

Genetic Polymorphisms in NAT1, NAT2, GSTM1, GSTP1 and GSTT1 and Susceptibility to Colorectal Cancer among Filipinos

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ABSTRACT

Objectives. Polymorphisms in metabolic genes which alter rates of bioactivation and detoxification have been shown to modulate susceptibility to colorectal cancer. This study sought to evaluate the colorectal cancer risk from environmental factors and to do polymorphism studies on genes that code for Phase I and II xenobiotic metabolic enzymes among Filipino colorectal cancer patients and matched controls.

Methods. A total of 224 colorectal cancer cases and 276 controls from the Filipino population were genotyped for selected polymorphisms in GSTM1, GSTP1, GSTT1, NAT1 and NAT2. Medical and diet histories, occupational exposure and demographic data were also collected for all subject participants.

Results. Univariate logistic regression of non-genetic factors identified exposure to UV (sunlight) (OR 1.99, 95% CI: 1.16-3.39) and wood dust (OR 2.66, 95% CI: 1.21-5.83) and moldy food exposure (OR 1.61, 95% CI: 1.11-2.35) as risk factors; while the NAT2*6B allele (recessive model OR 1.51, 95% CI: 1.06-2.16; dominant model OR 1.87, 95% CI: 1.05-3.33) and homozygous genotype (OR 2.19, 95% CI: 1.19-4.03) were found to be significant among the genetic factors. After multivariate logistic regression of both environmental and genetic factors, only UV radiation exposure (OR 2.08, 95% CI: 1.21-3.58) and wood dust exposure (OR 2.08, 95% CI: 0.95-5.30) remained to be

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significantly associated with increasing colorectal cancer risk in the study population.

Conclusion. This study demonstrated that UV sunlight and wood dust exposure play a greater role in influencing colorectal cancer susceptibility than genotype status from genetic polymorphisms of the GST and the NAT genes.

Key Words: colorectal cancer, genetic polymorphisms, xenobiotic metabolizing enzymes, Filipino, susceptibility

Introduction

Colorectal cancer is one of the leading cancers of the world. Based on incidence, it ranks fourth in men and third in women, with over one million new cases worldwide.¹ The majority (> 90%) of cancers of the colon and rectum are adenocarcinomas.² In the Philippines, the Philippine Society of Gastroenterology (PSG) stated that colorectal cancer is the number one cancer in the country as of March 2017. The data of PSG showed that among Filipinos, there are about 3000 new cases of colorectal annually and 67% of the reported cases die.³

The development of colon cancer can usually occur in one of three patterns: hereditary, familial and sporadic—majority of colorectal cancer cases (70-75%), however, are sporadic.¹ Disease etiology has been linked to both genetic and non-genetic factors. Factors pertaining to diet (intake of red/processed meats) and lifestyle (alcohol intake, obesity and physical inactivity) have been shown in several studies to be associated with risk for this disease.⁴⁻⁹

Population-based studies on different polymorphic genes have also shown that genetic variations influence susceptibility to certain types of cancer. Genes of interest include those that code for phase I and phase II xenobiotic metabolizing enzymes such as N-acetyltransferases (NATs) and Glutathione-S-Transferases (GSTs). NATs and GSTs are responsible for metabolizing diet- and environmentally-derived colorectal carcinogens such as heterocyclic and aromatic amines, and polyaromatic hydrocarbons.^{6,7,10} Heritable variations in such genes occurring as genetic polymorphisms can result in functional and allelic variants that lead to different rates of metabolic activation or detoxification of particular carcinogens thereby resulting in varied cancer susceptibility among individuals.^{9,10}

In humans, N-acetyltransferases (NATs) can be divided into two functional classes: NAT1 and NAT2. NAT1 and NAT2 perform N-acetylation, O-acetylation, and N, O-acetyltransfer which activate or deactivate aromatic and heterocyclic amines.^{6,7} NAT1 is ubiquitous and found in almost all tissues while NAT2 is primarily present in the liver.¹¹ Polymorphic variants of NAT1 and NAT2 give different rates of acetylation; slow acetylators (g.1095C>A for NAT1; g.341T>C, g.481C>T, g.803A>G, g.590G>A, and g.857G>A for NAT2) and fast acetylators (g.1088T>A and g.1095C>A, and 9del1065-1090 for NAT1).^{11,12} The rapid

acetylator phenotype has been associated with increased susceptibility to colorectal cancer.^{4,7,9} Similarly, significant associations have also been found between NAT1 and NAT2 rapid acetylator genotypes and colorectal cancer risk in previous population-based studies.¹³⁻¹⁵ Both of these genes were also found to be downregulated in colorectal cancer patients. The low expression of the genes initiates and aids in the development of colorectal cancer.¹⁶

