Xie and collaborators found that the CCND1 G870A polymorphism is associated with an increased risk of colorectal cancer (Xie et al., 2017). Bedewy suggested that homozygote A allele careers of the CCND1 gene are more susceptible to ALL cancer development, and CCND1 G870A polymorphism is a risk factor for breast cancer (Bedewy et al., 2013). However, some researches reveal no significant association between CCND1 G870A Polymorphism (Cyclin D1 Amplification) and the risk of breast cancer (Naidu et al., 2008; Takano et al., 1999; Krippl et al., 2003). A meta-analysis study on the Caucasian population showed a significant relationship between CCND1 gene variation and breast cancer risk (Soleimani et al., 2017). However, our results showed no significant association between Cyclin D1 (CCND1) G870A Polymorphism and risk of breast cancer. The controversial results may be due to sample size and other environmental and genetic factors among different populations.

Limitations of this study must be considered upon the interpretation of the results. First, the scale of the studied population was small. Second, this study evaluated the effect of one polymorphic site of the CCND1 gene on breast cancer susceptibility that does not represent the entire gene. Finally, as many factors influence the risk of breast cancer, more factors must be involved in future studies. This study is about breast cancer susceptibility among a limited population without considering some factors, such as familial history. Our goal plan is to conduct studies with different ethnicities and on bigger scales to identify genetic risks in the breast cancer population. This study represents an advance in biomedical science because it shows no association between CCND1 genetic variations and the risk of breast cancer.

Conclusion

Our study determined that there is not a significant association between breast cancer risks and CCND1 G870A Polymorphism among the Guilan province population of Iran. Further investigations are necessary to confirm the results of this study.

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