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Original Research

DNA repair and apoptosis: Roles in radiotherapy-related acute reactions in breast cancer patients

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Abstract: Normal tissue reactions are therapy limiting factor for the effectiveness of the radiotherapy in cancer patients. DNA repair and apoptosis are estimated to be critical players of adverse effects in response to radiotherapy. Our aim was to define the association of DNA repair (*ERCC1* and *XPC*) and apoptotic (*BCL2, CASP3* and *NFKB1*) gene expression, DNA damage levels, apoptosis changes and DNA repair gene variations with the risk of acute side effects in breast cancer patients. The study included 100 women with newly diagnosed breast cancer; an experimental case group (n=50) with acute side effects and the control group (n=50) without side effects. Gene expression was analyzed by reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR). Micronucleus (MN) and 8-hydroxy-2′-deoxyguanosine (8-OHdG) assays were performed to compare the DNA damage levels. Apoptosis was examined by TDT-mediated dUTP-biotin nick end-labeling (TUNEL) staining. *ERCC1* rs3212986 and *XPC* rs3731055 polymorphisms were genotyped by real-time PCR technique. No significantly correlation of DNA repair and apoptosis gene expression and DNA damage levels with acute side effects in response to radiotherapy. Also, there was no association between apoptosis levels and acute effects. *ERCC1* rs3212986 CC genotype showed a protective effect against radiotherapy-induced acute reactions ($p<0.001$; OR: 0.21; 95% CI= 0.08-0.52). Our results suggest that apoptosis and DNA damage levels are not associated with acute radiosensitivity. DNA repair may affect the risk of acute reactions. Further studies are needed to validate the current findings.

Key words: Acute side effects; Apoptosis; Breast cancer; DNA damage; DNA repair; ERCC1; Radiotherapy.

Introduction

Radiotherapy is one of the most effective clinical treatment strategy in many human cancers. However, effectiveness of the radiotherapy is commonly limited by adverse reactions also called as early or late side effects. Molecular and cellular mechanisms of radiotherapy-induced acute or late normal tissue reactions (radiation toxicity) still remain elusive and findings appear contradictory (1, 2). Thus, understanding the biological consequences of normal tissue reactions will provide a strong pathway for possible reduction or elimination of normal tissue toxicities to therapeutic radiation. It is considered that genetic alterations in cell cycle checkpoints, DNA repair, cell growth and apoptosis pathways can cause the risk of side effects (1).

Ionizing radiation (IR), an essential form of irradiation used in radiotherapy, leads to DNA damage to kill the cancer cells. DNA damage response (DDR) is one of the most critical factor for cellular radiosensitivity. Activation of DDR pathways such as DNA repair and apoptosis is not only involved in tumor sensitivity and resistance, also plays key role for the individual radiosensitivity of normal tissues (3). Cells with IR-induced damaged DNA activate different DNA repair pathways and proteins. Nucleotide excision repair (NER) proteins play a key role in repair of IR-related DNA damage (4). Excision repair cross-complementing group 1 (ERCC1) and xeroderma pigmentosum complementation group C (XPC) are critical proteins of NER pathway. *ERCC1* gene is localized on chromosome 19q13. ERCC1 protein is complexed with XPF and cleaved damaged DNA from 5' site (5). Chromosomal location of *XPC* is 3p25. XPC protein recognizes the region of damaged DNA (6). IR induces the activation of several DNA repair genes including *ERCC1* and *XPC* (7).

Several micro array studies revealed that apoptotic gene expression alterations associate with radiotherapy-induced normal tissue reactions (8, 9). Also, it has been shown that reduced levels of apoptosis correlate with clinical radiosensitivity (10). Numerous essential pro-apoptotic or anti-apoptotic genes such as *CASP3*, *NFKB1*, and *BCL-2* can play an important role in the development of normal tissue toxicity. *CASP3* gene is localized on chromosome 4q35.1. Casp3 protein activates caspase cascade for apoptosis induction. Chromosomal localization of *NFKB1* is 4q24. NFKB1, known to antagonize apoptosis, promotes cell survival and proliferation. Chromosome band of *BCL-2* is 18q21.33. Bcl-2 protein blocks the apoptotic death.

