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Lymph Node Metastasis Is Associated with Allelic Loss on Chromosome 13q12–13 in Esophageal Squamous Cell Carcinoma[1]

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ABSTRACT

Allelotype analysis of whole chromosomes showed that loss of heterozygosity (LOH) on 13q was exclusively associated with lymph node metastasis and poor prognosis in esophageal squamous cell carcinoma (ESC). To identify a locus responsible for lymph node metastasis, we performed fine deletion mapping on 13q by analyzing 60 ESCs with 18 polymorphic markers. Allelic loss was observed with at least one marker in 34 tumors (56.7%), and lymph node metastasis was significantly correlated with LOH ($P = 0.0053$). We found frequent loss at *D13S260* (43.7%), *D13S171* (38.6%), and *D13S267* (43.6%) on 13q12–13. Among these markers, LOH at *D13S171* showed a significant correlation with lymph node metastasis ($P = 0.0441$). Because these markers flank the *BRCA2* gene, we intensively examined a mutation of the gene through all coding exons and exon-intron junctions by PCR-single-strand conformational polymorphism analysis under two different assays. We found only seven nucleotide substitutions as normal polymorphic changes; tumor-specific mutations were not detected, and loss of expression was not observed, indicating that the *BRCA2* gene might not be a target of allelic loss in this region. Relatively frequent LOH was also found at the *RB1* locus (34.7%), but a significant correlation with lymph node metastasis was not observed ($P = 0.7430$). Protein expression of *RB1* was examined in 31 ESC cell lines, and loss of expression was infrequent (6.5%), indicating that inactivation of the *RB1* gene might not be responsible for lymph node metastasis. Taken together, allelic loss at 13q12–13 of the primary ESC was closely associated with lymph node metastasis, and unidentified tumor suppressor gene(s) in this region might be involved.

INTRODUCTION

Esophageal cancer is the sixth most common cancer among males in Japan, and its prognosis is relatively poor, despite the recent advances in diagnosis and treatment (1, 2). To improve the prognosis by appropriate clinical approaches, it is very important to precisely evaluate the progression of cancer, including lymph node metastasis, at diagnosis. Because recent molecular genetic studies have revealed accumulations of genetic alterations associated with the development of ESC[4] (3–10), it is also important to know the molecular biological mechanisms regulating lymph node metastasis.

We have revealed highly frequent allelic loss on chromosome 3p (41.1%), 5q (52.6%), 13q (52.7%), 17p (55.2%), and 18q (45.7%) by LOH analysis on whole chromosomes in ESC by a variable number of tandem repeat and restriction fragment length polymorphism markers (11). Among these chromosomal arms, LOH on chromosome 13q exclusively showed a significant correlation with lymph node metastasis ($P = 0.0103$) and poor prognosis ($P = 0.0062$; Ref. 11), suggesting the presence of the gene regulating lymph node metastasis. On 13q, several tumor suppressor genes have been identified, such as the retinoblastoma susceptibility gene (*RB1)* located on 13q14 and the inherited breast cancer susceptibility gene (*BRCA2)* located on 13q12–13 (12–16). Gross alterations and point mutations of the *RB1* gene were found in retinoblastoma, osteosarcoma, lung cancer, and breast cancer (14, 17, 18), and point mutations of the *BRCA2* gene were also detected in breast cancer, ovarian cancer, hepatocellular carcinoma, and pancreatic carcinoma (19–23). However, in ESC, somatic mutations of the *RB1* gene have been reported to be very rare (24), and thus far, inactivation of the *BRCA2* gene has not been examined.

To define the precise region of chromosome 13q involved in ESC, we examined 60 ESC specimens with 18 microsatellite markers on 13q and constructed a detailed deletion map. Two commonly deleted regions were identified; one is between *D13S260* and *D13S267*, which flank the *BRCA2* gene on 13q12–13, and the other is in the *RB1* locus. Here we show again that LOH on 13q is significantly associated with lymph node metastasis, although we have used a different series of tumor samples than the previous study. We also demonstrate that lymph node metastasis is significantly correlated with LOH on the *BRCA2* locus, whereas inactivation of the *BRCA2* and *RB1* genes is rare in ESC.

MATERIALS AND METHODS

Tumor Samples and Cell Lines. Tissue samples were obtained from patients with ESC who underwent surgery at the Kyoto University Hospital (40 cases) and the Aichi Cancer Center Hospital (20 cases). In each case, samples of the tumor and matched normal mucosal tissue were immediately stored at −80°C until examination. Thirty-one ESC cell lines (KYSE series) were established from the resected ESC specimens and maintained in Ham's F-12/RPMI 1640 containing 2% FCS (25). Genomic DNA was extracted from tissues and cell lines using the DNA Extraction Kit (Stratagene), and total RNA was extracted from cell lines using the RNeasy Total RNA kit (Qiagen).

