[CANCER RESEARCH 56, 3875–3878, September 1, 1996]

Advances in Brief

Inactivation of the *CDKN2* Gene by Homozygous Deletion and *de Novo* Methylation Is Associated with Advanced Stage Esophageal Squamous Cell Carcinoma[1]

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Abstract

We examined the genomic status of the *CDKN2* gene including *de novo* methylation of 5′ CpG islands in primary and metastatic tumor samples from 31 patients with esophageal squamous cell carcinoma. One somatic frame shift mutation (1 of 31; 3.2%) was identified by PCR-single strand conformational polymorphism analysis and DNA sequencing. Homozygous deletion and *de novo* methylation of the gene were confirmed in 5 (16%) and 6 (19%) of 31 patients, respectively. Homozygous deletion and *de novo* methylation were significantly associated with silencing of gene expression ($P < 0.01$). Aberrations of the *CDKN2* gene were detected in tumors with lymph node metastasis and muscular invasion (12 of 22; 54%) and in none of stage I tumors (0 of 9, 0%; $P < 0.05$). These results suggest that homozygous deletion and *de novo* methylation are predominant mechanisms of inactivation of the *CDKN2* gene and may be associated with metastatic and invasive phenotypes of esophageal squamous cell carcinoma.

Introduction

The *CDKN2* gene plays a key role in cell cycle regulation (1–3), and many groups have evaluated and discussed the significance of this gene in tumorigenesis. Reports of homozygous deletion of the *CDKN2* gene have accumulated in both cell lines and primary carcinomas (4). Recently, another mechanism of inactivation of the *CDKN2* gene, *de novo* methylation of the 5′ CpG island, has been reported to occur frequently in a variety of carcinomas (5–7). In ESCC,[3] it has been presumed that a point mutation followed by LOH is the predominant mechanism of inactivation of the *CDKN2* gene, similar to inactivation of the *p53* gene (8–10). The frequency of such mutations has varied among reports (8–11). Igaki *et al.* (10) have reported that homozygous deletion was detected in none of 25 primary ESCCs, although it has been found frequently in cell lines derived from esophageal carcinomas (12–14).

We searched for alterations of the *CDKN2* gene including *de novo* methylation in tumor samples from 31 patients with early or advanced stage ESCC, by using methods based on the PCR (PCR-SSCP, comparative multiplex PCR, and PCR-methylation assay). We found compelling evidence that homozygous deletions and *de novo* methylation occur frequently and are associated with lymph node metastasis and muscular invasion of ESCC.

Materials and Methods

Preparation of DNA and RNA. Thirty-one primary tumors from patients with ESCC were obtained from the surgical service of the Iwate Medical University School of Medicine. Twenty-two of them had lymph node metastasis and muscular invasion (stages II and III, according to the Tumor-Node-Metastasis staging system), and the remaining 9 did not (stage I). The histological type of all tumors was squamous cell carcinoma. Primary and matching metastatic tumors from lymph nodes and normal tissues were frozen immediately following surgery and stored at −80°C until use. Genomic DNA was extracted by proteinase K digestion and phenol/chloroform extraction. RNA was isolated from 10 of the 31 cases using Trisol reagent (Life Technologies, Inc.) and subjected to RT-PCR. Microscopic examination confirmed that all metastatic lesions contained >95% tumor cells.

PCR Amplification. Primer sequences for amplification of exon 1 and exon 2 of the *CDKN2* gene are shown in Table 1 (7, 15). Primer sets 1 and 3 were used for PCR-SSCP and comparative multiplex PCR (15), and set 2 was used for PCR-methylation assay (7). Set 4 was used for RT-PCR (7). One hundred ng of genomic DNA or cDNA were amplified in a total reaction volume of 20 μl containing 20 pmol of each primer, 1–1.5 mM $MgCl_2$, 10% DMSO, and 1 μl of [α-^{32}P]dCTP (3000 Ci/mmol, 10 Ci/ml). The PCR cycles and conditions, optimized for each assay, have been described previously (7, 15).

PCR-SSCP Analysis. Five μl of each PCR product were diluted 10-fold with gel-loading buffer [98% deionized formamide, 10 mM EDTA (pH 8.0), 0.025% xylene cyanol, and 0.025 bromphenol blue] and heated at 94°C for 2 min. For SSCP analysis, the samples were electrophoresed on a 6% neutral polyacrylamide gel, with or without 10% glycerol, at 20 to 40 W for 3 to 10 h.

