Prediction of Sensitivity of Rectal Cancer Cells in Response to Preoperative Radiotherapy by DNA Microarray Analysis of Gene Expression Profiles

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Abstract

Preoperative radiotherapy has been widely used to improve local control of disease and to improve survival in the treatment of rectal cancer. However, the response to radiotherapy differs among individual tumors. Our objective here was to identify a set of discriminating genes that can be used for characterization and prediction of response to radiotherapy in rectal cancer. Fifty-two rectal cancer patients who underwent preoperative radiotherapy were studied. Biopsy specimens were obtained from rectal cancer before preoperative radiotherapy. Response to radiotherapy was determined by histopathologic examination of surgically resected specimens and classified as responders or nonresponders. By determining gene expression profiles using human U95Av2 Gene Chip, we identified 33 novel discriminating genes of which the expression differed significantly between responders and nonresponders. Using this gene set, we were able to establish a new model to predict response to radiotherapy in rectal cancer with an accuracy of 82.4%. The list of discriminating genes included growth factor, apoptosis, cell proliferation, signal transduction, or cell adhesion–related genes. Among 33 discriminating genes, apoptosis inducers (lumican, thrombospondin 2, and galectin-1) showed higher expression in responders whereas apoptosis inhibitors (cyclophilin 40 and glutathione peroxidase) showed higher expression in nonresponders. The present study suggested the possibility that gene expression profiling may be useful in predicting response to radiotherapy to establish an individualized tailored therapy for rectal cancer. Global expression profiles of responders and nonresponders may provide insights into the development of novel therapeutic targets. (Cancer Res 2006; 66(7): 3370-4)

Introduction

Rectal cancer remains one of leading causes of cancer mortality. Preoperative radiotherapy has been widely used as a major treatment modality to improve local control of the disease as well as to improve survival (1–4). However, response to radiotherapy differs among individual tumors. If we can predict response to radiotherapy before treatment, other treatment modalities such as chemoradiotherapy can be considered for tumors that may not respond to radiotherapy alone. We have previously shown that a few molecular markers could be used to select good candidates among colon cancer patients for adjuvant chemotherapy (5). However, recent advances in expression genomics by DNA microarray have made it possible to analyze tens of thousands of genes at a time and have shown that expression profiles of cancer cells may be used for distinguishing responders and nonresponders to a given drug, as well as predicting toxicity and adverse effects of drugs (6). Until now, there have only been a limited number of studies of expression genomics aimed at predicting response to radiotherapy or chemoradiotherapy in cancer treatment (7, 8). In rectal cancer, this is the first report using DNA microarray for predicting response to radiotherapy. In this report, to predict response radiotherapy, we examined the gene expression profiles of rectal cancer cells by DNA microarray before preoperative radiotherapy was done. Our objective here was to identify a set of discriminating genes that could be used for characterization of responders and nonresponders to preoperative radiotherapy in rectal cancer. We were able to identify a novel set of genes of which the expression differed significantly between responders and nonresponders. Using this set of genes, we established a new model to predict response to radiotherapy with a high rate of accuracy. Furthermore, we did an ontology analysis of discriminating genes and showed a characteristic signature between responders and nonresponders. This may provide insights into the development of novel therapeutic targets in preoperative radiotherapy for rectal cancer.

Materials and Methods

Patient Samples

Informed consent was obtained from rectal cancer patients for the collection of specimens, and the study protocol was approved by the Ethics Committee of The University of Tokyo. Fifty-two rectal cancer patients who approved to receive preoperative radiotherapy were studied. We prospectively collected biopsy specimens during colonoscopic examination from rectal cancer before starting preoperative radiotherapy (Fig. 1). Paralleled tumor specimens were formalin fixed and paraffin embedded for histologic examination and other specimens were used for RNA extraction. Samples were used for RNA extraction when paralleled specimens contained at least 70% tumor cells as previously described (ref. 9; see Supplementary Fig. S1). Samples were snap-frozen in liquid nitrogen and stored at −80°C until use. All patients received a total dose of 50.4 Gy of radiation and underwent standardized curative resection, following an interval of 4 weeks after radiotherapy.