Susceptibility to acute reactions in breast cancer patients is not well understood. The aim of the current study is to define the biological consequences of DNA repair and apoptosis changes in lymphocytes of breast cancer patients.

Materials and Methods

Study population

This study included 100 women with breast cancer who were newly diagnosed at the Department of Clinical Radiation Oncology, Cerrahpasa School of Medicine, Istanbul University. Heparinized peripheral blood samples were collected from all the patients who had not any treatment before taking blood samples. These patients were treated with radiation at targeted-doses of 50-66 Gy (2 Gy dose fraction) by using 3D conformal radiotherapy at the Department of Clinical Radiation Oncology, Cerrahpasa School of Medicine. During six weeks, adverse side effects were followed weekly and graded by using common toxicity criteria (C.T.C.): Grade: 0 (no adverse effect), 1 (mild adverse effect), 2 (moderate adverse effect), 3 (severe side effect) and 4 (life-threatening adverse effect). Patients were divided into two groups according to grades: Patients with C.T.C. grades 2, 3, and 4 were the experimental case group, which consisted of 50 patients. Patients with C.T.C. grades 0 and 1 were the control group, which consisted of 50 patients. All of the patients of the experimental case group had acute skin reactions. Demographic and clinical characteristics of patients were obtained from the Department of Radiation Oncology. Informed consent was obtained from all patients. The study was conducted with approval of the ethical committee of our institution and in keeping with the guidelines of the Declaration of Helsinki.

Lymphocyte isolation from peripheral blood

Lymphocyte isolation is performed as described previously (11). Briefly, heparinized blood samples were diluted with phosphate buffered saline (PBS) in 1:1 ratio. Diluted blood samples were separated on histopaque (1077 g/ml, BioChrom AG, Berlin, GE) and then centrifuged at 600 x g, 21°C for 35 minutes. After centrifugation, supernatant was aspirated off and lymphocytes washed with PBS at 600 x g, 21°C for 10 minutes. Viable cell counting was determined with hemocytometer using trypan blue dye. Lymphocytes were suspended with the freezing medium which includes 10% dimethyl sulfoxide (DMSO, Applichem GmBH, Darmstadt, GE), 60% fetal bovine serum (BioChrom AG, Berlin, GE), and 30% RPMI 1640 medium (without L-glutamine, with 2g/l $NaHCO_3$, BioChrom KG, Berlin, GE) and aliquoted as $2x10^6$ lymphocyte/ml to each cryo tubes. Each aliquots are stored at -80°C for next analysis.

Cell culture

Lymphocytes were cultured in ready to use complete medium (with phytohemagglutinin (PHA) L, BioChrom AG, Berlin, GE) in a humidified atmosphere at 37°C in 5% CO_2.

Irradiation of lymphocytes

For gene expression analysis, culture cells were irradiated with *in vitro* 2 Gy γ-irradiation (cobalt 60 γ- rays) in 1.0 Gy min^{-1} dose rate at room temperature. Irradiated cells were incubated for 2 h., at 5% CO_2 in 37°C. For MN assay, culture cells were irradiated with *in vitro* 2 Gy γ-rays (cobalt 60 γ- rays) in 1.0 Gy min^{-1} dose rate at room temperature and followed by incubation for 72 h., at 5% CO_2 in 37°C (12). For apoptosis assay, culture cells treated with *in vitro* 2 Gy gamma-irradiation at room temperature and incubated for 24h., at 5% CO_2 in 37°C.

Reverse transcriptase- quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from cultured, irradiated cells by using GeneJET RNA Purification Kit (Thermo Sci.) 1 µg total RNA was transcribed into cDNA using Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, Mannheim, GE) as per the manufacturer's instructions. RT-qPCR analysis of each cDNA sample was performed using TaqMan probe technology on a LightCycler 1.5 detection system (Roche Diagnostics, Mannheim, GE) and normalized to ACTB. Relative mRNA expression alterations were assessed by using $2^{-\Delta Ct}$ threshold method.