Comparative Multiplex PCR for Detecting Homozygous Deletions and LOH of the *CDKN2* Gene. A primer set of exon 1 or 2 of the *CDKN2* gene and a set of the microsatellite markers as an internal control were included in the same PCR reaction as described above. Primers of *D9S171* (9p21), *IFNA* (9p22), and *DXS207* (Xp22.2) were obtained from Research Genetics (Huntsville, AL) and were used as controls. Samples of the amplified normal DNA were loaded on a polyacrylamide gel containing 7 M urea, along with the tumor DNA from primary and metastatic tumors. Using an image analysis system (Bio-Rad Labs, Hercules, CA), the ratio of the signal intensity of the *CDKN2* gene/control locus was calculated. A homozygous deletion was defined by the ratio of the signal in the tumor lane being less than 10% of that in the normal lane (16). This assay also defined LOH at *D9S171* (9p21) and *IFNA* (9p22) simultaneously.

PCR-Methylation Assay. We analyzed the methylation status of DNA by the modified PCR-based methylation assay (7). One μg of genomic DNA was digested for 4 h, with 10 units of methylation-sensitive (*Hpa*II, *Cfo*I, and *Sac*II; TaKaRa, Tokyo, Japan) and methylation-insensitive (*Msp*I) enzymes. DNA digestion was performed twice to rule out the possibility of an incomplete digestion. Each restriction site has been described previously (7). Fifty ng of digested DNA were amplified by PCR using the exon 1 primer of the *CDKN2* gene (set 2 in Table 1). The PCR mixture and conditions have been described previously (7). Labeling of the PCR products was performed directly with [α-^{32}P]dCTP during PCR. Five μl of PCR products were electrophoresed on a 5% polyacrylamide gel. Undigested and *Msp*I-digested DNAs were used as controls included for every site examined.

DNA Sequencing Analysis. The PCR products eluted from the shifted bands detected by SSCP analysis and those from the DNA of the primary

Received 5/21/96; accepted 7/16/96.

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[1] This work was supported by a Grant-in-Aid for Cancer Research (S6-32 and S7-1) from the Ministry of Health and Welfare, Japan.

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[3] The abbreviations used are: ESCC, esophageal squamous cell carcinoma; LOH, loss of heterozygosity; SSCP, single-strand conformational polymorphism; RT-PCR, reverse transcription-PCR.

Table 1 *Oligonucleotides and analysis methods for determining CDKN2 aberrations*

Set		Analysis
Set 1	GAA GAA AGA GGA GGG GCT G	PCR-SSCP, exon 1
	GCG CTA CCT GAT TCC AAT TC	
Set 2	AGC CTT CGG CTG ACT GGC TGG	PCR-methylation assay, exon 1
	CTG GAT CGG CCT CCG ACC GTA	
Set 3	CTG ACC ATT CTG TTC TCT CTG G	PCR-SSCP, exon 2
	CAT GGT TAC TGC CTC TGG TGC	
Set 4	AGC CTT CGG CTG ACT GGC TGG	RT-PCR, exon 1-2
	CTG CCC ATC ATC ATG ACC TGG	

tumors with LOH at *D9S171* or *IFNA* were sequenced directly using a terminator cycle sequencing kit (*Taq* DyeDeoxy; Applied Biosystems, Foster City, CA) and an automated DNA sequencer (model 373A; Applied Biosystems). The same primers (sets 1 and 3) were used in PCR amplification for SSCP analysis and cycle sequencing.

RT-PCR. Five hundred ng of total RNA were reverse transcribed using random hexamers and murine leukemia virus (Applied Biosystems, Foster City, CA). One hundred ng of cDNA were amplified as described previously (7). As an internal control, β-actin was also amplified (17). PCR products were electrophoresed on a 5% polyacrylamide gel, and the signal intensity was measured as described in the section on comparative multiplex PCR.

