Methylation of The Promoter of Survivin Gene May Affect Immunohistochemical Expression of Survivin Protein in Lung Cancers

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Cite this article as: *Budak M. Methylation of The Promoter of Survivin Gene May Affect Immunohistochemical Expression of Survivin Protein in Lung Cancers. Cerrahpasa Med J 2019; 43(2): 44-49.*

Abstract

Objective: In the present study, we aimed to investigate the promoter region of the survivin gene adenoma and squamous type lung cancer. Squamous cell carcinoma and adenocarcinoma are the two major histologic types of nonsmall cell lung cancer. Survivin is one of the most important apoptosis inhibitor proteins. As an inhibitor of programmed cell death, this protein is involved in chromosome separation during mitosis and is responsible for drug and radiation resistance against cancer treatments.

Method: We investigated the promoter region of the survivin gene by means of methylation-specific Polymerase Chain Reaction (PCR) and evaluated its impact on survivin protein expression following DNA isolation and bisulphite modification in paraffin-embedded normal and tumor tissues of lung cancer patients with squamous cell carcinoma and adenocarcinoma.

Results: We detected methylation in the promoter region of the survivin gene in normal and tumor tissues of 24 cases (27%) out of the 78 patients (adenocarcinoma, n=37; squamous cell carcinoma, n=41) included in our study. Among these cases, decrease in methylation by 20%–50% from normal tissues to tumors was observed. Twenty-one cases consisted of 11 adenomas (32% of adenoma cases) and 10 squamous cell tumors (27% of squamous cases).

Conclusion: These differences were statistically significant. In addition, a significant increase in survivin expression was observed immunohistochemically in adenoma and squamous type tumor tissues of lung. Survivin protein expression was found to be inversely related to promoter methylation. In this study, promoter methylation was observed for the survivin gene, which was considered an early potential marker in lung cancers. This data supports that further investigations may study survivin protein and methylation as a candidate marker for lung cancer.

Keywords: Survivin, methylation, lung cancer, squamous, adenocarcinoma

Cerrahpasa Med J 2019; 43(2): 44-49

Survivin Geninin Promotör Metilasyonu, Akciğer Kanserlerinde Immünohistokimyasal Olarak Survivin Gen Ekspresyonunu Etkileyebilir

Öz

Amaç: Bu çalışmada adenoma ve skuamöz tip dokulu akciğer kanser vakalarında survivin geninin promotör bölgesinin metilasyonunu ve gen ekspresyonuna etkisini araştırmayı amaçladık. Adenokarsinom ve skuamöz tip karsinomlar akciğer kanserlerini en sık rastlanan türleridir. Survivin geni en önemli apoptoz inhibitor proteinlerinden biridir. Bir programlı hücre ölümü engelleyici olan bu protein, mitoz esnasında kromozom ayrışmasında görev yapar ve kanserleşme sürecinde tedavilere karşı oluşan ilaç direncinden ve radyoterapik dirençten sorumludur.

Yöntem: Survin promotör metilasyonunu araştırmak için DNA'ya bisülfit modifikasyon sonrası metilasyon spesifik PCR ve survivin gen ekspresyonuna etkisini gösterebilmek için tümör dokularında immünohistokimyasal yöntem uygulanmıştır.

Bulgular: Çalışmamız sonucunda 78 (adenokarsinom n:37, skuamöz hücreli karsinom n:41) vaka arasından toplam 24 (%27) vakada survivin geninin promotör bölgesinde, normal ve tümör dokusunda metilasyon varlığı tespit edildi. Bu vakalarda normal dokudan tümöre doğru metilasyonda yaklaşık %20 ila %50 oranında bir azalma saptandı. Yirmi bir vakanın 11'i adenom (adenom vakalarının %32'si), 10'i skuamöz hücreli tümörlerde (skuamözlerin %27'si) görüldü.

Sonuç: Bu farklar istatistiksel olarak anlamlıydı. Ayrıca tümör dokularında immunohistokimyasal olarak survivin ekspresyonunda anlamlı bir artış gözlendi. Survivin protein ekspresyonunun promotör metilasyonu ile ters korelasyon sergilediği kaydedildi.

