Bcl-2 Oncoprotein Positivity and High MIB-1 (Ki-67) Proliferative Rate Are Independent Predictive Markers for Recurrence in Prostate Carcinoma

Albert A. Keshgegian MD, PhD,[1,2] *Elaine Johnston,*[1] *and Avital Cnaan, PhD*[3]

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Abstract

Predicting the clinical behavior of prostate carcinoma can be difficult; one approach is to identify molecular prognostic markers. We evaluated proliferative rate (MIB-1 antibody) and expression of bcl-2, *p53, and retinoblastoma (pRB) proteins, which have cell cycle–related functions, in 208 consecutive radical prostatectomy specimens. Values were correlated with histopathologic parameters (Gleason tumor score, tumor amount, capsule invasion, and involvement of surgical margins, seminal vesicles, or lymph nodes) and with recurrence-free survival (4-year median follow-up). A high MIB-1 proliferative rate was associated with all of the measured histopathologic parameters, p53 overexpression with tumor amount, and pRB expression with positive lymph nodes. pRB and p53 expression levels were not associated with differences in recurrence-free survival. A high MIB-1 proliferative rate and* bcl-2 *positivity were associated with increased recurrence, both considered individually, and also independently and additively when examined together and with the most predictive histopathologic factors (Gleason tumor score and seminal vesicle involvement). MIB-1 proliferative rate and* bcl-2 *positivity may prove to be useful markers for poor prognosis in prostate carcinoma.*

Prostate carcinoma is the most common cancer and the second leading cause of death due to cancer in men.[1] The advent of serum screening with prostate-specific antigen has led to increased public and physician awareness and to the discovery of more tumors at an earlier stage. There is controversy whether all of these tumors would eventually grow and become life-threatening or whether some or most of these are indolent tumors in elderly men, for whom the treatment may pose more risk than the tumor.[1] Although histopathologic grade provides some information,[2] additional means of distinguishing biologically aggressive from indolent carcinomas at the time of diagnosis would be clearly beneficial. Increasing attention has turned to molecular markers as a possible means of obtaining such information. One group of molecular markers that is of interest is the group that measures proliferative capacity of the tumor or is otherwise involved in cell cycle regulation.

Proliferative activity has been measured by determining the S-phase fraction of DNA by flow cytometry, but the significance of such measurement is unclear.[3] Proliferating cell nuclear antigen expression is probably not related to prognosis.[4] Assessment of argyrophilic nucleolar organizer regions may be helpful[5] but is technically demanding and time consuming. Antibodies to the Ki-67 nuclear antigen, including the MIB-1 antibody that can be used on formalin-fixed material, also have shown association with prognosis.[6–8]

The *p53* gene is a tumor-suppressor gene active in cell cycle regulation that has been studied in a variety of tumors.[9] A gene mutation in tumors renders the protein product ineffective, apparently contributing to the malignant phenotype; however, the aberrant protein product often has a longer half-life than the wild type protein and is detectable by immunohistochemistry.[9] The overexpression of p53 protein

in prostate carcinoma has been reported to be associated with high histopathologic grade and poor prognosis.[10–12]

Bcl-2 oncoprotein seems to be an inhibitor of apoptosis,[13] with diminished cell death in tumors possibly contributing to their growth. Although originally identified in lymphomas and leukemias, bcl-2 also has been found to be expressed in epithelial malignant neoplasms.[13] In prostate carcinoma, expression of bcl-2 has been associated with androgen independence[14,15] and with decreased survival in 1 report[16] but not others.[12,17]

The retinoblastoma gene product (pRB) is another protein involved in cell cycle regulation; it represses the expression of genes required for cell division.[18] Retinoblastoma gene mutations and the lack of expression of the gene product are associated with development of a cancer or increased aggressiveness of a preexisting cancer.[18] There is evidence that some prostate carcinomas can have abnormalities of the retinoblastoma gene and decreased or absent expression of pRB protein[19,20] along with a poor prognosis.[12]

Most previous studies have examined only 1 or 2 of these molecular markers, and some have used small numbers of cases. For the present study, we assembled a series of 208 consecutive radical prostatectomy specimens that had undergone standardized fixation and histologic processing. We evaluated the association of several molecular markers known to be involved in proliferation and cell cycle regulation—Ki-67 (MIB-1), bcl-2, p53, and pRB—with histopathologic factors and recurrence-free survival. In this combined analysis, we determined which of these markers showed independent predictive value that may prove to be clinically useful for patient prognosis.