Results

A shifted band was detected in 1 of the 31 primary tumors by SSCP analysis (Fig. 1*A*). The tumor showed loss of heterozygosity at *D9S171* (Fig. 1B). The mutation was a one-base deletion at nucleotides 210–212 (CCC to CC; GenBank accession no. L27211) in exon 2 of the *CDKN2* gene and resulted in a truncated gene product (Fig. 1*C*). The PCR products of exons 1 and 2 of the *CDKN2* gene in five primary tumors exhibiting LOH at the *D9S171* or *IFNA* were sequenced directly, and no intragenic mutation was detected. Homozygous deletions were confirmed in 5 of the 31 patients (16%). All homozygous deletions were detected in metastatic lymph nodes (Fig. 2), whereas none of the primary tumors revealed homozygous deletions. *De novo* methylation was detected in 6 of 31 (19%) patients (Fig. 3). One of the six *de novo* methylations was found only in a metastatic lesion (ESQ-32), but the remaining five revealed *de novo* methylation in both primary and metastatic lesions (Fig. 3). We confirmed the methylation status of five cases including ESQ-32 by Southern blotting. The results were concordant with those of the PCR-methylation assay (data not shown). *De novo* methylation status and homozygous deletion were significantly associated with loss of mRNA expression of the *CDKN2* gene ($P < 0.01$; Fig. 3; Table 2). All three types of aberrations (point mutation, homozygous deletion, and *de novo* methylation) of the *CDKN2* gene were detected in tumors with lymph node metastasis and muscular invasion (12 of 22; 54%). The incidence of any alterations was significantly higher in tumors with lymph node metastasis and muscular invasion than in stage I tumors ($P < 0.05$; Table 3).

Discussion

We have found that inactivation of the *CDKN2* gene is frequently a result of homozygous deletion or *de novo* methylation. This finding is consistent with the report by Merlo *et al.* (5) and Herman *et al.* (6), which included squamous cell carcinoma of the head and neck. Previous reports have not examined the methylation status of the *CDKN2* gene in ESCC (9–14), and in addition, have failed to detect homozygous deletions (10). Although we also failed to detect homozygous deletions in primary tumors, a decrease in the signal intensity of the *CDKN2* gene was found in two of the five primary tumors exhibiting a homozygous deletion in metastatic lesions (20% decrease in ESQ-46 and 40% in ESQ-66). Metastatic tumors in lymph nodes exhibited an expansive growth pattern allowing easy extraction of tumor cell DNA, avoiding contamination by nonneoplastic cells. In contrast to multiplex PCR, the PCR-methylation assay is more sensitive (7). Positive signals were detected in five of six primary tumors exhibiting *de novo* methylation in metastatic lesions. Although the possibility remains that the PCR-methylation assay can give false-positive results, we performed DNA digestion twice to rule out the possibility of an incomplete digestion. Expression of mRNAs were well associated with the methylation status of the gene. These findings suggest that both homozygous deletion and *de novo* methylation occur in primary and metastatic lesions of ESCC.

Homozygous deletions were found more frequently in late-stage tumors. Walker *et al.* (18) have reported that homozygous deletions occurred in 13 of 25 (52%) grade III and 27 of 46 (58%) grade IV astrocytomas but in no grade II tumors. Okamoto *et al.* (19) have demonstrated that homozygous deletions occurred in 4 of 22 metastatic non-small cell lung cancers but in none of the primary tumors. The same results were confirmed in a large number of lung cancers by Nakagawa *et al.* (20) Kelly *et al.* (21). Loss of CDKN2 protein expression was confirmed immunohistochemically in 19 of 37 (52%) invasive and 10 of 14 (72%) metastatic melanomas but not in *in situ* lesions (22). Our study has also demonstrated that homozygous deletion and *de novo* methylation of the *CDKN2* gene were frequently detected in tumors with lymph node metastasis and muscular invasion. These observations suggest that disruption of the *CDKN2* gene produced by a homozygous deletion or *de novo* methylation might confer a growth advantage and play an important role in the acquisition of metastatic potential.

Global changes in DNA methylation patterns are known to occur during tumorigenesis resulting in inactivation of other genes such as

