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ORIGINAL ARTICLE

An A/C germline single-nucleotide polymorphism in the *TNFAIP3* gene is associated with advanced disease stage and survival in only surgically treated esophageal cancer

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Prognostication of disease relapse and survival is essential for cancer patients and genetic variations in cancer patients may serve as important indicators. A single-nucleotide polymorphism (SNP) mapping to the tumor necrosis factor, alpha-induced protein 3 (*TNFAIP3*) gene at position 138241110 displays three genotypes (AA, AC and CC). The aim of this study was to evaluate the potential prognostic value of the TNFAIP3-SNP in esophageal cancer (EC). Genomic DNA was extracted from peripheral blood leukocytes of 173 patients who underwent complete surgical resection for EC and did not receive any neoadjuvant or adjuvant therapy. For SNP detection, a 260- bp fragment was PCR amplified, purified and sequenced with tested primers. The product was analyzed by automatic DNA sequencer.The TNFAIP3 genotypes were correlated with clinico-pathological parameters, tumor cell dissemination in bone marrow and clinical outcome. The C-allele carrier presented with higher disease stage ($P<0.001$). This was predominantly because of the presence of lymph node metastasis ($P<0.001$). The recurrence rate was higher in C-allele carriers (AC and CC genotype; $P=0.004$). Kaplan–Meier plots for disease-free ($P=0.017$) and overall survival ($P<0.001$) displayed a gene dosage-associated outcome with AA genotype patients presenting the longest and CC genotype patients the poorest survival. In disease stage-adjusted multivariate analysis the TNFAIP3-SNP was identified as an independent prognostic factor for survival (hazard ratio 1.9; $P=0.008$). The TNFAIP3-SNP allows risk stratification of EC patients and may be a useful tool to identify patients eligible for multimodal therapy concepts.
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INTRODUCTION

Owing to early tumor cell dissemination and metastasis a poor survival is linked to esophageal cancer (EC). The leading cause of death in EC is disease relapse, either at a local site or becasue of a distant metastasis.[1] Clinical prognostic indicators of overall survival (OS) are the depth of tumor infiltration, lymph node infiltration and distant metastasis. A disease relapse develops in 20% of histologically verified lymphnode-negative patients and in up to 90% of lymphnode-positive patients.[2–9] An adequate risk-stratification before initiation of a particular therapy since multimodal therapy concepts have been introduced and increasingly applied in the treatment of EC and in order to follow up tumor patients is enormously important.[10,11] Identification of patients who would truly benefit from a multimodal therapy approach and exclusion of those running the risk of over-treatment remains a challenge.

Minimal disease residuals which are not detectable by conventional diagnostic tools may remain present but unnoticed after complete surgical resection, this makes the prediction of the clinical course of EC patients difficult.[12–18] Ideal prognostic markers should be easy to determine, be independent of tumor tissue availability and harbor genomic stability that remains unbiased by the type of specific tumor therapy. Unfortunately clinically useful markers are missing in EC yet. Important prognostic indicators of clinical outcome in cancer patients might be genetic germline variations.[19–21]

Prior studies identified genetic variations of the *TNFAIP3* gene (tumor necrosis factor, alpha-induced protein 3) as risk alleles for multiple autoimmune diseases.[22–28] The cytoplasmic protein A20 is encoded by the *TNFAIP3* gene. A20 is a key regulator of cell survival, inflammation and immunity.[29,30] The oncogenic role of A20 is

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tissue-specific, its pro- and antitumor properties have been characterized in lymphoma and solid tumor patients.[29,31–34]

The *TNFAIP3* gene is located on chromosome 6 and displays polymorphic activity.[29] Although many studies have been conducted in inflammatory diseases, the role of TNFAIP3 genetic variations in oncogenic setting has not been evaluated yet.