Anahtar Sözcükler: Survivin, metilasyon, akciğer kanseri, skuamöz, adenokarsinom

Cerrahpaşa Tıp Derg 2019; 43(2): 44-49

Received/Geliş Tarihi: 5 July 2019 **Accepted/Kabul Tarihi:** 17 September 2019
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DOI: 10.5152/cjm.2019.19003

Types of lung cancer are among the cancers with the highest incidence globally. Approximately 90% of these cases consist of nonsmall cell lung cancers and adenocarcinomas [1]. Lung cancers occur worldwide with a high mortality rate and approximately

80% of these cancers are classified as nonsmall cell lung cancer (NSCLC). NSCLCs have two subtypes; nonsquamous and squamous [2, 3]. NSCLCs are further histologically classified as adenocarcinoma and squamous cell carcinoma. Adenocarcinomas and squamous carcinomas account for about 50% of all NSCLCs [2].

Survivin is a member of the family of proteins referred to as IAPs, which inhibit apoptosis by controlling the apoptotic mechanism at a critical stage. It is a protein that is particularly responsible for the development of resistance to anticancer treatments. The survivin gene is localized on chromosome 17 and produces a 16.5 kDa protein. Several studies to date have investigated this gene with an attempt to improve treatment efficacy in cancer. It is one of the research topics of interest, especially in studies on methylation, an epigenetic mechanism [4, 5].

Methylation is a mechanism characterized by the addition of a methyl group to the 5-carbon of cytosine, mainly in CpG gene sequences located in the promoter regions of genes. Studies have shown that a large proportion of mammalian genomes is in methylated state [6, 7].

Changes in methylation affect the expression of genes, and thus may either inactivate or activate genes. In this study, we aimed to determine possible methylation of the survivin gene in the promoter region of normal and tumor tissues in adenocarcinoma squamous cell cancer and cancers through by methylation-specific PCR, and to investigate the effect of survivin gene on the expression of the survivin protein.

Material and Methods

This study is a retrospective cross-sectional on survivin promoter methylations and survivin protein with immunohistochemical analysis of lung cancer cases was performed. Seven hundred and eighty slide samples in total were prepared for the study from 78 formalin-fixed paraffin-embedded tissue blocks with 10 each from histologically proven and graded as early, moderate, and advanced any lung tumor tissues and 5 of normal lung tissue in same cases. All tissues used in this study were obtained so that both paraffin biopsy and tissue archive of patients diagnosed with lung cancer in the Trakya University School of Medicine University Department of Pathology were used from 2007 to 2015 from the Pathology Department of Trakya University Hospital and the study was initiated upon the necessary permission from the local ethics committee. All of the tissues were evaluated pathologically by a medical pathologist according to the international lung cancer committee standards. Age, gender, histological type, and disease stage of the 78 cases included in the study are presented in Table 1-3.

DNA isolation from paraffin-embedded tissues

Sections from selected tissues were obtained as described previously, and DNAs were isolated [8, 9].

Table 1. Clinical characteristics of patients

Patient Information	Normal Cases (n=78)		Tumor Cases (n=78)	
	N	%	N	%
Age, years (mean±SD)	61±11			
≤53	43	55	43	55
>59	35	45	35	45
Gender				
Female	10	13	10	13
Male	68	87	68	87
Grade				
1			52	66
2			8	10
3			18	23

Table 2. Clinical characteristics of the patients with adenocarcinoma

Patient Information	Normal Cases (n=37)		Tumor Cases (n=37)	
	N	%	N	%
Age, years (mean±SD)	58±11			
≤59	24	54	24	54
>59	13	41	13	41
Gender				
Female	5	13.5	5	13.5
Male	32	86.5	32	86.5
Grade				
1			21	57
2			2	5.4
3			14	37.6

These DNAs were then analyzed at 280 nm and 260 nm wavelength via spectrophotometric method after DNA quantities were determined; and the samples were stored at +4 °C for further analysis.

Bisulphite modification was performed in accordance with the Sigma-Aldrich DNA modification kit protocol.