LOH Analysis. Eighteen polymorphic microsatellite markers on 13q reported in the CEPH/Généthon linkage map and the RBi2 marker in the *RB1* locus were used to detect LOH. From centromere to telomere, they consist of: *D13S175; D13S283; D13S221; D13S1244; D13S217; D13S289; D13S260; D13S171; D13S267; D13S220; D13S218; D13S263; D13S328; RBi2; D13S273; D13S272; D13S176*; and *D13S269* (26–28). PCR mixtures contained 25 ng of genomic DNA, 10 pmol each of forward primer labeled with [γ^{32}P]ATP and reverse primer, 1.5 mM $MgCl_2$, 200 μM deoxynucleotide triphosphates, and 0.5 unit of Taq DNA polymerase. After denaturing at 94°C for 5 min, PCR was performed with 35 cycles of 94°C for 40 s and 54°C to 65°C for 30 s, with a final extension at 72°C for 2 min. After PCR amplification of the DNA samples from tumor tissue and matched normal tissue, products were electrophoresed through 6% denaturing polyacrylamide gels. Gels were dried and exposed to X-ray film. LOH was scored when one allele in the tumor was absent or <50% as dense as the corresponding normal tissue in heterozygotes (Fig. 1).

Mutation Screening and Direct Sequencing Analysis of the *BRCA2* Gene. Mutations in all 26 coding exons of the *BRCA2* gene were screened by PCR-SSCP. PCR primers including intron/exon boundaries have been described previously (19, 21). PCR was carried out in a 25-μl reaction mixture containing 25 ng of genomic DNA; 10 pmol of each primer; 200 μM each of dATP, dGTP, and dTTP; 20 μM dCTP; 0.1 μCi of [α^{32}P]dCTP; and 0.5 unit

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[4] The abbreviations used are: ESC, esophageal squamous cell carcinoma; LOH, loss of heterozygosity; SSCP, single-strand conformational polymorphism; RT-PCR, reverse transcription-PCR; pRB, retinoblastoma protein; TFII H, transcription factor II H.

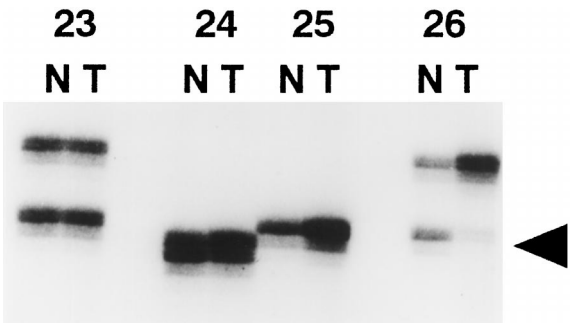

Fig. 1. Examples of LOH and retained heterozygosity at the *D13S171* locus. *N*, normal tissue; *T*, tumor. The tumor numbers are shown at the *top*. Tumors 23 and 24 retained heterozygosity. Tumor 26 showed LOH, and the *arrowhead* indicates allelic loss. Tumor 25 was not informative because normal DNA showed homozygosity

of Taq polymerase under the same conditions as LOH analysis. A 2-μl aliquot of amplified product was diluted with 18 μl of denaturing loading buffer (95% formamide, 20 mM EDTA, 0.05% xylene cyanol, and 0.05% bromphenol blue). Diluted samples were heat-denatured at 80°C for 5 min and loaded onto a 6% nondenaturing polyacrylamide gel with Tris-borate EDTA gel with 10% glycerol and onto a 5% nondenaturing polyacrylamide gel with Tris-2-morpholineethanesulfonic acid-EDTA as described previously (29). After electrophoresis (40 W, 25°C), gels were dried and exposed to X-ray film. Samples with shifted bands were further analyzed by direct DNA sequencing using a DNA sequencing kit (Sequencing PRO; Toyobo).

RT-PCR Analysis of the *BRCA2* Transcript. Expression of the *BRCA2* gene in 31 ESC cell lines was analyzed by RT-PCR analysis. Total RNA from the ESC cell lines was reverse-transcribed using the SUPER SCRIPT Preamplification System (Life Technologies, Inc.) for first-strand cDNA synthesis. PCR amplification on this cDNA was then performed using primer pairs including all coding regions, as described previously (Refs. 19, 22, and 30; Table 1), in a Perkin-Elmer 9600 thermal cycler for 45 cycles of 94°C for 15 s, 50°C to 60°C for 15 s, and 72°C for 30 s. PCR products were analyzed by electrophoresis on a 2% agarose gel. The integrity of the RNA was evaluated by amplification of human glyceraldehyde-3-phosphate dehydrogenase mRNA.

Western Blot Analysis of pRB. To determine whether the *RB1* gene was inactivated in ESC, we analyzed the expression of pRB in 31 ESC cell lines by Western blotting. Total proteins were prepared with sample buffer [62.5 mM Tris (pH 6.8), 10% glycerol, and 2.3% SDS], and the protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories). Fifteen μg of protein for each sample were separated by 7.5% SDS-PAGE and transferred onto a nitrocellulose membrane (Hybond-C pure; Amersham). The membranes were incubated with anti-pRB monoclonal antibody G3-245 Rb (PharMingen) and anti-TFIIH antibody TFIIHp89 (S-19) (Santa Cruz Biotechnology) as a control for nuclear protein. Bands were visualized by Western Blot Chemiluminescence Reagent Plus (New England Nuclear Life Science Products).