In this study, we assessed the prognostic value of the TNFAIP3 single-nucleotide polymorphism (SNP) rs610604 at position 138241110, the rs610604 is located in intron 6 of the *TNFAIP3* gene and encodes A20, a TNF-α-inducible zinc finger protein that temporally limits immune responses by inhibiting NF-κB activation and terminating NF-κB mediated responses, recent studies could show that this SNP has a significant influence in inflammatory diseases.[35] The study population was homogenous and contained EC patients treated by surgery only. We correlated our results to clinic-pathological parameters, the presence of disseminated tumor cells (DTC) in bone marrow as an indicator of early haematogenous spread and to clinical outcome.[18,36]

MATERIALS AND METHODS

The study was approved by the Medical Ethical Committee of Hamburg, Germany. All patients enrolled in this study underwent esophageal resection at the department of general, visceral and thoracic surgery at the University Medical Center Hamburg-Eppendorf. Only patients with histopathological proven EC after complete resection (R0) were included into the study. In total, 173 patients were eligible. None of the patients received neoadjuvant or adjuvant treatment. Informed consent was obtained from all patients before including them in a prospective database. The sixth edition of the American Joint Committee on Cancer (AJCC) was used for classifying the disease stage.

DNA genotyping

Blood samples were obtained on the day before the operation and whole blood leukocyte DNA was extracted and purified by standard procedures using QIAmp tissue kit (QIAGEN GmbH, Hilden, Germany). For detection of the SNP in intron 6 at position 138241110 (A>C), a 260- bp fragment was PCR-amplified with primers 5′-AGTTAGCTTCATCCAACCTGA and 5′-G AAGTCTTAGCAAACTAACT. PCR was performed in a 50-μl reaction mix consisting of 0.5 μl extracted DNA, 5 μl 10× PCR Buffer IV (ABgene, Epsom, UK), 4 μl deoxyribonucleotide triphosphate (1.25 mM), 0.4 μM each primer and 1.25 U Taq-polymerase. PCR conditions consisted of 35 repeated cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. Amplified fragments were purified by QIAquick PCR Purification Kit (QIAGEN GmbH) and sequenced with nested primers: 5′-CTTCATCCAACCTGAAGACCA. Sequencing was processed in 20 -μl mix containing 3.0 -μl DNA fragment, 2.0- μl buffer (ABI 5× sequencing buffer; Applied Biosystems, Foster City, CA, USA), 4.0 -μl ABI BigDye di-deoxyterminator sequencing mix version 1.1 (Applied Biosystems) and primer. Synthesis program was denaturation at 95 °C for 2 min followed by 30 cycles at 95 °C for 45 s, 50 °C for 10 s and 60 °C for 4 min. The product was then analyzed by automatic DNA sequencer ABI3110 (Applied Biosystems).

Disseminated tumor cell detection

DTC detection in the bone marrow has been previously described in detail according to international standard.[37,38] Briefly, on the day of surgery, 4–8 ml bone marrow aspirates from the iliac crest of the patients were obtained. The aspirates were collected in heparin, and mononuclear cells were isolated by density-gradient centrifugation through Ficoll-Hypaque (Pharmacia, Freiburg, Germany) at 400×*g* for 30 min. To apply cells to a glass slide, they were then subjected to cyto-centrifugation at 150×*g* for 3 min at room temperature. We used monoclonal antibody A45-B/B3 (IgG1; Micromet, Munich, Germany) to detect tumor cells in bone marrow. It detects an epitope on various cytokeratins, including cytokeratins 8, 18 and 19. As a control antibody, unspecific IgG1 was used. Visualization was performed by the APAAP technique. Counterstaning was done with Mayer's haematoxylin. To screen immuno-stained bone marrow slides for DTC, we used the Automated Cellular Imaging System (ChromaVision Medical Systems Inc., San Juan Capistrano, CA, USA).

Statistical analysis

SPSS for Windows (Chicago, IL, USA) was used for statistical analysis. To describe patient baseline characteristics, descriptive statistics were used. The X^2-test was used to evaluate a potential association between the TNFAIP3 genotype and clinico-pathological parameters. Survival analysis of the patients were plotted by the Kaplan–Meier method and analyzed using the log rank test. Results are presented as median survival with 95% confidence interval (95% CI) and number of patients at risk. In case if the median survival was not reached, mean values are presented and specifically indicated. The OS was computed as the time period from the date of surgery to either the date of death or last follow-up, whichever occurred first. The disease-free survival (DFS) was defined as the time period from the date of surgery to the date of recurrence, last follow-up or date of death, whichever occurred first. Patients alive without recurrence at the follow-up date were censored. Cox regression hazard model was used for multivariate analysis to assess the independent influence of TNFAIP3-SNP and other covariates on survival and tumor recurrence. Results are presented as hazard ratio (HR) and 95% CI. Significant statements refer to *P*-values of two-tailed tests that were <0.05.