Another gene superfamily investigated in the study includes the Glutathione-S-transferase genes (*GSTM1*, *GSTP1*, *GSTT1*), which code for both cytosolic and membrane-bound proteins that are primarily involved in cellular detoxification by neutralizing the electrophilic sites of endogenous and exogenous compounds through conjugation with glutathione. This mechanism prevents the attack of potential reactive electrophiles on macromolecules.^{17,18} For the *GSTT1* and *GSTM1* genes, a polymorphic gene deletion which results in a lack of conjugation activity has been hypothesized to influence cancer initiation as a result of an impaired ability to detoxify electrophilic carcinogens. An increase in colorectal cancer risk has been reported previously among individuals having a homozygous deletion of *GSTM1* and *GSTT1*.¹⁹⁻²⁵ For *GSTP1*, the c.313A>G polymorphism has also been noted to be a significant modifier for colorectal cancer susceptibility.²⁶ Previous studies have demonstrated that this particular polymorphism—which causes an Ile105Val substitution in the encoded product— exhibit altered catalytic activity and structural stability which affect over-all metabolic detoxification, contributing to individual cancer susceptibility.^{27,28}

This study focused on genetic polymorphisms of the GST and the NAT genes as possible modifiers of colorectal cancer risk in the Filipino population. More specifically, this study sought to accomplish the following: a) to evaluate the colorectal cancer risk from environmental factors in addition to past medical, family, reproductive, and socio-demographic history; b) to do polymorphism studies on Phase I and II metabolic enzymes - *GSTP1*, *GSTT1*, *GSTM1*, *NAT1* and *NAT2* in colorectal cancer patients and their controls; and c) to evaluate the colorectal cancer risk from specific polymorphisms, comparing cancer cases and their controls.

Methods

Subject Population

This case-control study is comprised of 224 confirmed colorectal cancer cases, and 276 healthy controls. The patients were recruited from five tertiary hospitals (Philippine General Hospital, *Ospital ng Maynila* Medical Center, East Avenue Medical Center, Jose Reyes Memorial Medical Center and Medical Center Manila. The inclusion criteria were the presence of histopathologically confirmed colorectal cancer (any age, any stage, any Eastern

Cooperative Oncology Group) and no history of chemotherapy or radiotherapy prior to his/her enrolment to the study. The inclusion criterion for the controls was the absence of any prior history of cancer. The controls (age-5 years-interval-matched; clinically free of cancer; sex-matched) were randomly selected from the same hospitals as the cases during the same time period. Classification was verified by obtaining the histopathology result of the case patient's tissue and blood was taken for genetic analysis. Ethical committee approval was obtained prior to study initiation and informed consents from the subject participants were acquired.

Data Collection

All subject participants were interviewed by trained health workers using a questionnaire and standardized interview and measurement techniques. Information including age, gender, occupation, tobacco and betel nut chewing habits, family history of colorectal cancer, oral contraceptive use, diet (consumption of alcohol, canned meat, fish sauce (*patis*), shrimp paste (*bagoong*), vegetables, scalding hot food, preserved foods, smoked foods, salted foods), and occupational exposure (exposure to moldy food, pesticides, vinyl chloride, benzene, UV sunlight, coal carbonization, and wood dust). The questionnaire and the interview technique were pre-tested among a group of Filipino patients and were modified accordingly. All data collected from the subject participants were treated as confidential.

DNA Isolation

Genomic DNA was extracted from whole blood samples of enrolled patients using the QIAamp® Blood Midi Kit Spin Protocol (Qiagen GmbH, Hilden, Germany). The DNA extracts were subsequently stored at -20°C .