Table 3. Clinical characteristics of the patients with squamous cell carcinoma

Patient Information	Normal Cases (n=41)		Tumor Cases (n=41)	
	N	%	N	%
Age, years (mean ±SD)	55±11			
≤59	22	54	22	54
>59	19	41	19	41
Gender				
Female	7	17	17	17
Male	36	83	36	83
Grade				
1			23	56
2			3	7
3			15	37

Methylation analysis of the promoter of survivin

After sodium bisulphite modification of genomic DNA, samples were treated with bisulphite for methylation analysis of the survivin gene. By doing so, all unmethylated cytosines were transformed to uracil. Because this modification converts cytosines to thymine in unmethylated regions, the MethPrimer V1.1 beta (available on www.urogene.org) program was used for the region thought to be altered this way in order to determine the CpG sequences in the promoter of survivin and the methylation-specific primer sequences, and MSP (methylation-specific PCR) was performed by means of the protocols hereunder to investigate methylation (Figure 1).

Methylation-specific PCR (MSP) protocols

Methylation-specific primers for the promoter of the survivin gene region were as follows: Forward: TTTTATTTTTAGAAGGTCGCGG, Reverse: AAACTACCAAACAAAAAACAACGTC; Product Size: 164 bp, Tm: 77.4. Unmethylation-specific primers for the promoter of the survivin gene region were: Forward: TTTATTTTTAGAAGGTTGTGG; Reverse: AAACTACCAAACAAAAAACAACATC; Product Size: 163 bp, Tm: 72.3. PCR conditions consisted of initiation at 95 ˚C for 10′ followed by 95 ˚C 45′′, 55 ˚C 30′′, and 72 ˚C 30′′ x35 cycles, finally terminating at 72 ˚C for 5′. Methylated and unmethylated PCR media were: PCR buffer 1x; MgCl2: 2 mM; DMSO: 5% (v/v); dNTP: 12.5 mM; Primer Forward: 10 nM; Primer Reverse: 10 nM; Taq Polymerase: 1 U (5U/µL)(Fermentas; Massachusetts, USA) with thermocycler

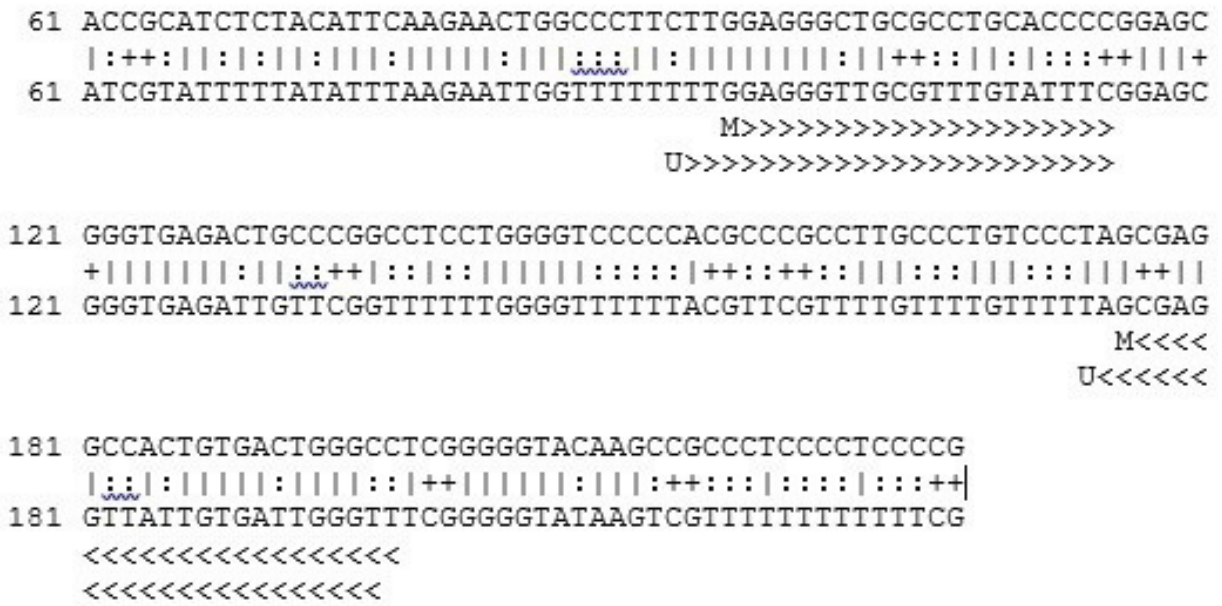

Figure 1. Upper row: Original sequence, Lower row: Bisulphite-modified sequence

(For display purposes, assuming all CpG sites are methylated) ++ CpG sites, :::: Non-CpG "C" converted to "T", M >>>>>> Left methylated-specific primer, M<<<<<< Right methylated-specific primer, U >>>>>> Left unmethylated-specific primer, U<<<<<< Right unmethylated-specific primer.