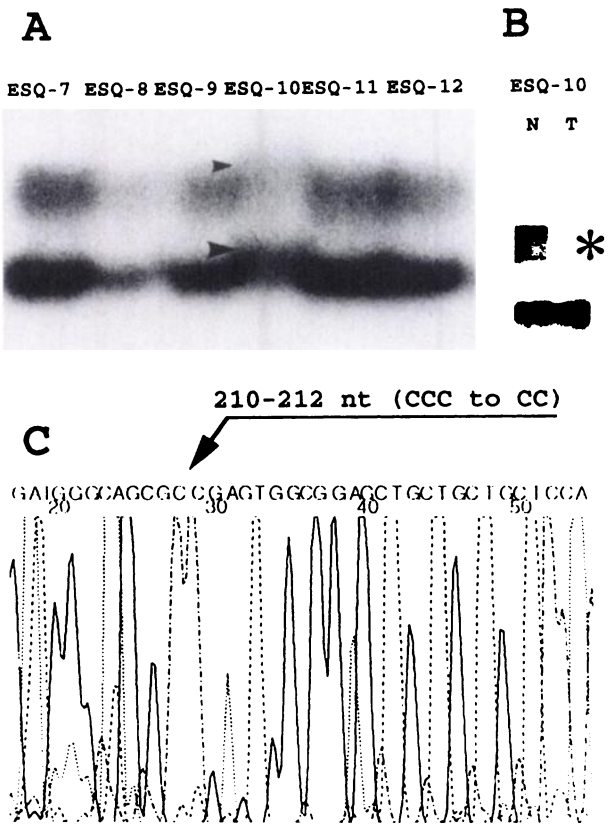


Fig. 1. SSCP analysis of exon 2 of the *CDKN2* gene in primary ESCCs. *A*, mobility shifts (*arrowheads*) detected in ESQ-10 (*A*). *B*, microsatellite analysis at *D9S171* in ESQ-10. *, presence of LOH. *N*, normal DNA; *T*, tumor DNA. *C*, sequencing histogram of exon 2 of the *CDKN2* gene. A one-base deletion at 210–212 (CCC to CC) is shown.

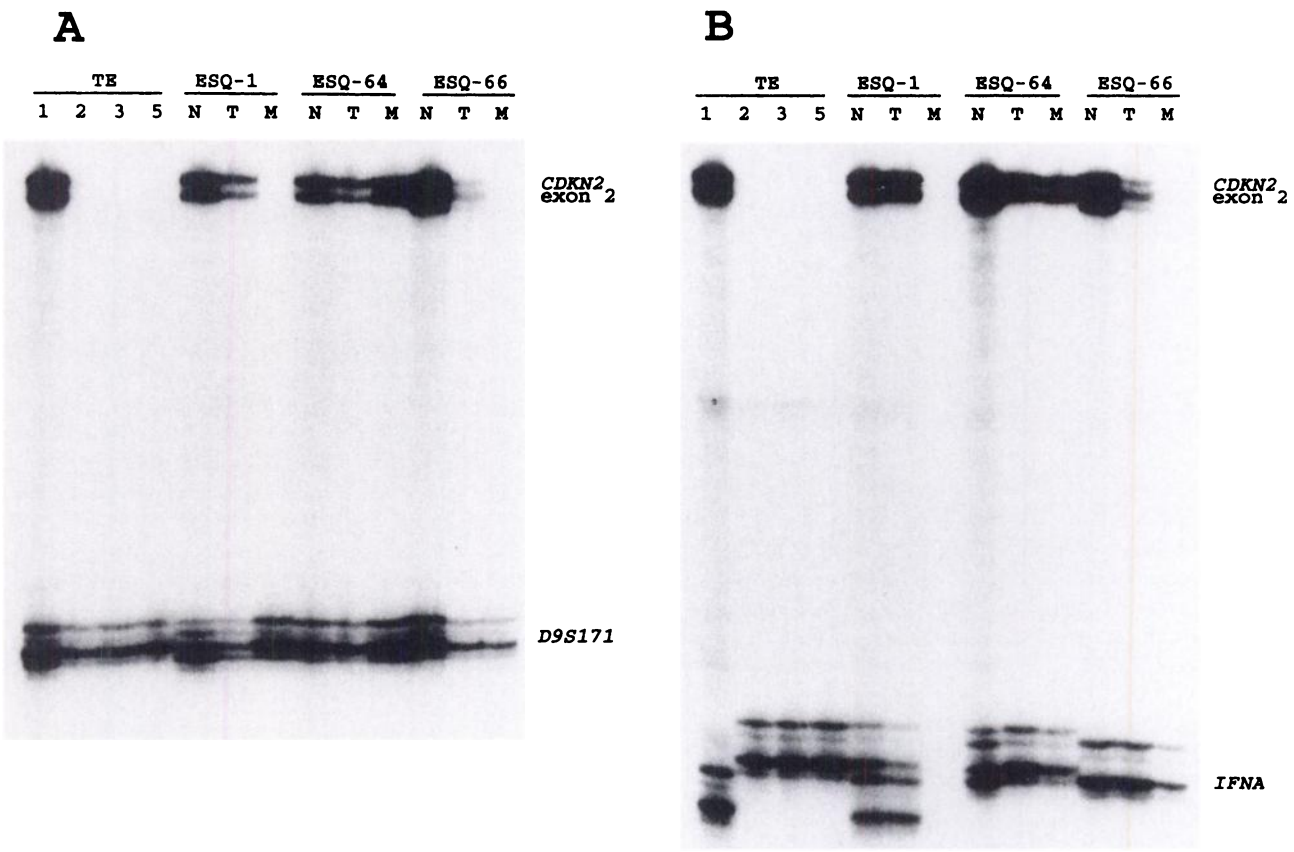
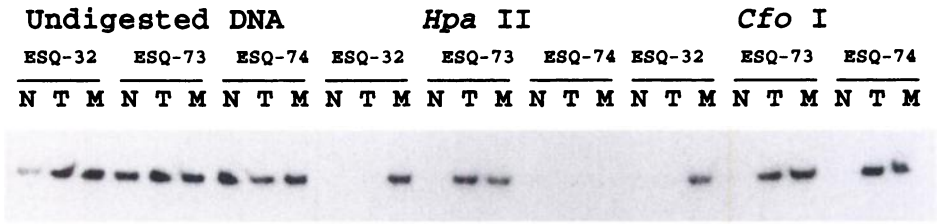