Genotyping

Polymorphisms for *GSTM1*, *GSTT1*, *GSTP1*, *NAT1* and *NAT2* genes were detected using polymerase chain reaction (PCR) and PCR-restriction fragment length polymorphism (RFLP). Genotyping methods by PCR-RFLP were verified by direct sequencing.

***GSTM1* and *GSTT1*.** The presence of at least one allele of *GSTM1* and *GSTT1* was determined using standard PCR protocol.^{28,29} As an internal control, exon 7 of the *CYP1A1* gene was co-amplified for each reaction using previously published primers.²⁹

***GSTP1*.** The c.313A>G polymorphism in exon 5 was ascertained using PCR-RFLP with *Alw26I*.³⁰

***NAT1*.** Detection of *NAT1* alleles was performed using PCR-RFLP with *MboII* for *NAT1*4*, and *NAT1*11*. Allele-

specific PCR was performed using *NAT1*3*-specific and *NAT1*10*-specific primers to distinguish between the two alleles.³¹ As an internal control for allele-specific PCR, β -globin was co-amplified using previously published primers.

***NAT2*.** The PCR-RFLP strategy by Hubbard et al. was used to genotype *NAT2*.³²

Statistical Analysis

For the environmental variables, statistical analysis was done using Stata Program version 9.0, while SPSS 14.0 software was used for statistical analysis of genetic variables. Age-and sex-matched pairs were analyzed including multiple controls per case within an age-group. Comparison of characteristics between groups was carried out through χ^2 tests for genetic variables, McNemar's test for environmental variables, and independent t-tests for continuous (environmental) variables. Univariate conditional logistic regression analyses for environmental and genetic variables were initially carried out separately using simple conditional logistic regression to assess the significance of each independent variable or risk factor for cancer by cancer site. All environmental factors that were significant at 0.2 level in the univariate analyses are identified as the initial set of candidate predictors for colorectal cancer. They are then included in the multivariate conditional logistic regression model to generate adjusted ORs and corresponding p-values. All environmental factors that were significant in the multivariate analysis were included in multivariate logistic regression with statistically significant genetic variables (with ORs that are significant at $\alpha=0.05$) to test for gene-environment interactions.

For diallelic genes *GSTP1*, *CYP1A1* and *CYP2E1* genotypes were tested for Hardy-Weinberg Equilibrium (HWE). Only four of 26 known *NAT1* alleles and six of 62 known *NAT2* alleles were tested, thus genotype frequencies for both genes are expected to deviate from HWE.

Results

A total of 500 patients with 224 cases and 276 controls were available for analysis (Table 1). Among the cases, there were 131 males and 93 females. The control population had 131 males and 145 females. Median age range for the whole group was 50-54 years old; 50-54 for the controls and 55-59 for the cases. Univariate logistic regression analyses of non-genetic factors identified exposure to moldy food (≥ 1 /month) (OR 1.61; 95% CI 1.11-2.35), UV sunlight exposure (≥ 7 /month) (OR 1.99; 95% CI 1.16-3.39), and wood dust occupational exposure (OR 2.66; 95% CI 1.21-5.83) as significant risk factors (Table 2). These three variables remained significant environmental risk factors after multivariate analysis (Table 3). Among the genetic factors,

Table 1. Age and sex distribution of colorectal cancer cases and controls

Age	Control		Case		Total
	Male	Female	Male	Female	
<20	0	2	0	1	3
20-24	2	2	1	2	7
25-29	6	4	3	4	17
30-34	10	6	9	3	28
35-39	16	12	9	4	41
40-44	13	18	12	4	47
45-49	13	16	11	10	50
50-54	19	23	21	15	78
55-59	19	24	12	16	71
60-64	11	13	18	13	55
65-69	13	14	18	10	55
70-74	6	8	12	3	29
75-79	2	3	4	6	15
>79	1	0	1	2	4
Total	131	145	131	93	500

Table 2. Age-matched univariate analysis for epidemiological factors and colorectal cancer