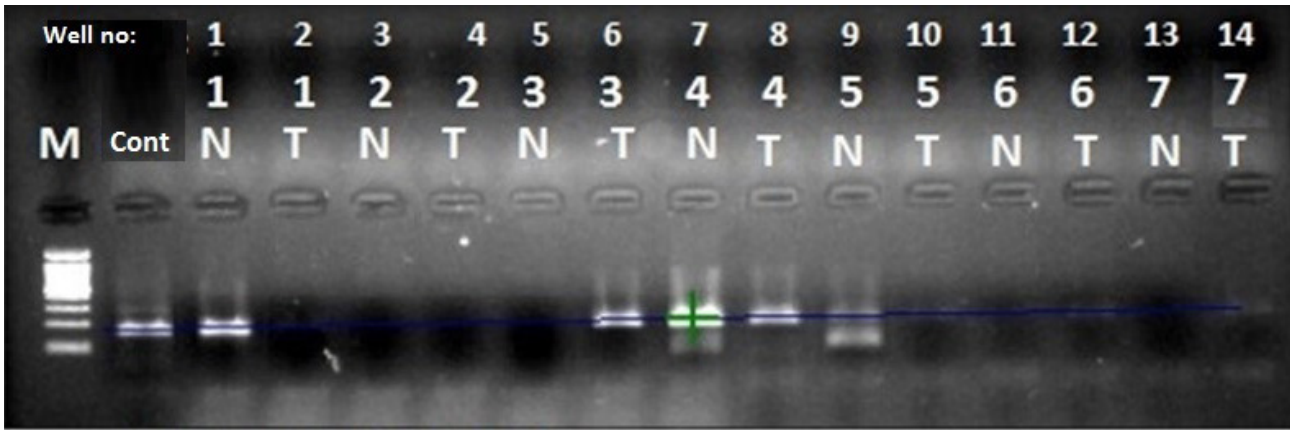

Figure 2. N, Normal; T, Tumor; Unmethylation-specific PCR result of exon 1 of survivin; Line 1: M: 100 bp marker; Line 2: (+) Control DNA, normal, and tumor pairs of the same cases in Lines 3–12, full non-methylation-PCR signal in N sample and T sample of the cases in Lines 3–12. Methylation-specific PCR result of survivin promoter; Line 1: M: 100 bp marker, Line 2: (+) Control DNA; methylation (–) in N sample; (–) in T sample of the cases in Lines 3–12

(Bioneer exicyclerTM 96, Daejeon, Republic of Korea). Template DNA 100 ng was filled up to 50 µL with dH2O. Methylated and unmethylated human DNAs were used as positive and negative control DNAs (S8001 | CpGenome™ Human Methylated & unmethylated DNA Standard Set, Fermentas, Massachusetts, USA). These DNAs underwent modification prior to PCR [8].

Polymerase Chain Reaction was performed with the above mentioned samples using primers designed as methylation-specific and unmethylation-specific. PCR products were subsequently assessed under ultraviolet (UV) light on 2% agarose gel [10], tumors and normal tissues were compared for methylation, and methylation rates were determined.

Immunohistochemical detection

We used an immunohistochemical method to detect the survivin protein. For this purpose, we obtained samples of 5 µL (+/–2 µL) on average on polylysine slides from 85 cases. Using an auto-analyzer (Ventana, Roche, USA), all of the sections were treated with survivin protein antibody using antibody detection kits (ultraView Universal DAB Detection Kit; Roche, Basel, Switzerland and Novus, Centennial, Colorado USA). For positive control, Glia astrocytes were used as recommended by the manufacturer's company data sheet. Internal control was used as negative control and unstained interstitial regions on case slides were accepted as negative. During the evaluation, the most dense areas on case slides were assessed semi-quantitatively and survivin presence was determined as % (per cent). According to this approach, interpretation was as follows: 0%–5% negative (–); 6%–25% (+)26%50% (++); 51%–75% (+++); 76%–100% (++++) [8] (Figure 2).