Response to Radiotherapy

Response to radiotherapy was determined by histopathologic examination of surgically resected specimens based on a semiquantitative

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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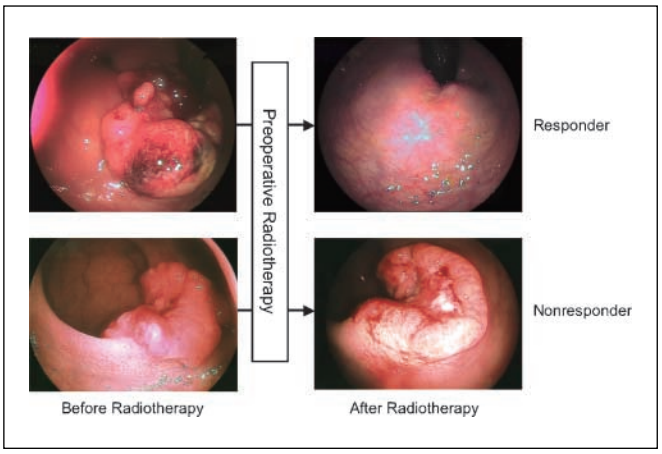

Figure 1. Endoscopic view of rectal cancers before and after preoperative radiotherapy. Specimens were obtained during colonoscopic examination before preoperative radiotherapy.

classification system as described in detail previously (10). Tumors were classified as "responder" when assigned to the regression grade 2 or 3, and "nonresponder" when grade 0 or 1.

RNA Isolation and Microarray Procedures

Tissues were lysed and total RNA was extracted using the Sepasol-RNA I (WAKO, Osaka, Japan) according to the instructions of the manufacturer. Total RNA was then purified using an RNeasy column (Qiagen, Austin, TX). Total RNA (5 μg) was reverse transcribed to cDNA using T7-(dT)24 primer. Biotin-labeled cRNA was synthesized from cDNA using the MEGAscript In Vitro Transcript Kit (Ambion, Austin, TX). cRNA was fragmented to an average size of 50 to 100 nucleotides by incubating at 95°C for 35 minutes in 40 mmol/L Tris-acetate (pH 8.1) containing 100 mmol/L potassium acetate and 30 mmol/L magnesium acetate, and then hybridized to human U95Av2 GeneChip (Affymetrix, Santa Clara, CA). The hybridized cRNA probes to oligonucleotide arrays were stained with streptavidin R-phycoerythrin (Molecular Probes, Eugene, OR) and were scanned using a confocal scanner (Affymetrix). The scanned data obtained from each microarray were normalized to correct for small differences in the amounts of each of the cRNA probes applied to the microarray and were processed for signal values using Affymetrix software (LIMS 5.0; the entire microarray data set is available at http://www.ncbi.nlm.nih.gov/geo/info/linking.html under the data series accession number GSE3493).

Statistical Analysis

Class prediction. Comparative analysis between expression profiles for responders and nonresponders was carried out on GeneSpring software version 7.2 (Silicongenetics, Redwood, CA). Gene expression data, when classified as either flag-P or flag-M in >50% of all samples, were loaded into the software and then normalized in two ways: "per chip normalization" and "per gene normalization." For "per chip normalization," all expression data on a chip were normalized to the 50th percentile of all values on that chip. For "per gene normalization," the data for a given gene were normalized to the median expression level of that gene across all samples. To identify genes that were differentially expressed between the two groups, the data sets were assigned to either responders or nonresponders. Next, samples from 52 patients were divided into a training set (35 samples) and a testing set (17 samples). We used only training samples (35 samples) in the original statistical analysis to distinguish a gene set and to build a prognostic signature. The testing set was used for independent validation. We first collected 35 training samples to build a predictive model. After establishing a predictive model, we prospectively collected 17 samples for validation of the model. We classified samples into training and test samples in exact chronological order. All test samples were obtained from the 17 patients who were treated after the initial 35 patients for training samples had been treated. The expression profiles were compared using unpaired t tests (with Welch's correction for unequal variances) and fold change value to identify gene markers that can best discriminate between responders and nonresponders. Two-dimensional hierarchical clustering was then applied to the log-transformed data with average-linkage clustering with standard correlation as the similarity metric for the discriminating genes that we identified as differentially expressed between responders and nonresponders. Variation of multigene expression between responders and nonresponders was also compared by principal component analysis. We then carried out supervised class prediction using the k-nearest-neighbor method and a leave-one-out cross-validation with the discriminating genes in 35 training samples. We then did validation test by applying the same method to 17 test samples (11, 12).