Genotyping

The SNPs in *ERCC1* (rs3212986) and *XPC* (rs3731055) genes were determined by melting curve analysis in LightCycler 1.5 (Roche Diagnostics, Mannheim, Germany). Primers and probes were designed and synthesized by TibMolBiol (Berlin, Germany). PCR reaction and melting curve analysis were performed in 10 µl volumes in LightCycler capillaries: 100 ng of genomic DNA, 0.5 µM of each primer, 0.15 µM of each hybridization probe, 3 mM of $MgCl_2$, and 1x LightCycler FS DNA Master HybProbe mix (Roche, Mannheim, Germany).

Micronucleus assay

Briefly, 2 Gy γ-irradiated lymphocytes were incubated with complete medium (with phytohemagglutinin (PHA) L, BioChrom AG, Berlin, GE) in a humidified atmosphere containing 5% CO_2 at 37°C for 72 h. Cytochalasin B (final concentration 6 µg/ml) was added to the culture after 44 h and the cells were further incubated for 28 h. Cells were harvested, treated with Carnoy's fixative (methanol:glacial acetic acid, 3:1, v/v) and fixed for 10 min. Cell suspensions were then placed on glass slides drop by drop and glass slides were incubated at room temperature overnight. Glass slides were stained with 5% Giemsa solution. The MN frequencies in 1000 binucleated cells per sample were scored under a light microscope (13).

8-OHdG Assay

Cells treated with 2Gy γ-irradiation incubated containing 5% CO_2 at 37°C for 24h. Post 24h of incubation, quantitative oxidative DNA damage in cells was determined by ELISA 8-hydroxy-2'-deoxyguanosine (8-OHdG) kit (OxiSelect™ Oxidative DNA Damage ELISA Kit (8-OHdG Quantitation), Cell Biolabs, INC.) according to the manufacturer's protocol.

TUNEL staining for apoptosis detection

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, a technique, in which DNA fragmentation is detected by labeling the terminal end of nucleic acids, has also been performed to confirm the apoptosis of cells.

For TUNEL staining, cell slides were stained with the

reagents supplied by ApopTag Peroxidase In Situ Apoptosis Detection Kit (ApopTag Plus, In Situ Apoptosis Detection Peroxidase kit, S7101-KIT, Chemicon). TUNEL staining procedures were carried out following the manufacturer's instructions. Briefly, cells treated with 2 Gy gamma-irradiation and incubated for 24h. After incubation, cells were suspended in 4% formaldehyde and fixed at room temperature for 30 min. Fixative solution was removed by centrifugation, 3000xg; 1 min. Cell pellet washed with PBS (pH 7.4) once and incubated in TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (TdT) for 1 h in a humidified chamber at 37^0C. Then 3,3-diaminobenzidine (DAB) chromogen was applied. DAB substrate was used to detect sites of in situ apoptosis under a light microscope. Methyl green was used as counterstaining. For negative control, TdT was omitted from the reaction mixture. The apoptotic signal was recorded as positive when stained the nucleus brown. Apoptotic cell counting (TUNEL) for all groups was performed under high-power fields (original magnification 40×) with a light microscope (Leica DM 2500 light microscope, Wetzlar, Germany).

Statistical Analysis

Statistical analyses were performed using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). Normal distribution of data was analyzed by Kolmogorov-Smirnov normality test. Continuous values were analyzed by Student's t test, and variables showing unequal variance or departing from normal distribution were analyzed by Mann-Whitney U test. Genotype and allele frequencies were analyzed by Pearson's Chi-square (χ^2) or Fisher's exact test (two-sided). The odds ratio (OR) and their 95% confidence intervals (CIs) were calculated to estimate the strength of the association between polymorphism genotype alleles and experimental cases and controls. All data are presented as mean plus or minus the standard error (S.E.) or standard deviation (S.D.). The level of significance was accepted as $p<0.05$.

Results

Demographic and clinical characteristics of breast cancer patients

Basic demographic and clinical characteristics of experimental and control patients are shown in Table 1. There were not any significant associations between these two groups in terms of demographic and clinical characteristics. Whereas, clinical characteristics, including nodal status and HER2 should be considered a borderline result between experimental case and control groups in terms of statistical significance.

Gene expression levels

We did not find any significant difference of DNA repair and apoptotic mRNA expression levels between experimental case and control groups ($p>0.05$) (Table 2).