RESULTS

Characterization of the study population

A total of 173 patients were included in this study. The median age of the study population was 62.8 years (range = 34.5–84.7). Majority of the patients were males (82.1%). All patients underwent complete esophageal resection with histologically proven tumor free margins and without evidence of distant organ metastases. Owing to the presence of nonregional lymph-node metastases, 12 (6.9%) patients were classified as M1a positive (stage IVa). Adenocarcinoma (AC) was histologically proven in 78 (45.1%) and squamous cell carcinoma (SCC) in 95 (54.9%). None of the patient received neoadjuvant or adjuvant therapy. Almost 90% ($N=156$) of the patients were also evaluated for DTC which were detected in 37.2% ($N=58$) of these patients. The AA genotype was found in 22 (12.7%), the AC in 119 (68.8%) of the patients, and the remaining 32 (18.5%) patients displayed the CC genotype. Table 1 depicts the tumor-specific characteristics of the entire study population.

TNFAIP3-SNP and clinic-pathological parameters

The three genotypes were correlated to clinic-pathological parameters (Table 1). A significant correlation was found between AJCC disease stage and the TNFAIP3-SNP ($P<0.001$) with almost 80% of the homozygous C-allele carriers presenting advanced disease stage, whereas the homozygous A-allele carriers displayed only disease stage $\leqslant$II. A subanalysis at the TNM level revealed no association of the TNFAIP3-SNP with tumor size but with lymphatic tumor spread ($P<0.001$). Majority of the C-allele carriers displayed lymph node-positive disease, whereas none of the homozygous A-allele carrier presented lymph nodes metastasis ($P<0.0001$). In line with this finding, all patients with stage IVa disease (M1a, nonregional lymph node metastasis) belonged to the CC genotype ($P<0.001$).

Disseminated tumor cells in the bone marrow as marker of early haematogenous spread and more aggressive tumor biology were found in almost one third of all genotype patients and no significant correlation between TNFAIP3-SNP and DTC in bone marrow could be drawn ($P=0.759$).

The tumor recurrence rate was, however, higher in C-allele carriers (>50%), whereas <25% of the homozygous A-allele carrier experienced a disease relapse ($P=0.004$).

Table 1 Patient characteristics and correlation of TNFAIP3-SNP with clinico-pathological parameters

		TNFAIP3 -genotype			
Variables	*All*	*AA*	*AC*	*CC*	P-*values*
Total	173 (100)	22 (100)	119 (100)	32 (100)	—
Age (years)					
⩽60	76 (43.9)	12 (54.5)	45 (37.8)	19 (59.4)	0.052
>60	97 (56.1)	10 (45.5)	74 (62.2)	13 (40.6)	
Sex					
Male	142 (82.1)	18 (81.8)	94 (79.0)	30 (93.8)	0.154
Female	31 (17.9)	4 (18.2)	25 (21.0)	2 (6.2)	
AJCC stage[a]					
Stage I	6 (3.5)	2 (9.1)	1 (0.8)	3 (9.4)	<0.0001
Stage IIa	36 (20.8)	20 (90.9)	12 (10.1)	4 (12.5)	
Stage IIb	35 (20.2)	0 (0.0)	35 (29.4)	0 (0.0)	
Stage III	76 (43.9)	0 (0.0)	71 (59.7)	5 (15.6)	
Stage IVa	20 (11.6)	0 (0.0)	0 ((0,0)	20 (62.5)	
Tumor size					
pT1	8 (4.6)	2 (9.1)	3 (2.5)	3 (9.4)	0.201
pT2	49 (28.3)	7 (31.8)	37 (31.1)	5 (15.6)	
pT3	102 (59.0)	13 (59.1)	69 (58.0)	20 (62.5)	
pT4	14 (8.1)	0 (0.0)	10 (8.4)	4 (12.5)	
Nodal status					
Negative	47 (27.2)	22 (100)	14 (11.8)	11 (34.4)	<0.0001
Positive	126 (72.8)	0 (0.0)	105 (88.2)	21 (65.6)	
Metastasis					
Negative	161 (93.1)	22 (100.0)	118 (99.2)	21 (65.6)	<0.0001
Positive	12 (6.9)	0 (0.0)	1 (0.8)	11 (34.4)	
Grading					
G1	4 (2.3)	2 (9.0)	2 (1.7)	0 (0.0)	0.174
G2	75 (43.4)	10 (45.5)	53 (44.5)	12 (37.5)	
G3	94 (54.3)	10 (45.5)	64 (53.8)	20 (62.5)	
Histological subtype					
SCC	95 (54.9)	13 (59.1)	66 (55.5)	16 (50.0)	0.786
AC	78 (45.1)	9 (40.9)	53 (44.5)	16 (50.0)	
Disseminated tumor cells					
No	98 (62.8)	14 (73.7)	65 (61.3)	19 (61.3)	0.579
Yes	58 (37.2)	5 (26.3)	41 (38.7)	12 (38.7)	
Recurrence					
Negative	76 (44.7)	17 (38.8)	45 (43.8)	14 (43.8)	0.004
Positive	94 (55.3)	5 (61.2)	71 (61.2)	18 (56.2)	