Gene functional category analysis. Gene Ontology categories were analyzed by the BioScript Library tool on GeneSpring 7.2. Genes were classified according to their annotated role in biological processes, molecular function, and cellular components from Gene Ontology (The Gene Ontology Consortium). This program identifies genes belonging to different Gene Ontology categories and also calculates the statistical significance of nonrandom representation (i.e., overlapping P values). Using hypergeometric probability, the overlapping P values was calculated by

$$P = \frac{\sum_{i=k}^{n} \binom{m}{i} \binom{u-m}{n-i}}{\binom{u}{m}}$$

where the probability of overlap corresponding to k or more genes between a gene list of n genes is compared against a gene list of m genes when randomly sampled from a universe of u genes.

Results

Gene expression profiling: class comparison between responders and nonresponders. Gene expression profiling was established using DNA array in all samples consisting of training and test samples. Among the 35 training samples, 7 were classified as responders and 28 as nonresponders according to the histologic examination of surgical specimens. On the other hand, 6 were classified as responders and 11 as nonresponders among the testing samples. There was no significant difference between responders and nonresponders in clinicopathologic factors such as gender, age, histologic classification, preoperative tumor stage, and so on. To identify molecular signatures of responsiveness to preoperative radiotherapy, gene expression profiles were compared between responders and nonresponders (see Supplementary Table S1). Using class-comparison analysis, we identified a list of 33 genes that were differentially expressed at significant levels ($P < 0.05$) between responders and nonresponders (Table 1). Twenty genes showed higher and 13 genes lower expression in responders as compared with nonresponders. The list of genes included a member of growth factor (*TDGF3*), rho family genes (*RAC2*), as well as genes related to apoptosis (*LUM, THBS2, LGALS1, CYPD*, and *GPX2*), signal transduction (*TYRO3*), cell adhesion (*THBS2*), or cell proliferation (*PSPHL*). Among five genes related to apoptosis, lumican (*LUM*), thrombospondin 2 (*THBS2*), and galectin-1 (*LGALS1*), which induce apoptosis, showed significantly higher expression in responders than in nonresponders. On the other hand, two genes, cyclophilin 40 (*CYPD*) and glutathione peroxidase 2 (*GPX2*), which inhibit apoptosis, showed higher expression in nonresponders than in responders. Results of a hierarchical cluster analysis of the 33 genes are presented in Fig. 2. Responders and nonresponders were clustered into two distinct groups. We also used 33 discriminating genes to generate

Table 1. List of 33 discriminating genes between responder and nonresponder to preoperative radiotherapy