Factor	OR	95% CI
Family history of colorectal cancer, 1st degree	1.48	0.69, 3.16
Current Smoker	1.44	0.85, 2.44
Ex-smoker	1.23	0.73, 2.07
Passive smoking	0.92	0.62, 1.36
Current drinker, alcohol	1.05	0.63, 1.76
Ex-drinker, alcohol	1.06	0.62, 1.80
Consumption of canned meat (daily-2/month)	1.47	0.98, 2.20
Consumption of fish sauce (<i>patis</i>) (daily-2/month)	1.33	0.92, 1.94
Consumption of shrimp paste (<i>bagoong</i>) (daily -2/month)	1.40	0.97, 2.02
Vegetable Consumption (≥ 1 /week)	0.86	0.49, 1.49
Consumption of scalding hot-food (> 5 days/month)	1.07	0.72, 1.58
Consumption of preserved food (nitrite-treated) (>5 days/month)	1.34	0.87, 2.07
Consumption of smoked food (> 5 days/month)	1.35	0.93, 1.96
Consumption of salted food eater (> 5 days/month)	0.85	0.55, 1.29
Moldy food exposure (≥ 1 / month) ^a	1.61	1.11, 2.35
Pesticide exposure (≥ 1 /week)	1.12	0.42, 2.99
Vinyl chloride occupational exposure	2.49	0.96, 6.47
Benzene occupational exposure	2.35	1.00, 5.50
UV sunlight exposure (≥ 7 /month) ^a	1.99	1.16, 3.39
Coal carbonization occupational exposure	2.17	0.38, 12.38
Wood dust occupational exposure ^a	2.66	1.21, 5.83
Betel quid chewing	3.36	0.34, 33.59
Oral contraceptive use (≥ 1 / year)	1.42	0.75, 2.66

Source: Ngelangel CA, Javelosa MA, Cutiongco-de la Paz, EM and the Philippine Cancer Genetics Study Group. Epidemiological Risk Factors for Cancers of the Lung, Breast, Colon-rectum & Oral cavity: A Case-Control Study in the Philippines. *Acta Medica Philippina*. 2009; 43(4):29-34.

^aEnvironmental factors that remained significant after age and sex-matched multivariate logistic regression: moldy food exposure OR 2.47 (95% CI: 1.11-5.48); UV sunlight exposure OR 1.75 (95% CI 1.01-3.03); wood dust occupational exposure OR 1.48 (95% CI: 1.01-2.17)

univariate logistic regression revealed that only the NAT2*6B allele and the NAT2*6B homozygous genotype were found to significantly increase colorectal cancer susceptibility in this study (Table 4).

Table 3. Multivariate logistic regression analyses of genetic and environmental factors

Variable*	Odds ratio	OR 95%CI
Constant	--	--
Age	1.02	1.01, 1.04
UV radiation	2.08	1.21, 3.58
Wood dust exposure	2.24	0.95, 5.30

*The following variables were removed from the model after backward logistic regression: sex, frequency of moldy food exposure and NAT2*6B allele. $N = 406$; $-2LL = 542.1$; $R^2 = 0.05$.

Table 4. Age- and sex-adjusted univariate logistic regression analyses by colorectal cancer status*

Variable*	Odds ratio (OR)	OR 95% CI
GSTM1 null	0.95	0.66, 1.37
GSTT1 null	1.08	0.75, 1.54
GSTP1 c.313G homozygote	1.10	0.24, 4.96
GSTP1 c.313G allele (dominant)	1.07	0.75, 1.53
GSTP1 c.313G allele (recessive)	1.06	0.59, 1.91
GSTP1 c.313G allele	1.07	0.75, 1.53
NAT1*3 allele	1.58	0.61, 4.06
NAT1*4 allele	0.94	0.65, 1.35
NAT1*10 allele	1.32	0.86, 2.05
NAT1*11 allele	1.09	0.39, 3.05
NAT2*4 allele	0.85	0.59, 1.23
NAT2*5B allele	0.57	0.29, 1.11
NAT2*5C allele	0.82	0.23, 2.95
NAT2*6B homozygote	2.19	1.19, 4.03
NAT2*6B heterozygote	1.38	0.95, 2.02
NAT2*6B allele (dominant)	1.51	1.06, 2.16
NAT2*6B allele (recessive)	1.87	1.05, 3.33
NAT2*7A allele	0.89	0.62, 1.29

*Formula: Colon cancer status ~ constant + $\beta_{\text{Variable}} \times \text{Variable}$