Genotyping

Table 3 shows the genotype distributions of *ERCC1* rs3212986 (C>A) and *XPC* rs3731055 (C>T) promoter gene polymorphisms in experimental case and control groups. We found a significantly difference between two groups in terms of *ERCC1* rs3212986 polymorphism ($p<0.001$). *ERCC1* rs3212986 CC genotype plays a pro-

Table 1. Patients' characteristics.

	Experimental Case n(%)	Control n(%)	*P*
Age / years,			
Mean ± S.E.	48.7 ± 1.6	49.7 ± 1.5	0.76[a]
Range	23-74	30-70	
Tumor type, n %			
Ductal	41 (82)	34 (68)	0.25[b]
Lobular	3 (6)	4 (8)	
Other	6 (12)	12 (24)	
Tumor size, cm, n %			
<3	34 (68)	30 (60)	0.53[c]
≥3	16 (32)	20 (40)	
Histological grade, n %			
G1	0 (0)	1 (2)	0.28[b]
G2	9 (18)	14 (28)	
G3	41 (82)	35 (70)	
Nodal status, n %			
N0	27 (54)	17 (34)	0.07[c]
N+	23 (46)	33 (66)	
ER, n %			
Positive	39 (78)	37 (74)	0.81[c]
Negative	11 (22)	13 (26)	
PR, n %			
Positive	37 (74)	34 (68)	0.66[c]
Negative	13 (26)	16 (32)	
Her2, n %			
Positive	10 (20)	19 (38)	0.08[c]
Negative	40 (80)	31 (62)	

ER, Estrogen receptor; *PR*, Progesterone receptor; *Her2*, Human epidermal growth factor receptor. S.E., Standard error. [a]Mann-Whitney U test. [b]Fisher's exact test. [c]Pearson's Chi-square test

Table 2. Relative gene expression levels in experimental case and control groups.

Gene Expression	Experimental Case*	Control*	*P*[a]
BCL2	0.88 ± 0.06	0.78 ± 0.06	0.14
CASP3	1.30 ± 0.38	1.4 ± 0.36	0.77
NFKB1	6.56 ± 1.09	6.71 ± 0.77	0.37
ERCC1	0.80 ± 0.14	0.82 ± 0.13	0.19
XPC	1.50 ± 0.14	1.58 ± 0.13	0.43

The results are presented as mean ± S.E. [a]Mann Whitney U-test. *The values indicate the mean gene expression levels of irradiated cells

Table 3. Distributions of XPC and ERCC1 genotypes in experimental case and control groups.

		Experimental Case (n)	Control (n)	*P*	OR (95% CI)
ERCC1	**CC**	19	37	Ref.	
rs3212986	**CA**	25	10	<0.001[a]	0.21 (0.08-0.52)
	AA	6	3	0.08[b]	0.26 (0.06-1.14)
XPC	**CC**	49	49	Ref.	
rs3731055	**CT**	1	1	1.00[b]	1.00 (0.06-16.4)
	TT	0	0		

[a]Pearson's Chi-square test. [b]Fisher's exact test

tective role against acute side effects in breast cancer patients. However, we did not find any significant association of *XPC* genotype distributions in experimental case and control groups (p=1.00).

Micronucleus frequencies and serum 8-OHdG Levels

We did not find any significant difference of MN frequency between experimental cases (Mean±S.D; 6.8±4.2) and controls (Mean±S.D; 6.9±2.6) (p=0.96). Also, we did not observe any significant difference of serum 8-OHdG levels between experimental case (Mean±S.D; 1.2±0.6 ng/ml) and control groups (Mean±S.D; 1.1±0.6 ng/ml) (p=0.57).

Apoptosis analysis

When we analyzed the apoptosis levels of patients with and without acute side effects, we observed increased apoptotic cell counts in experimental case (Mean±S.D; 6.9±4.8) (Fig. 1) compared to control (Mean±S.D; 4.8±4.2) (Fig. 2). But, this difference was not statistically significant (p=0.23). Cells with nuclei that stained dark brown were considered to be TUNEL-positive. Counterstaining: Methyl green.