Probe ID	Fold change (nonresponder/responder)	*P, t* test	Gene symbol	GenBank	Gene name
41470_at	2.839	0.0231	*PROML1*	AF027208	prominin-like 1
33703_f_at	2.398	0.00548	*PCK1*; *PEPCK1*; *PEPCKC*	L12760	phosphoenolpyruvate carboxykinase gene
40386_r_at	1.854	0.0418	*TDGF3*; *CR-3*; *TDGF1*; *TDGF2*; *CRIPTO*; *CRIPTO-3*	M96956	teratocarcinoma-derived growth factor 3
948_s_at	1.83	0.0363	*PPID*; *CYPD*; *CYP-40*	D63861	DNA for cyclophilin 40
38072_at	1.639	0.0223		AL031432	contains two novel genes, ESTs, GSSs and CpG islands
32225_at	1.589	0.000905		X04297	ATPase α subunit; mRNA for Na, K-ATPase α-subunit
35194_at	1.567	0.0288	*GPX2*	X53463	glutathione peroxidase 2 (gastrointestinal)
40487_at	1.564	0.00856	*MC7*	W26634	likely orthologue of mouse brain cDNA 7
32878_f_at	1.484	0.0211		AA524802	ESTs similar to H3 histone
37061_at	1.448	0.0446	*CHIT1*	U29615	chitinase 1
35384_at	1.427	0.0466	*HRH1*	Z34897	histamine receptor H1
41225_at	1.403	0.0379	*DUSP3*	AL049417	EST
38736_at	1.402	0.0471	*WDR1*	AL050108	WD repeat domain 1
32749_s_at	0.687	0.0339	*FLNA*; *ABPX*; *FLN1*; *NHBP*; *ABP-280*	AL050396	EST similar to Endothelial actin-binding protein
32720_at	0.681	0.00122	*APG12L*	AA151716	APG12 autophagy 12-like
120_at	0.677	0.00232	*ITGA1*; *VLA1*	X68742	mRNA for integrin, α subunit
32736_at	0.646	0.0065	*RAC2*	W68830	ras-related (rho family, small GTP binding protein Rac2)
2086_s_at	0.632	0.0112	*TYRO3*	D17517	TYRO3 protein tyrosine kinase
38420_at	0.63	0.0362	*COL5A2*	Y14690	collagen, type V, α 2
33412_at	0.622	0.03	*LGALS1*	AI535946	lectin, galactoside-binding, soluble, 1 (galectin 1)
40438_at	0.614	0.00524	*PPP1R12A*	D87930	protein phosphatase 1, regulatory (inhibitor) subunit 12A
41618_at	0.584	0.0464	*COL17A1*	M91669	collagen, type XVII, α 1
753_at	0.583	0.0128	*NID2*	D86425	nidogen 2 (osteonidogen)
36176_at	0.547	0.00877	*TBCC*	U61234	tubulin-specific chaperone c
36239_at	0.546	0.0335	*POU2AF1*	Z49194	POU domain, class 2, associating factor 1
40694_at	0.524	0.0423	*KRT20*	X73502	cytokeratin 20
32307_s_at	0.469	0.017	*COL1A2*	V00503	collagen, type I, α 2
38038_at	0.468	0.00964	*LUM*	U21128	lumican
37208_at	0.464	0.0182	*PSPHL*	AJ001612	phosphoserine phosphatase-like
658_at	0.445	0.0287	*THBS2*	L12350	thrombospondin 2
32488_at	0.421	0.02	*COL3A1*	X14420	collagen, type III, α 1
32305_at	0.42	0.00409	*COL1A2*	J03464	collagen, type I, α 2
32306_g_at	0.378	0.0058	*COL1A2*	J03464	collagen, type I, α 2

a three-dimensional (from 33-dimensional) plot of the data (Fig. 3). The three axes are the first three principal components fitted to the responder and nonresponder molecular profile data. Principal component analysis–based multidimensional scaling visualization separated nonresponder (*Yellow*) and responder (*Red*) samples into linearly separable gene expression data space.

Gene functional category analysis. To investigate the biological functions involved in the discriminating genes, we did Gene Ontology category analysis. Categories of cell growth, signal transduction, cell differentiation, and receptor activity showed a higher proportion than other categories. When selected, discriminating genes were compared with all genes of which expression profile could be evaluated; some categories, such as cell adhesion molecule activity, showed a significantly higher proportion among selected 33 genes.

Gene expression profiling: class prediction of responders and nonresponders. We next examined whether the expression profiling is useful in predicting response to radiotherapy. Using training samples, we carried out supervised class prediction with the *k*-nearest-neighbor method and a leave-one-out cross-validation with the 33 differentially expressed gene list. The accuracy of class prediction by the *k*-nearest neighbor method was 88.6% (31 of 35 correct calls). There were four misclassifications in the training samples, two of which belonged to responders and two to nonresponders. The sensitivity and specificity of the present model in the training set were 71.4% and 92.9%, respectively. Furthermore, because the positive and negative predictive values are also important in a clinical setting, we calculated the positive and negative predictive values. The positive and negative predictive values of the present model were 71.4% and 92.9%, respectively. Next, to further test the validity of our data analysis, we did class

prediction using test sample sets. With the *k*-nearest-neighbor method, there were three misclassifications in the test samples, which belonged to responders. The sensitivity, specificity, positive predictive value, and negative predictive value in the test samples were 50.0%, 100%, 100%, and 78.6%, respectively. We confirmed that class prediction was correct in 82.4% of test samples.