Materials and Methods

Patients

The study included 208 consecutive men who had undergone radical prostatectomy for prostate carcinoma, who did not have another concurrent malignant neoplasm, and for whom follow-up information was available from the Bryn Mawr Hospital Tumor Registry (Bryn Mawr, Pa). The following information was obtained: length of follow-up in months; presence of recurrence; time to recurrence, if present; patient survival status at last follow-up; and presence of carcinoma at death. Recurrence was defined by the attending physician and could include clinical evidence and increasing serum concentrations of prostate-specific antigen. For data analysis of time to recurrence, failure was defined as recurrence (22 patients) or never disease-free (6 patients) after radical prostatectomy. The mean follow-up time for the 208 patients was 4 years, ranging from 4 patients with follow-up of 1 year or less up to 9 years.

Histopathologic Parameters

Prostate glands were inked and completely submitted for routine histopathologic examination, with mapping diagrams of the submitted sections. Seminal vesicles were removed and submitted separately. The number of submitted sections containing carcinoma, compared with the total number of sections represented by the entire gland, was quantitated as a percentage to provide a semiquantitative estimate of the amount of carcinoma. In 14 cases in which the entire gland had not been submitted, this information was not assessed. The median percentage of sections involved by tumor per prostate gland was 36%. Amounts of tumor greater than 36% were classified as high and amounts less than or equal to 36% as low. Carcinomas were graded and scored according to the Gleason system.[21] Each prostate gland also was assessed for extension of tumor to an inked specimen margin (which does not necessarily imply invasion through the capsule or residual tumor in the patient[2]), as well as overt invasion through the capsule into periprostatic tissue. Seminal vesicles and lymph nodes were examined for tumor involvement except in 1 case in which these tissues were not received with the specimen.

Immunohistochemistry

One block of tumor considered to be most representative was selected from each case for immunohistochemical staining. Ten percent neutral-buffered, formalin-fixed (overnight), paraffin-embedded blocks were cut at 4-µm sections and deparaffinized and rehydrated according to standard methods. Before immunostaining, antigen retrieval was performed by placing rehydrated slides into H.I.E.R. (heat induced-epitope retrieval) Buffer (containing sodium citrate) (Ventana Medical Systems, Tucson, Ariz) and microwaving in an 800-W oven for a total of 15 minutes on high power. Following pretreatment, the slides cooled for 20 minutes at room temperature.

The following primary antibodies and dilutions and incubations were used: nuclear antigen Ki-67 (clone MIB-1, Immunotech, Westbrook, Me), 1:50, 25 minutes; bcl-2 (Clone 124, DAKO, Carpinteria, Calif), 1:50, 25 minutes; p53 (clone DO-7, DAKO), 1:40, 25 minutes; and pRB (clone G3–245, BioGenex, San Ramon, Calif) 1:20, 25 minutes. All antibodies were diluted with the ChemMate Antibody Dilution Buffer (Ventana Medical Systems). The following tissues were used as positive and negative (omitting the primary antibody) controls: MIB-1 and bcl-2, human tonsil; p53, human breast carcinoma; and pRB, human colon carcinoma. Staining was performed on the automated Biotek Techmate 500 (Ventana Medical Systems) stainer, using a standardized staining protocol (MIP-2) and the ChemMate Secondary

Detection Kit-Peroxidase/DAB (Catalog no. SDK605), and ChemMate Standard Buffer Kit (Catalog no. SDK601A) developed by the manufacturer. The slides were counterstained with hematoxylin provided in the ChemMate Standard Buffer Kit according to the MIP-2 staining protocol.