Abbreviations: AC, adenocarcinoma; AJCC, American Joint Committee on Cancer; SCC, squamous cell carcinoma.
Round parentheses indicate percentages.
P indicates significance according to X^2-test.
[a]According to the 6th AJCC staging.

TNFAIP3-SNP and clinical outcome

Three (1.7%) patients died perioperatively and were excluded from the survival analyses. The median follow-up time was 17 months (range 3–120). During the observation period 94 (55.3%) patients experienced a relapse and in addition to the three perioperative deaths another 107 (62.9%) patients died.

The TNFAIP3 genotype had a significant impact on DFS and OS. Kaplan–Meier analyses demonstrated a distinctly decreasing DFS ($P=0.017$) and OS ($P=0.001$) between AA, AC and CC genotype patients (Figures 1 and 2; Table 2).

Prognostic value of TNFAIP3-SNP

Multivariate analyses according to the Cox regression hazard model using age, sex, tumor differentiation, disease stage and TNFAIP3 genotype were performed to evaluate the prognostic value of TNFAIP3-SNP in only surgically treated EC patients. Besides disease stage the TNFAIP3-SNP was found to be a strong disease-stage independent prognosticator with marked increased risk for poor OS (HR 1.9, $P=0.008$) in C-allele carriers compared with AA patients (Table 3).

Despite the close correlation between TNFAIP3-SNP and positive nodal status as well as disease recurrence the TNFAIP3-SNP could not be verified as an independent marker for recurrence in this study cohort.

DISCUSSION

We were able to show that the TNFAIP3-SNP is an independent prognosticator of clinical outcome in EC patients. A key finding of our study was that the TNFAIP3-SNP significantly correlated with the lymphatic tumor spread and disease relapse. The DFS and OS declined significantly between AA, AC and CC genotype patients indicating toward a gene dosage effect. In line with this finding was the disease stage distribution pattern among the three genotype patients. Our data indicate that the prognostic value of TNFAIP3-SNP applied to AC and SCC, the two tumor main histological subtypes, in EC. Our study cohort is unbiased by potential interactions of a systemic chemotherapy or radiotherapy and reflects the natural course of the disease after complete resection. Hence, it is ideal for prognostic studies.

Genome-wide association studies have identified germline polymorphisms associated with individual susceptibility to different cancer types.[19–21,39–41] In addition, genomic variations have also been analyzed in pharmaco-dynamic and pharmacokinetic studies to identify patients likely to develop toxicity or resistance toward specific drugs.[42–46] However, the prognostic value of germline polymorphisms in cancer is poorly understood yet.[47,48] Polymorphisms in germline DNA represent inherited stable genetic markers that are unbiased by genomic instability occurring in cancer tissue and are easily accessible.

A recent study by Lurje *et al.*[49] reported multiple angiogenesis pathway polymorphisms in EC and verified the utility of SNPs for risk stratification in EC patients treated by surgery alone.