Discussion

Environmental Factors

Among the non-genetic variables, diet is the strongest and most likely contributor to colorectal cancer susceptibility; high intake of meat and fat, and low intake of fruits, vegetables are some dietary factors that have been reported to increase the likelihood of developing this particular cancer type.^{1,7,14} Lifestyle factors such as the lack of physical activity, obesity and alcohol consumption have also been identified as significant risk factors.^{5,7} Recent research has shown that the microbiome in the colon modulates the dietary metabolites that act as carcinogens.⁸ However, we were unable to detect significant interactions between the abovementioned colorectal cancer-associated variables and colorectal cancer risk in our study population; and the environmental factors that were identified to be significant in this study (moldy food exposure, occupational exposure to UV light and wood dust) have not been commonly associated with colorectal cancer, though they have been documented in the past as potential carcinogens and risk modifiers for other cancer types.^{8,11,33} The clinical significance of these findings is uncertain because of the fact that the lower limits of the 95% CI for the three factors are very close to the null value of one, and also because of the difficulty of

accurately ascertaining the level of consumption and exposure of subject participants in a retrospective case-control study such as this one.

Genetic Factors

Correlating acetylation status with colorectal cancer risk, the rapid acetylator genotype/phenotype has been hypothesized to cause an increase in colorectal cancer as a result of the higher NAT enzyme activity which activates dietary heterocyclic amines and other carcinogenic intermediates, leading to DNA adduct formation.^{7,9} Studies on animal models have also reported a higher incidence of colon aberrant crypt foci among rapid versus slow acetylators after administration of aromatic amine carcinogens.¹¹ Likewise, a number of population-based studies among African Americans, Caucasian, Chinese, Hungarian, Japanese and Portuguese subject participants, as well as one meta-analysis noted a significant excess of NAT2 rapid acetylation genotypes among colorectal cancer patients.^{4,13,34-37} Two separate meta-analysis studies by Wang et al (meta-analysis of 4 studies of Japanese (2,217 CRC cases, 3,788 controls) and 3 studies of African Americans (527 CRC cases, 4,527 controls) and Ananthkrishnan et al (meta-analysis of 11 studies (8,290 CRC cases and 9,115 controls)), and a study by Procopciuc et al (using 150 Romanian CRC cases and 162 controls; with PCR-RFLP as genotyping method) also showed the association of processed meat and red meat intake to colorectal cancer being significant for patients with NAT2 rapid acetylator phenotypes.^{4,7,9} Sørensen et al found an increase in risk among NAT1 fast acetylators in a prospective study among 379 German CRC cases and 769 sub-cohort members using direct sequencing, while the NAT1*10 allele—which is suggested to be a rapid acetylator allele that has been noted to increase risk for this cancer type.^{14,15} However, a number of previous studies have been unable to detect any significant interactions between NAT1 and NAT2 genotypes and colorectal cancer susceptibility.^{6,14,35} A systematic review of 73 studies done by Ananthkrishnan et al, also noted that NAT1 polymorphisms have significant association to pancreatic cancer and not colorectal cancer.⁷

The results of this study show that there is no statistically significant relationship between specific NAT1 genotypes and the risk of colorectal cancer in our subject population. Carriers of the NAT2*6B allele, which results in a slow acetylator phenotype, have demonstrated an increased risk for colorectal cancer. This finding diverges from earlier mentioned reports that linked fast acetylators with increased colorectal cancer risk. After adjustment for non-genetic factors, however, NAT2*6B allele was dropped from the model. This suggests that the NAT2*6B allele may not be as significant a modifier of colorectal cancer risk as that of environmental exposure. A recent report, using 150 Romanian CRC cases with PCR-RFLP and sequencing as

genotyping methods, has shown that NAT2*6B allele increases risk for sporadic colorectal cancer (OR=5.16; $p < 0.0001$) among Romanians.⁹

As for the GSTs, the null *GSTM1* and *GSTT1* genotypes, and the *GSTP1* c.313A>G polymorphism was evaluated as risk modifiers for colorectal cancer. The c.313A>G transition in *GSTP1* results in an isoleucine to valine substitution at codon 105, close to the enzyme's binding site for electrophilic substrates.²⁶ The 105val allele has also been associated with reduced *GSTP1* enzyme activity.²⁷ Similarly, reduced detoxification activity is also expected from the homozygous deletion of *GSTM1* or *GSTT1*, since the null genotype for either gene leads to a lack of catalytic activity of their respective enzyme products.^{19,24} All three GST variants have been hypothesized to increase colorectal cancer susceptibility as a result of the slower deactivation rate of potential carcinogens.