The procedure for MIB-1 was similar to that used in a previous report from this laboratory, which showed good correlation between MIB-1 positivity and prognosis in breast carcinoma.[22]

Positivity for antigens was determined by counting the number of positive cells in 500 tumor cells, in multiple representative high-power fields across the slide, consciously choosing fields that contained high and low numbers of positive cells when variability was present. Results for MIB-1 were expressed as the percentage of positive cells. Results for bcl-2, p53, and pRB were expressed semiquantitatively according to the following categories: negative, no positive tumor cells; up to 5% positive tumor cells; 6% to 20%; 21% to 50%; more than 50%. For MIB-1, p53, and pRB staining was nuclear and for bcl-2, cytoplasmic. Any degree of staining was considered positive.

Statistical Analysis

Descriptive statistics were calculated for numeric variables. MIB-1 and amount of tumor had skewed distributions. To achieve normality, all parametric analyses with these 2 variables were performed on the square root transformed data. One-way analysis of variance was used to assess the possible associations between MIB-1 and histopathologic factors. Kruskal-Wallis tests were used to examine the possible associations between categorical antibody results (bcl-2, pRB, p53) and histopathologic factors.

Kaplan-Meier survival curves were calculated to show overall recurrence-free survival probabilities and recurrence-free survival probabilities in various subgroups. Log-rank tests were used to compare differences between recurrence-free survival curves. Multivariable survival analyses were done using a Cox regression model and an all subsets approach on all significant factors. Statistical significance was declared if the P value was .05 or less. Statistical analysis was performed using Stata 5.0. (Stata, 1997. Stata Statistical Software, Release 5.0, College Station, Tex).

Results

Histopathologic Prognostic Factors

The distribution of Gleason tumor scores was as follows: 5 or less, 42 (20.2%); 6, 60 (28.8%); 7, 65 (31.3%); and 8 or more, 41 (19.7%). Capsule invasion was present in 97 (46.6%), and the margins were involved in 123 (59.1%) of 208 cases. The seminal vesicles were involved in 27 (13.0%) and lymph nodes in 8 (3.9%) of 207 cases (data not available for 1 case).

The range of MIB-1–positive tumor cells (nuclei) was 0% to 28.8%, with a median of 6.4% and mean of 7.7%. Bcl-2 positivity was identified in the cytoplasm of tumor cells, as well as in the cytoplasm of some benign ducts and in lymphocytes, which served as additional internal controls. p53 stained the nuclei of tumor cells, as well as occasional benign epithelial cells. Nuclear positivity for pRB approximately correlated between benign and malignant cells.

The range of values for bcl-2, pRB and p53 is shown in **Table 1**. There were 28 cases (13.4%) showing any positivity for bcl-2. All cases contained at least some tumor cells positive for pRB and almost all (97%) had some positivity for p53. Most tumors contained 1% to 5% cells that were pRB positive and 1% to 20% that were p53 positive.

In studying associations between molecular markers and histopathologic parameters, higher MIB-1 positivity was observed for a higher Gleason tumor score (P = .0001), larger amount of tumor (P = .0016), capsule invasion (P = .0061), involved margin (P = .0042), seminal vesicle involvement (P = .0053), and lymph node involvement (P = .0001). pRB was associated only with lymph node positivity (P = .04), which was more likely in tumors with greater rather than less pRB expression. There was no significant association of bcl-2 or p53 with any histopathologic parameter.

When molecular markers were compared with each other, the only significant associations identified were between MIB-1 and pRB (P = .02) and between pRB and p53 (P = .02); in both instances, higher amounts of pRB expression were observed in tumors with higher degrees of MIB-1 or p53 positivity.

Recurrence-Free Survival

Table 2 shows the relationship of individual histopathologic factors and molecular markers with recurrence-free survival. Among the histopathologic variables, there was a significant association between prognosis and Gleason

Table 1
Frequency Distribution of Positivity for bcl-2, pRB, and p53*

Positivity	bcl-2	pRB	p53
0	180 (86.5)	0 (0.0)	6 (2.9)
≤5	17 (8.2)	137 (66.1)	79 (38.0)
6–20	4 (1.9)	46 (22.1)	92 (44.2)
21–50	4 (1.9)	22 (10.6)	21 (10.1)
>50	3 (1.4)	3 (1.4)	10 (4.8)

*Positivity is given as the percentage of tumor cells; other data are given as number (percentage).
pRB = retinoblastoma gene product.