Genome-wide association studies have identified several SNPs of the *TNFAIP3* gene as risk alleles for multiple inflammatory diseases including systemic lupus erythematosus, morbus crohn, psoriasis and rheumatoid arthritis.[22,23,25,50–54] The potential for oncogenic transformation is at least inherited by released inflammatory mediators, growth factors and cytokines triggered by a chronic inflammatory response.[55–58] In contrast to many lymphomas, which have inactivating A20 mutations, the A20 expression is up regulated in several solid tumors.[26,31,33,34] A20 is a key mediator in cell survival and represents an ideal candidate gene for prognostic studies. However, the tissue-specific role of this protein pinpoints the problems associated with most prognostic markers. Firstly lymphoma and solid tumors contain a different expression pattern. Secondly, an access to tumor tissue is necessary for the evaluation of the protein itself as a prognostic marker. Thirdly, other mutations of the gene of temporary nature due

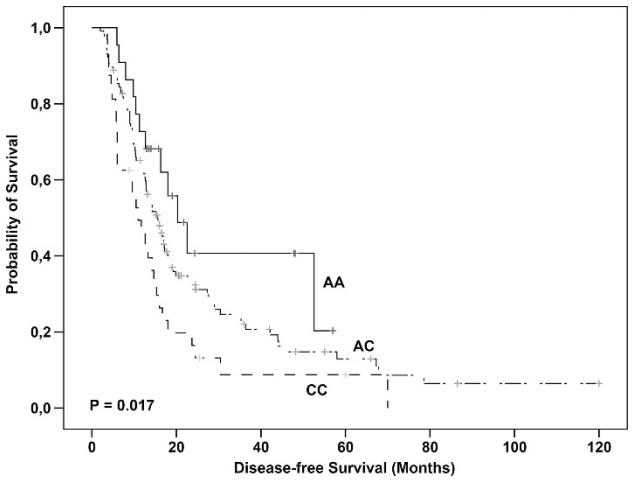

Figure 1 Kaplan-Meier plots of disease-free survival in relation to TNFAIP3 genotype showing a significant difference of survival according to the genotype ($P=0.017$). A full color version of this figure is available at the *Journal of Human Genetics* journal online.

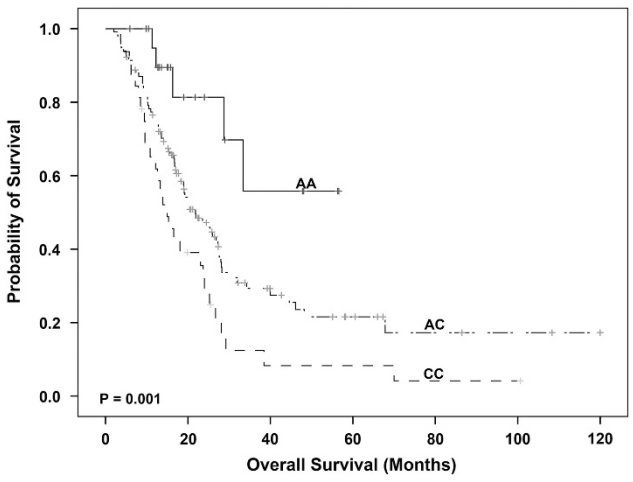

Figure 2 Kaplan-Meier plots of overall survival in relation to TNFAIP3 genotype showing a significant difference of survival according to the genotype ($P=0.001$). A full color version of this figure is available at the *Journal of Human Genetics* journal online.

Table 2 Survival according to TNFAIP3 genotype

		Disease-free survival			*Overall survival*		
Genotype	N	*Median survival (months)*	*95% confidence interval*	P-*values*	*Median survival (months)*	*95% confidence interval*	P-*values*
All	170	15.16	12.64–17.68	0.017	23.07	18.10–28.04	0.001
AA	22	20.3	13.32–27.28		42.14*	32.03–52.26	
AC	116	15.56	12.74–18.38		21.84	16.07–27.61	
CC	32	10.96	6.88–15.04		14.96	10.56–19.36	