Discussion

Our study first established a new model using global gene profiling to predict response to radiotherapy in rectal cancer. Responders and nonresponders could be predicted with an accuracy rate of 88.6% with the *k*-nearest-neighbor method. We further validated the ability of this model to predict response by using a new set of test samples, giving an accuracy rate of 82.4%. There have only been a few studies aimed at predicting response to radiotherapy using gene expression profiling in the treatment of cancer (7, 8). In rectal cancer, Ghadimi et al. (8) recently reported that gene expression profiling is useful in predicting response to preoperative chemoradiotherapy. However, considering that the postoperative morbidity rate is higher in chemoradiotherapy than in radiotherapy alone and chemoradiotherapy needs continuous infusion of a chemotherapeutic agent lasting several days, radiotherapy seems to be a more feasible modality in conducting neoadjuvant therapy for rectal cancer. It is suggested that the present model may be used to offer a new strategy for tailored therapy in rectal cancer. If response to radiotherapy is expected, then the patient can receive radiotherapy, thereby eliminating the risk of side effects due to chemoradiotherapy. On the other hand, if response to radiotherapy is not expected, chemoradiotherapy may be offered as an alternative neoadjuvant modality.

By comparing gene expression profiling between responders and nonresponders, we identified 33 novel genes of which the expression differed significantly between responders and nonresponders. Among the 33 genes, 20 genes showed higher and 13 genes lower expression in responders as compared with nonresponders. Gene Ontology category analysis identified nonrandom enrichment of a variety of biological process categories, including transcription, cell growth, signal transduction, and apoptosis. It has been reported that induction of apoptosis is an important factor in determining the response to radiotherapy (13). Among 33 discriminating genes, there were five apoptosis-related genes (lumican, thrombospondin 2, galectin-1, cyclophilin 40, and glutathione peroxidase 2). Lumican, a member of the small leucine-rich proteoglycan family, induces apoptosis by increasing Bax expression and suppresses cell proliferation (14). Its reduced expression has been reported to be associated with poor outcome of invasive carcinoma (15). Thrombospondin 2, a potent endogenous inhibitor of tumor growth and angiogenesis, inhibits cell proliferation and induces apoptosis (16). Thrombospondin 2–deficient mice are characterized by accelerated and enhanced skin carcinogenesis whereas stable overexpression of thrombospondin 2 in human squamous cell carcinomas potently inhibits tumor growth (17). Galectin-1 is a member of the mammalian β-galactoside-binding proteins and known to initiate cell apoptosis (18). Its apoptotic potential has been shown in