Statistical Analysis

Statistical analysis of the study was conducted using Statistical Package for the Social Sciences version 20 program (IBM Corp.; Armonk, NY, USA). Continuous variables were expressed as mean ± standard deviation (SD) and qualitative variables as a percentage. MethylationSpecific Polymerase Chain Reaction results of statistical analyses done were compared between the patient and the control group and their immnuhistochemical result by the $\chi 2$ test, $p<0.05$ was considered statistically significant [11].

Results

1–3 µL bisulphite-modified DNA was used per methylated and unmethylated PCR reaction. MSP and un-MSP products were assessed on 2% agarose gel stained with ethidium bromide (Figure 2, 3). The expected band size was 117 bp for MSP and un-MSP in the promoter of the survivin gene. Furthermore, immunohistochemical staining and analyses were performed on slides with sections derived from the samples used for PCR. Increased survivin protein expression was detected, showing reverse correlation with methylation of the promoter of survivin (Figure 4).

Expression of survivin in squamous cell carcinoma and adenocarcinoma

In samples obtained from patients with squamous cell cancer, which is the nuclear staining more dense the cytoplasmic (Figure 3). Weak-nuclear and cytoplasmic survivin expression was seen in alveolar epithelium (Figure 4). Furthermore, methylation in the promoter region of the survivin gene was observed in both bronchial and squamous tumor tissues of the same cases (Figure 2A, B).

Survivin protein was positive 12 of 14 in grade 3 adenocarsinoma tissues (85%), whereas in the group of 23 in grade 1-2 of adenocarsinoma tissues, survivin was positive 15 of them (65%). The statistical analysis

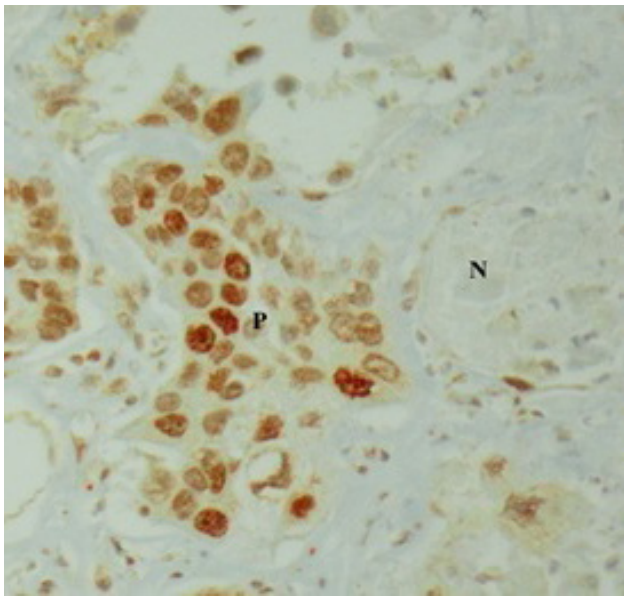

Figure 3. Heterogenic-strong-nuclear and few delicate shades of cytoplasmic survivin expression in squamous cell lung cancer. Immunohistochemical staining of survivin, ×100

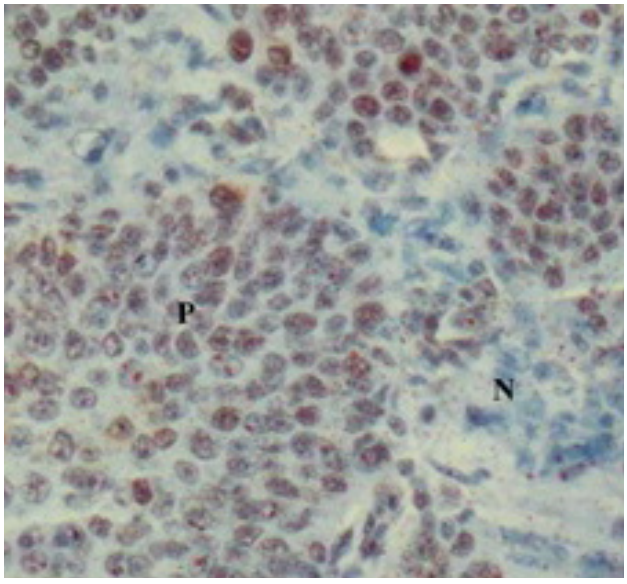

Figure 4. Few delicate shades of nuclear and cytoplasmic survivin expression in alveolar epithelium. Immunohistochemical staining of survivin, ×100

showed a significantly elevated survivin expression in high-grade adenocarsinoma while comparing to grade 1 lesions (p=0.01). Survivin protein expression was positive 11 of 15 in grade 3 (85%); whereas in the group of 21 low-grade squamous tissues was positive in nine of them (73.3%). Statistical analysis showed a significantly elevated survivin expression in grade 3 as compared to grade 1 and 2 squamous cell lesions.