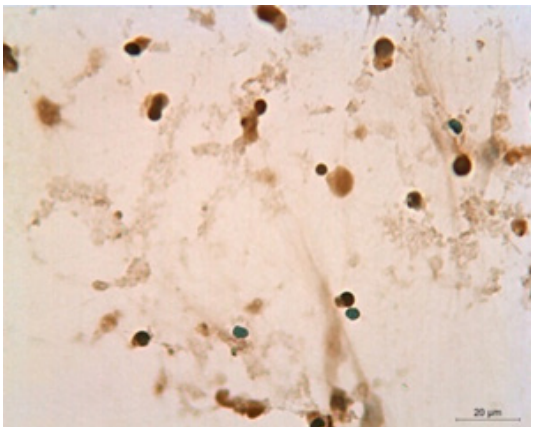

Figure 1. TUNEL staining of an experimental case. Bar, 20 µm.

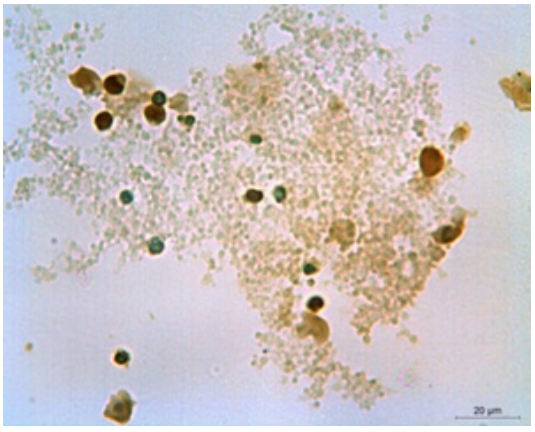

Figure 2. TUNEL staining of a control. Bar, 20 µm .

Discussion

Understanding the genetic factors in radiotherapy-related normal tissue reactions may provide an important information to reduce or prevent adverse reactions in breast cancer patients. Recently, several studies defined some factors affecting the risk of adverse reactions. However, the relationship between DNA repair and apoptotic pathways and the risk of acute side effects is not well understood.

The present study showed that radiotherapy-induced acute normal tissue reactions were not associated with the levels of apoptosis-related *BCL2, CASP3, NFKB1* and DNA repair-related *ERCC1* and *XPC* gene expression and apoptosis in breast cancer patients. Also, MN and 8-OHdG DNA damage levels seemed almost similar in experimental case and control groups. In addition to these results, we found that *ERCC1 rs3212986* CC genotype might protect patients against early adverse effects.

Radiation therapy increases the apoptosis (14). If apoptotic cells are not removed, it can develop toxicity and adverse effects due to presence of intracellular contents (15). Thus, apoptosis studies can give promising results to define the risk of radiation-related normal tissue reactions. Several studies determined altered apoptosis profiles after in vitro radiation in various cancer patient cohorts with enhanced radiotoxicity (16, 17). Ozsahin et al. found a reduced apoptotic response in cancer patients with radiation-induced adverse effects (18). However, similar to our findings, some research groups did not find any correlation between clinical radiation sensitivity and apoptosis levels (17, 19).

DNA damage levels and DNA repair activity are pro-

posed mechanisms to provide information on the development of radiotoxicity. MN levels, one of the cytogenetic DNA damage assays, are frequently used to assess the radiosensitivity *in vitro*. Several studies have investigated the possible association between MN frequency and normal tissue toxicity in various cancer patient cohorts (20, 19). Especially, many studies showed positive correlation in breast cancer patients (21, 22). In accordance with our current results, some studies did not find any significant correlation (19, 23). Also, our previous study on a small size of breast cancer patients with and without acute effects did not show markedly differences in terms of MN frequency (24).

Furthermore, different DNA damage markers, such as 8-OHdG levels and especially γ-H2AX/53BP1 foci numbers or intensity are analyzed to define the risk of radiotherapy-related side effects. IR can produce 8-OHdG which is repaired by NER (25). It is proposed as a marker of radiosensitivity (26). Our current data suggested that 8-OHdG levels did not correlate with acute adverse effects. However, in a study by Haghdoost et al., radiosensitive breast cancer patients showed increased levels of 8-OHdG (27). Werbrouck et al. did not find any association between γ-H2AX/53BP1 foci formation and normal tissue reactions in gynecological cancer patients (28). Conversely, the study of Vandevoorde et al. showed significantly positive correlation between DNA damage levels (γ-H2AX/53BP1 focus levels) and adverse effects (29). In accordance with Vandevoorde and colleagues' findings, the other study reported the association of DNA damage levels (γ-H2AX/53BP1 foci numbers) with normal tissue reactions in breast cancer patients (22).