P according to the log rank test

Table 3 Multivariate analysis for recurrence and overall survival

	Disease-free survival			*Overall survival*		
Variables	*Hazard ratio*	*95% confidence interval*	P-*values*	*Hazard ratio*	*95% confidence interval*	P-*values*
Age (years)						
⩽60 vs >60	0.78	0.52–1.19	0.25	0.83	0.56–1.22	0.34
Sex						
Male vs female	0.84	0.49–1.47	0.55	0.71	0.41–1.26	0.25
AJJC stage						
I & IIa vs IIb. III. IVa	3.46	1.73–6.92	<0.0001	2.52	1.42–4.50	0.002
Grading						
G1&2 versus G3	1.21	0.81–1.80	0.37	1.15	0.78–1.70	0.48
TNFAIP3						
CC & AC vs AA	1.30	0.80–2.09	0.29	1.68	1.12–2.51	0.012

Abbreviations: AJJC, American Joint Committee on Cancer; TNFAIP3, tumor necrosis factor, alpha-induced protein 3.
P indicates significance according to Cox regression analysis

to instability of tumor DNA may be caused by chemotherapy or radiotherapy. Finally, protein expression analysis based on immunohistochemistry has limited reproducible capacity.

The incidence of EC is steadily increasing,[59] over the last decade a change in the therapy of patients with EC has emerged. For many years, the standard therapy for locally advanced EC has been surgical resection only. However, the overall outcome for EC patients after resection remains poor. A never-ending debate regarding the current standard of care for the management of EC is still ongoing.[60–62] A major problem is considered in identifying patients who would truly

benefit from a multimodal therapy concept, e.g. neoadjuvant chemo-radiotherapy followed by surgery. Besides accurate preoperative staging the assessment of response to neoadjuvant therapy is crucial but currently unsatisfying. Clinical useful markers are missing in EC. The endoscopic ultrasound is considered to be the most accurate imaging modality but has several limitations since it is observer and experience-level dependent.[17,63] Furthermore, the morphology of the tumor results in different staging. In our experience the endoscopic ultrasound has an accuracy of 60% only.[12]

Functional or biological consequences of the most germline genetic variants are still unknown.[64] However, altered binding affinity for transcription factors OCT1, RUNX2 and C/EBPb as a result of identified breast cancer susceptibility SNPs in intron 2 of FGFR2 gene are related to an increased FGFR2 expression.[65] Such associations have already been shown for other genes.[66–68] Thus, such association between TNFAIP3-SNP and protein expression can be assumed but still has to be shown.

To our understanding identification of objective, reproducible and observer independent tools are essential to allocate patients into different risk profiles and optimize use of multimodal therapy and avoidance of overtreatment.

The level of significance for the association of the SNP to tumor progression observed in our study is rather high. The number of patients for a genetic study seems small to evaluate the disease stage independent value as a prognostic marker. The study population represents a large homogeneously, only surgically treated cohort reflecting the natural course of the disease after complete resection. The 12 patients with nonregional lymph node metastasis did not receive any adjuvant therapy since complete resection was achieved in those patients and currently there is no evidence for adjuvant therapy in EC.[11,69,70]

A weak point of our study remains the retrospective nature despite collection of the blood samples preoperatively and entering the patient data into a prospective database. Furthermore, we did not evaluate the TNFAIP3 protein expression as a function of TNFAIP3-SNP. In addition, we cannot implicate any role of the TNFAIP3-SNP in development of EC since we did not perform a genome-wide association study but only evaluated the prognostic role of the TNFAIP3-SNP on clinical endpoints in EC, According to our findings the C-allele seems to accelerate aggressive biological tumor behavior in EC. but the biological and physiological role of this polymorphism in normal and tumor tissue remains at present unknown.

Furthermore we evaluated the prognostic role of TNFAIP3-SNP in only surgically treated EC patients. Esophageal cancer patients with neoadjuvant or curative radio-chemotherapy may display a different association between TNFAIP3-SNP and clinical outcome.

In conclusion, our data pinpoint toward TNFAIP3-SNP as an independent prognostic marker for OS in EC. The possibility of preoperative determination of the TNFAIP3 genotype allows allocation of patients to different risk profiles and may help to tailor customized therapies and follow-up. Further studies with focus on functional impact of the TNFAIP3-SNP and evaluation in patients undergoing multimodal therapy are needed to clarify the biological importance of these findings.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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