Fig. 2. Autoradiographies of multiplex PCR using *D9S171* (*A*) or *IFNA* (*B*) and exon 2 of the *CDKN2* gene. Homozygous deletions are observed in TE-2, TE-3, and TE-5 [Homozygous deletion was confirmed in these cell lines by Igaki *et al.* (12)] and in metastatic tumors of ESQ-1 and ESQ-66. LOH at *D9S171* and *IFNA* are detected in the primary tumor of ESQ-1 and at *IFNA* in primary and metastatic tumors of ESQ-64. *N*, normal DNA; *T*, primary tumor DNA; *M*, metastatic tumor DNA.



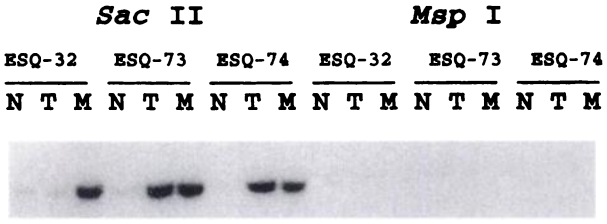


Fig. 3. PCR-methylation assay and RT-PCR expression analysis of the *CDKN2* gene in esophageal squamous cell carcinoma. *De novo* methylation is visible by dense signals in materials digested with methylation-sensitive enzymes (*Hpa*II, *Cfo*I, and *Sac*II). No positive signal is detected in materials digested with a methylation-insensitive enzyme (*Msp*I). *De novo* methylations occur at primary and metastatic tumors in ESQ-73 (*Hpa*II, *Cfo*I, and *Sac*II) and ESQ-74 (*Cfo*I and *Sac*II). In ESQ-32, *de novo* methylation occurs in only metastatic tumor (all enzymes). Expression of *CDKN2* mRNA is decreased in all tissues affected by *de novo* methylation.

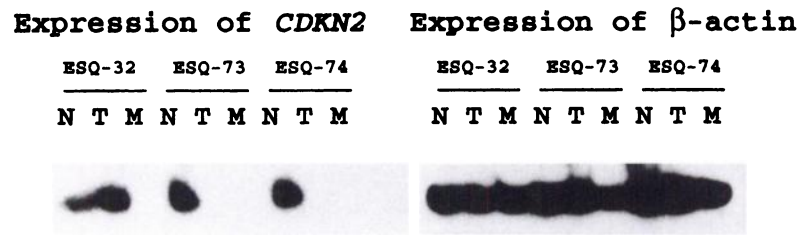


Table 2 *Correlation of CDKN2 gene expression with homozygous deletion or de novo methylation in ESCC*

	Homozygous deletion or *de novo* methylation	
	+	−
CDKN2 expressed	0	4
CDKN2 not expressed	6[a]	0[b]

[a] Including four methylated and two homozygous deleted samples.
[b] $P < 0.01$.

Table 3 *Correlation of CDKN2 aberrations with tumor stage of ESCC*

	CDKN2 aberrations	
	Present	Absent
Stage I	0	9
Stage II or III	12	10[a]

[a] $P < 0.05$.

p15 (7, 23), *E-cadherin* (24, 25), and *VHL* (26) in several human malignancies. We also examined the methylation status of *p15*, which was not always concordant with that of the *CDKN2* gene (data not shown). It is important to determine mutational, deletional, and methylation status of such genes and to examine the significance of these aberrations found in ESCC.

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Cancer Res 1996;56:3875-3878.