It is well known that patients with ataxia telangiectasia (AT) and other chromosomal breakage syndromes have genetic defects in DNA repair pathway. And these patients show hyper sensitivity to ionizing radiation (30, 31). In light of this evidence, it is also focused on defining the prediction to radiotoxicity in terms of DNA repair function. It is estimated that susceptibility to radiotherapy-induced adverse effects can be due to expression levels and genetic variants of DNA repair genes (32). Our previous results reported that reduced BER pathway DNA repair gene XRCC1 expression was associated with acute side effects in breast cancer patients (24), while the current study indicated that NER pathway DNA repair genes *ERCC1* and *XPC* expression did not significantly correlate with acute side effects. There are many studies which investigated the association of DNA repair gene expression levels with normal tissue reactions. Hümmerich et al. showed that increased mRNA expression of several DNA repair-related genes (*ERCC1, RAD52, ATM*, and *TP53*) play a protective role against acute side effects in prostate cancer patients (33). In contrast to these results, Wiebalk et al. found that increased XPC mRNA expression might be a biomarker for the risk of acute side effects in prostate cancer patients (7).

All these contradictory results can be explained by numerous factors, such as characteristics or numbers of the study groups and differences in the experimental set up.

Furthermore, functional genomic approaches, such as microarray technology were performed for defining the molecular mechanisms of normal tissue sensitivity to radiotherapy. These studies aimed to detect target genes and expression levels of these genes which are possibly responsible for radiation therapy-induced normal tissue reactions. Particularly, DNA repair, apoptosis, cell cycle, and growth factor genes were investigated in these studies. Gene expression profiles were obtained from in vitro irradiated lymphoblastoid cell lines or peripheral blood lymphocytes. These studies showed that the alterations of DNA repair, cell cycle, apoptosis, or RNA processing-related gene expression levels could affect the risk of radiotherapy-related normal tissue reactions (8, 34).

Studies investigating the association between DNA repair gene polymorphisms and development of acute side effects reported conflicting results. Mangoni et al. showed that the risk of acute toxicity was markedly increased variant carriers of *XRCC3* (double strand break repair gene) Thr241Met (35). Also, it is reported that increased risk of radiosensitivity was related with *XRCC3* polymorphisms (36). Two studies found an association of *XRCC1* 399Gln variant with increased risk of side effects (37, 38). However, Sterpone and colleagues did not find any relation with acute normal tissue reactions in terms of *XRCC1* polymorphism (39). In our current study, we found a significant association of *ERCC1*, functions in NER pathway, promoter polymorphism with acute side effects, but no relation for *XPC*, functions in NER. However, other research group suggested a predictive role of *XPC* polymorphism (Lys939Gln, rs2228001) for acute toxicity in bladder cancer patients (40). These all results demonstrated that numerous DNA repair pathway gene polymorphisms might be critical factor for adverse effects, but it is needed further studies to define their roles clearly.

In conclusion, our findings revealed that variation in the *ERCC1* gene is associated with the risk of acute side effects in breast cancer patients. However, we did not find any significant correlation between DNA repair and apoptotic gene expression levels and radiotherapy-induced normal tissue reactions. Also, we observed that DNA damage levels did not affect the development of radiosensitivity. Furthermore, we found a borderline significance for HER2 and Nodal status between experimental case and control groups. These two clinical parameters could show statistical significance in future studies with larger sample size. Small study group is limiting factor for the current study.

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Conflict of interest
The authors declare that they have no potential conflict of interest relevant to this article.

Author's contribution
BB and MG wrote the manuscript. MA and NB provided the materials. BB, TM, and MB performed the experiments. BB, MG, and MT analyzed the data. All authors discussed and edited the manuscript. All authors read and approved the final manuscript.

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