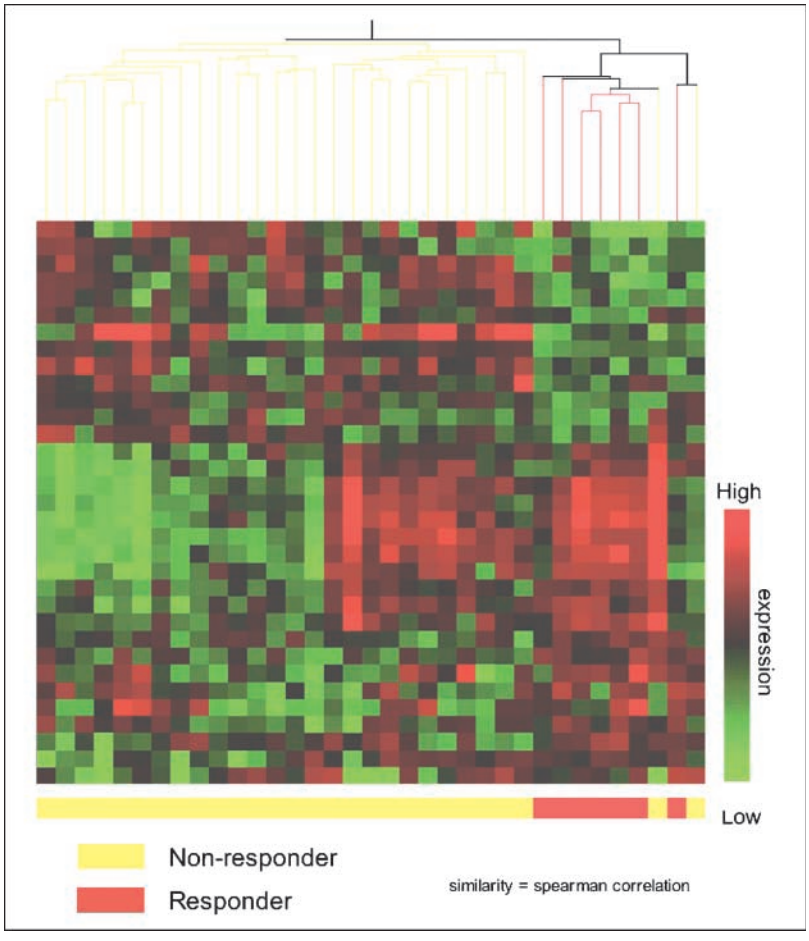

Figure 2. Supervised clustering of rectal cancer and 77 genes. Two-way hierarchical clustering was used to order samples (*columns*) and array targets (*rows*). *Red,* overexpression; *green,* underexpression. *Bottom, yellow,* nonresponders; *red,* responders. Samples were classified correctly into responders and nonresponders except for two nonresponder cases.

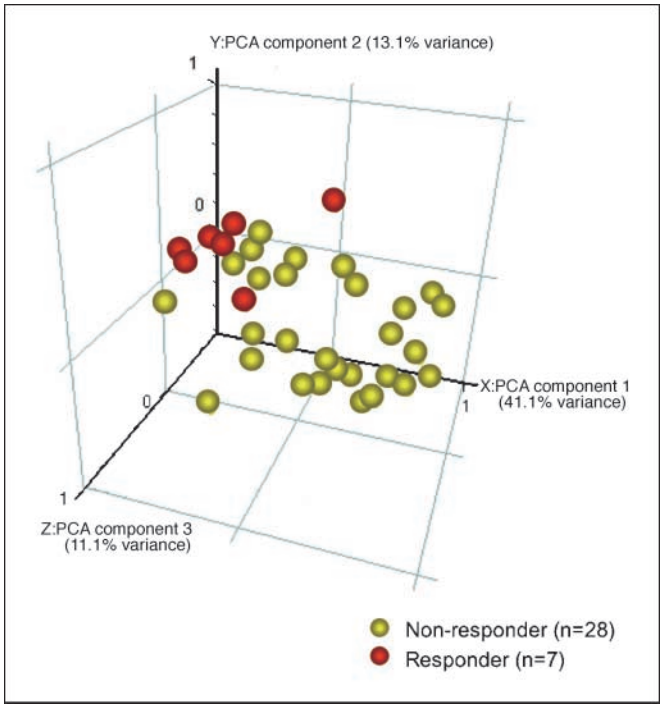

Figure 3. Discriminating genes were used to generate a three-dimensional (from 33-dimensional) plot of the data. The three axes are the first three principal components fitted to the responder and nonresponder molecular profile data. The cumulative proportion of the variance captured by each principal component axis is principal component axis 41.1%; principal component axis 13.1%; and principal component axis 11.1%. Principal component analysis–based multidimensional scaling visualization separated nonresponder (*Yellow*) and responder (*Red*) samples into linearly separable gene expression data space.

epithelial tumor cell lines as well as in human colon cancer cell lines (18). All three of these genes (lumican, thrombospondin 2, and galectin-1), which induce apoptosis, showed significantly higher expression in responders than in nonresponders. On the other hand, cyclophilin 40 and glutathione peroxidase have been reported to have inhibitory effects on apoptosis (19). Previous studies showed that glutathione peroxidase overexpression inhibits apoptosis in doxorubicin-treated human breast carcinoma cells (19). In addition, glutathione peroxidase–deficient fibroblasts show enhanced sensitivity to oxidant-induced apoptosis (20). Both of these two genes with inhibitory effects on apoptosis showed significantly lower expression in responders than in nonresponders. Considering that all three apoptosis inducers showed higher expression in responders whereas two apoptosis inhibitors both showed higher expression in nonresponders, it is suggested that apoptosis-related genes play an important role in determining the response to radiotherapy.

The present study suggests the possibility that gene expression profiling may be useful in predicting response to radiotherapy to establish an individualized tailored therapy for rectal cancer. However, because the number of patients, especially responders, was limited in the present study, large prospective trials will be needed to confirm the validity of the present predictor. We believe that global expression profiles of responders and nonresponders may provide insights to the development of novel therapeutic targets.

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