Table 2
Observed and Expected Recurrence Events by Prognostic Factors

Factor	N	Recurrence		P
		Observed	Expected	
Gleason tumor score				
≤5	42	6	5.48	.0049
6	60	2	8.53	
7	65	8	8.48	
≥8	41	12	5.51	
Amount of tumor (n = 194)				
Low	95	10	13.14	.21
High	99	16	12.86	
Capsule invasion				
No	111	10	15.36	.041
Yes	97	18	12.64	
Margins involved				
No	85	9	11.95	.26
Yes	123	19	16.05	
Seminal vesicle involved (n = 207)				
No	180	16	24.86	<.00005
Yes	27	12	3.14	
Lymph nodes positive				
No	199	24	26.7	.015
Yes	8	4	1.3	
MIB-1				
≤6.4%	106	8	14.79	.0099
>6.4%	102	20	13.21	
bcl-2				
Negative	180	20	24.55	.0086
Positive	28	8	3.45	
pRB				
≤5%	137	18	18.41	.87
>5%	71	10	9.59	
p53				
≤5%	85	7	11.1	.11
>5%	123	21	16.9	

pRB = retinoblastoma gene product.

tumor score, capsule invasion, and seminal vesicle or lymph node involvement, but not amount of tumor or involvement of surgical resection margins. For Gleason tumor score, there is an increased recurrence rate associated with tumors with a score greater than 7.

The association of molecular markers with recurrence-free survival was studied in a dichotomous fashion. For MIB-1, the median value was chosen as the cutoff. For bcl-2, because of the relatively small number of positive tumors, any staining was regarded as positive. For p53, 5% or greater tumor cells was considered positive, and for pRB, less than 5%. Among the molecular markers, high levels of MIB-1 and positivity for bcl-2 showed a significant association with increased recurrence. There was no significant relationship between pRB or p53 and recurrence-free survival. Similar analysis for these markers at a 20% cutoff also failed to show statistical significance (pRB, $P = .36$; p53, $P = .29$).

Figure 1 shows Kaplan-Meier recurrence-free survival curves for MIB-1 and bcl-2, as well as a combined analysis of MIB-1 and bcl-2. Higher MIB-1 and positive bcl-2 are associated with poorer recurrence-free survival ($P = .0099$ and .0086, respectively). When classified according to positivity for both markers (Figure 1, C), patients whose tumors have high levels of MIB-1 and positivity for bcl-2 have a significantly poorer prognosis than patients whose tumors have low MIB-1 levels and bcl-2 negativity ($P<.00005$). Patients whose tumors have a high MIB-1 level or bcl-2 positivity have an intermediate prognosis, significantly worse than the low MIB-1, negative bcl-2 group ($P = .036$) but better than the high MIB-1, positive bcl-2 group ($P = .016$).

Cox models were fit to examine the relation of multiple variables jointly and for time to recurrence **Table 3**, combining the most significant histopathologic factors (Gleason tumor grade, seminal vesicle involvement) and the significant molecular markers (MIB-1 and bcl-2). Although lymph node involvement also is strongly prognostic, there were only 8 patients (3.8%) in this series, so this finding is considered a weaker result. Gleason tumor grade and seminal vesicle involvement are closely associated; thus when they are both in the same model, one loses significance. Therefore 2 different models are presented, combining MIB-1 and bcl-2 with tumor grade or seminal vesicle involvement as the

explanatory variables. In both of these models, all of the variables examined retain a significant association with recurrence-free survival, even in the presence of the other variables. Specifically, high and MIB-1 levels and bcl-2 positivity are associated with increased recurrence rate, even when examined together with the other molecular marker and with Gleason tumor grade or seminal vesicle involvement.