In our previous study, survivin-positive nuclear staining was observed in 83% of squamous carcinomas, and cytoplasmic survivin expression was detected in 33% of same carcinomas. Expression of nuclear and cytoplasmic survivin was significantly higher in squamous carcinoma tissue as compared to alveolar epithelium ($p<0.01$). Nuclear survivin expression was significantly higher than cytoplasmic survivin expression as compared to normal tissues. Exon 1 methylation was not seen in the study [8]. However, the rate of methylation in the promoter region of survivin was found to be inversely proportional to expression of survivin methylation.

In addition, survivin promoter methylation was found to be present in approximately 70% of adenocarcinoma tumors, and un-methylation was observed in 30%–40% of the same tumors. While 70% un-methylation and 20% methylation were seen in normal lung tissues of the same cases, the level of survivin protein expression was increased, showing an inverse correlation with methylation ($p<0.05$).

Discussion

Survivin may be a good chemotherapeutic or radiotherapeutic target, especially owing to its role as inhibitor in apoptosis mechanisms. Studies on this gene are of interest, both for those investigating epigenetic properties and those seeking to clarify epigenetic inhibition mechanisms. The presence of an inverse relationship between apoptosis and anti-apoptosis signaling pathways, especially in the development of cancer, reveals the importance of activation or inhibition of these pathways [12-15].

While exon 1 methylations are seen in various genes, the survivin gene lacks exon 1 methylation and expression studies have shown that the promoter region of survivin affects gene expression. Furthermore, our study has demonstrated that different rates of methylation may be observed both in adenocarcinoma and squamous cell carcinoma of the lung. Although this may suggest that survivin gene activation inactivates anticancer treatments, thereby shortening life expectancy in lung cancers, there is currently only limited information in the literature in this regard.

Previous studies have simulated methylation with SurKex oligonucleotides for the methylation of the survivin gene promoter, showing the effect on survivin expression as well as on histone acetylation. Moreover, survivin methylation may not always show a negative effect on survivin expression and, interestingly, even increase the expression in endometrial tumors; however, the present study demonstrates decreased expression of survivin at different rates in both two most common types of lung cancer [16, 17]. Our study is one of the rare studies showing these methylations together in lung cancers and gene expression of the survivin promoter region.

In summary, further studies on survivin gene expression and inhibition mechanisms are expected to increase alternative therapeutic options in cancers where this gene is known to be active. In addition, we believe that silencing the survivin gene in the future may improve efficacy of currently available cancer treatments, and also show favorable effects on life expectancy and quality of life among patients with cancer. Survivin promoter methylation regions are likely to be promising therapeutic targets in the future.

Ethics Committee Approval: Ethics committee approval was received for this study from the Ethics Committee of Trakya University School of Medicine.

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

Acknowledgement: I thanked to Dr. Omer Yalcin for pathology examinations.

Conflict of Interest: The author has no conflicts of interest to declare.

Financial Disclosure: This study was supported by The Scientific and Technological Research Council of Turkey (Project No: 3501-113S158).

Etik Komite Onayı: Bu çalışma için etik komite onayı Trakya Üniversitesi Tıp Fakültesi Etik Kurulu'ndan alınmıştır.

Hasta Onamı: N/A.

Hakem Değerlendirmesi: Dış bağımsız.

Teşekkür: Patolojik incelemeler için Dr. Ömer Yalçın'a teşekkür ederim.

Çıkar Çatışması: Yazar çıkar çatışması bildirmemiştir.

Finansal Destek: Bu çalışma Türkiye Bilimsel ve Teknolojik Araştırma Kurumu tarafından desteklenmiştir (Proje No: 3501-113S158).

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