Studies have shown that the *GSTM1* null variant increases cancer risk among Caucasian, Chinese, Indian, Kazakh, Romanians, Russian, Saudi Arabians, Spanish and Turkish populations; while the *GSTT1* homozygous deletion has also been identified as a significant genetic risk factor for Caucasian, Chinese, Indian, Spanish and Turkish population-based studies.^{9,10,20-22,38-43} In two separate meta-analyses done by Li et al (using 33 studies with 8,502 CRC cases and 13,699 controls) and Liu et al (using 19 articles containing 3,217 cases), the null genotype of *GSTM1* was significantly associated with susceptibility to colorectal cancer and neurotoxicity, respectively, in Asians.^{23,24} It was also shown to be associated with poor overall survival and poor disease-free survival.²⁵ The heterozygous genotype of *GSTT1* was seen to be correlated with cancer susceptibility in Kazakh and Russian populations (OR = 1.98; 95% CI = 1.30 - 3.00; $p = 0.001$).^{21,25} Additionally, the combination of the null genotypes of *GSTM1* and *GSTT1* increases the risk of colorectal cancer among Chinese (OR, 1.95; 95% CI, 1.33-2.85; $p < 0.001$), ethnic Kashmiri (OR = 3.5, 95% CI: 0.9-13.8; $p = 0.06$) and Pakistani populations.^{10,17,19} Then again, the systematic review done by Liu et al also concluded that *GSTM1* and *GSTT1* have no association with chemotherapy-related toxicities and tumor response.²⁴

Among the three GST genes, *GSTP1* in particular, is a potential biomarker because it is widely expressed in the colon.³⁹ However, reports on the effect of the *GSTP1* c.313G on colorectal cancer risk have been inconsistent; with one study identifying the homozygous variant genotype as a risk factor, while another study finding the homozygous variant genotype to be protective.^{32,37} However, many other molecular epidemiology reports have also found no statistically significant associations with polymorphisms in the *GSTM1*, *GSTP1*, or *GSTT1* genes with colorectal cancer—these is similar to the findings of our own study among Filipino subject participants.^{10,18,20,21,24,34,41,43,44} The association of *GSTP1* c.313G to tumor response, progression and overall

survival after chemotherapy in colorectal and gastric cancers have also been demonstrated in a systematic review by Shen et al in 2016 but this was not part of this study.²⁷

As a case-control epidemiological study, there are inherent methodological limitations particularly pertaining to the evaluation of non-genetic factors as markers of colorectal cancer risk in the study population. Although the study was able to detect novel interactions between environmental variables and cancer susceptibility, a comprehensive prospective study coupled with a refinement in data collection techniques are needed to validate the findings.

For the genetic factors, the lack of significant associations does not necessarily discount the potential of these metabolic gene polymorphisms as colorectal cancer biomarkers. A more comprehensive study having a greater population size and allelic/genotype coverage of the candidate genes is recommended to better understand the individual and combinatorial effects of the different gene variants on cancer risk. To evaluate the interaction of environmental and genetic factors, stratified analysis with regard to epidemiologic subgroups is suggested in order to better identify at-risk subgroups in the population.

Conclusion

Among the non-genetic factors investigated in the study, only exposure to moldy food, occupational exposure to wood dust as well as to UV sunlight were found to increase susceptibility to colorectal cancer. As regards genetic polymorphisms in the xenobiotic metabolizing enzyme genes, only the *NAT2*6B* allele and *NAT2*6B/NAT2*6B* genotype were found to be a significant modifier of disease risk. Multivariate analysis of both environmental and genetic factors, however, suggests that UV sunlight and wood dust exposure play a greater role in influencing colorectal cancer susceptibility than *NAT2* genotype status. A comprehensive prospective study using a greater population size and allelic/genotype coverage of the candidate genes is recommended to validate the findings and to provide more insights on the gene-environment and gene-gene interactions of the environmental variables and different gene variants on colorectal cancer risk.

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Statement of Authorship

All authors have approved the final version submitted.

Author Disclosure

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