Discussion

As part of the analysis of molecular markers, accepted histopathologic markers of prognosis were determined for correlation. As expected, a strong association between prognosis and Gleason tumor score, seminal vesicle involvement, or lymph node involvement was observed.[2] Similarly, a weaker, but still significant, association between prognosis and invasion through the capsule also was identified.[2] In the present study, neither the presence of tumor at the surgical margin nor a semiquantitative estimate of tumor volume correlated significantly with prognosis. Although other investigators have found correlation between these parameters and prognosis, the meaning of positive margins and methods of estimation of tumor volume are controversial areas.[2]

For the molecular markers, previous studies have reported average rates of MIB-1 positivity from 3.4% to 10%, with a range of positivity up to 33%.[6,23,24] The results of the present study are in accord with these previous reports. Comparisons of positivity rates for the other markers are more difficult, owing to varying criteria that have been applied for positivity, as well as to the use of different antibodies and other methodologic variables. The 13% positivity rate for bcl-2 obtained in this study is somewhat lower than other reports of 25% to 62%,[14,15,25,26] but similar to the 12.6% obtained in 1 study.[12] On the other hand, the rate of p53 positivity, with almost all cases showing some staining, and more than half of the cases showing more than 5% positivity, is higher than in most previous reports.[10–12,27–30] We did not find any tumors with complete lack of pRB expression, which is at variance with some studies[12,19,20] but similar to the findings of 2 preliminary reports.[31,32]

MIB-1 positivity was strongly associated with all histopathologic parameters studied. Association with Gleason score and extension of tumor outside of the prostate also have been reported previously.[8] pRB expression (rather than lack of expression) was associated weakly with lymph node positivity. Bcl-2 and p53 expression were not associated with any histopathologic parameters. A number of studies have reported an association between 1 of these molecular markers and 1 or more histopathologic parameters,[10–12,17,25,28,29] but there does not seem to be a consensus in the literature.[15,16,33]

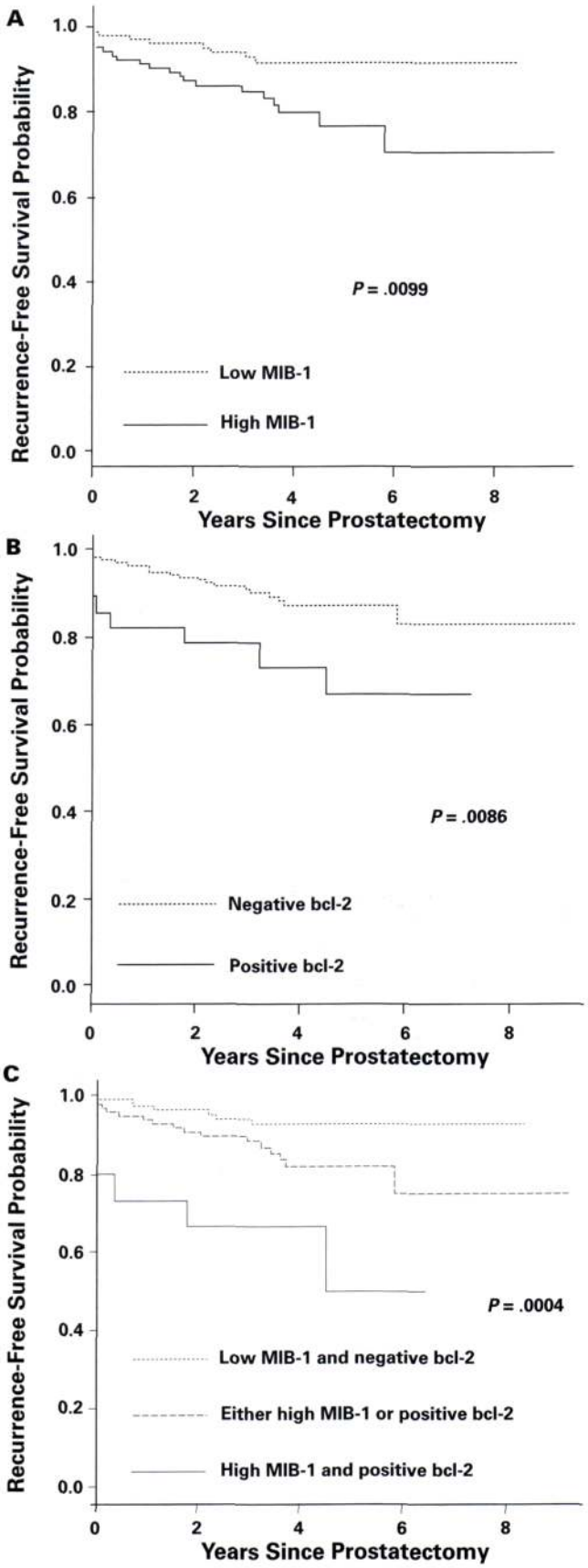

Figure 1 Relationship of recurrence-free survival with MIB-1 proliferative rate and bcl-2 positivity (Kaplan-Meier survival curves). A, MIB-1; B, bcl-2; C, MIB-1 and bcl-2.

Table 3
Model of Time to Recurrence Predicted by Multivariable Models

Variables in Model	Relative Risk	95% Confidence Interval of Relative Risk	*P*
Model 1			
Seminal vesicle involved	6.4	2.9–14	<.0005
MIB-1	2.4	1.1–5.5	.037
bcl-2	3.9	1.7–9.3	.002
Model 2			
Gleason tumor grade	2.7	1.2–5.7	.012
MIB-1	2.4	1.0–5.5	.042
bcl-2	2.7	1.2–6.2	.016

Of the molecular markers studied, p53 expression showed no significant association with recurrence-free survival, despite analysis using 2 different cutoff values for positivity. Previous reports have suggested that p53 overexpression may not be prognostically significant[34] or may be significant only in small subsets of prostate carcinoma[10–12,35] and more frequent in tumors that are already metastatic.[36,37]

The lack of pRB expression would be expected to correlate with poor prognosis. In this series, no tumors had totally absent pRB expression, and there were no substantial differences in expression between tumor cells and adjacent benign tissue. Assigning cutoffs at 5% and 20% tumor cell positivity showed no association in either case with recurrence-free survival.

On the other hand, a high MIB-1 proliferative rate and bcl-2 overexpression were strongly associated with decreased recurrence-free survival, not only when considered individually, but also when considered together and in models with the most predictive histopathologic factors, Gleason tumor score and seminal vesicle involvement. It is interesting that a marker for proliferation (MIB-1) and an inhibitor of apoptosis (bcl-2), which also would contribute to increased net proliferative capacity, show independent, additive, association with decreased recurrence-free survival.

An association between high proliferative rate and poor prognosis has been reported previously, using other antibodies directed against the Ki-67 antigen,[7] as well as MIB-1.[6] An association between bcl-2 overexpression and prognosis also has been reported, as well as an additive effect of high Ki-67 (MIB-1) labeling index and bcl-2 positivity.[16] However, in that study[16] bcl-2 did not provide additional prognostic information in multivariable analysis, whereas in the present study, the association of MIB-1 and bcl-2 with prognosis remained significant, even in the presence of other variables. This suggests that a high MIB-1 proliferative fraction and bcl-2 positivity could be used together as prognostic markers, in addition to determinations such as histopathologic tumor grade.

The known heterogeneity of prostate carcinomas is a potential limitation in this type of approach. The present study used tissue from radical prostatectomy specimens (rather than biopsies), so that there would be enough tissue to perform multiple immunohistochemical studies. Representative areas of tumor were chosen for study. In addition to heterogeneity of histopathologic tumor appearance, heterogeneity in intratumor distribution of p53 gene mutations has been reported.[38] Indeed, we observed heterogeneity of molecular markers in the single tissue block chosen for study, which we addressed by applying a consistent evaluation method. Ideally, one would want to apply prognostic marker analysis at the time of original diagnosis by prostate biopsy to help determine whether radical prostatectomy or other definitive therapeutic procedures with potentially severe adverse effects should be performed. Whether the findings from studies of radical prostatectomy tissues can be confirmed and extended to prostate biopsy specimens is a question for future study.

From the [1]John S. Sharpe Research Foundation and [2]Department of Pathology, The Bryn Mawr Hospital, Bryn Mawr, Pennsylvania; and the [3]Division of Biostatistics and Epidemiology, Department of Pediatrics, The Children's Hospital of Philadelphia, Philadelphia, Pennsylvania.

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Address reprint requests to Dr Keshgegian: Department of Pathology, The Bryn Mawr Hospital, 130 S Bryn Mawr Ave, Bryn Mawr, PA 19010.

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