Statistical Analysis. The χ^2 test and Fisher's exact test were used for statistical analysis of the relationship between LOH and clinicopathological parameters.

RESULTS

Deletion Mapping on 13q. Fig. 1 shows an example of LOH analysis at the *D13S171* locus. A summary of deletion mapping and the location of the 18 markers used in this study are shown in Fig. 2. Allelic loss was observed in 34 of 60 ESCs (56.7%) for at least one locus on 13q. Six of 60 tumors (10%) revealed LOH at only a single locus. Four of 60 tumors (6.7%) showed LOH at all informative loci, which might mean a complete loss of the long arm of chromosome 13. Replication error was detected at two loci, *D13S217* and *D13S220*, in only one tumor. The frequency of LOH ranged from 10.4% at *D13S175* to 43.9% at *D13S260* (Table 2). Two commonly deleted regions were revealed: (*a*) the proximal segment located within a 3-cM interval between *D13S260* and *D13S267* containing the *BRCA2* gene; and (*b*) the distal region located within a 2-cM interval flanked by *D13S328* and *D13S273* containing the *RB1* gene.

We examined the relationship between LOH on 13q and several clinicopathological parameters including lymph node metastasis, depth of tumor invasion, histopathological grading, and clinical staging (Tables 3 and 4). Among the parameters, only lymph node metastasis was significantly associated with LOH on 13q ($P = 0.0053$; Table 4). Moreover, a significant association was also identified between the degree of lymph node metastasis and 13q loss ($P = 0.0431$; Table 4). We then investigated the correlation between LOH at each locus and lymph node metastasis and found a significant association only at *D13S171* ($P = 0.0441$; Table 4). These results suggest that the tumor suppressor gene, which might regulate lymph node involvement in ESC, is located in the vicinity of *D13S171*, which flanks the *BRCA2* tumor suppressor gene.

Screening of Mutations and Expression of the *BRCA2* Gene in ESC. To determine whether the *BRCA2* gene was the target of 13q loss in ESC, we screened 40 tumors obtained at the Kyoto University Hospital for mutations of the gene through all coding exons and exon-intron junctions by PCR-SSCP analysis using 56 pairs of primers. We detected shifted bands by using seven primer pairs, and in all cases, the same mobility shift was also observed in the corresponding normal tissues, indicating that these were not tumor-specific mutations but rather normal polymorphisms (Table 5). Sequencing analysis revealed that one of these polymorphisms was a new one with an A to G transversion at nucleotide 1365 in exon 10, but the others had been reported previously. These results suggest that tumor-specific alteration of the *BRCA2* gene is infrequent in ESC.

Because transcriptional block by aberrant methylation of the $p16^{INK4}$ and *FHIT* genes was frequent in ESC (31, 32), mRNA expression of the *BRCA2* gene was examined in 31 cell lines by RT-PCR (Fig. 3). Although loss of expression with 5′ CpG island methylation of the $p16^{INK4}$ and *FHIT* genes was frequently observed in those cell lines (31, 32), there was no aberrant expression of the *BRCA2* gene.

Table 1 *BRCA2 RT-PCR primers*

Exon	Forward (5′→3′)	Reverse (5′→3′)	Annealing
Exon 2–8	ACTTATTTACCAAGCATTGGA	AGCAGTAGTATCATGAGGAAAT	55°C
Exon 7–11	AGCTACACCACCCACCCTTAGTTC	GACATAAGGAGTCCTCCTTC	50°C
Exon 10–11	AAGCCTCTGAAAGTGGACTG	CAAGATCCTGAGAGATTACTG	60°C
Exon 11	GCTCTTTTGGGACAATTC	TGGTTTGAATTAAAATCCTGC	55°C
Exon 11	GTCATATAACCCCTCAGATG	ACAAGGTTTTTATCATTATTG	55°C
Exon 11	CTGCCCCAAAGTGTAAAGAAAT	TATGAAGCTTCCCTATACT	55°C
Exon 11–14	CACCTTGTGATGTTAGTTTG	GGTTGGTCTGCCTGTAGTAAT	55°C
Exon 11–18	TTCAACAAGACAAACAACAGT	GCTGTGTCATCCCTTTCCAT	55°C
Exon 16–20	GGAATACAGTTGGCTGATGGT	CCTCCACATATTTTGCTGCTTCCT	60°C
Exon 19–24	CCCTATACAGTGGATGGAGAAGAC	AATCCTATTAGGTCCACCTC	55°C
Exon 22–27	GGAAGTTGCGTATTGTAAGC	AAGCGTCAATAATTTATTGTC	55°C

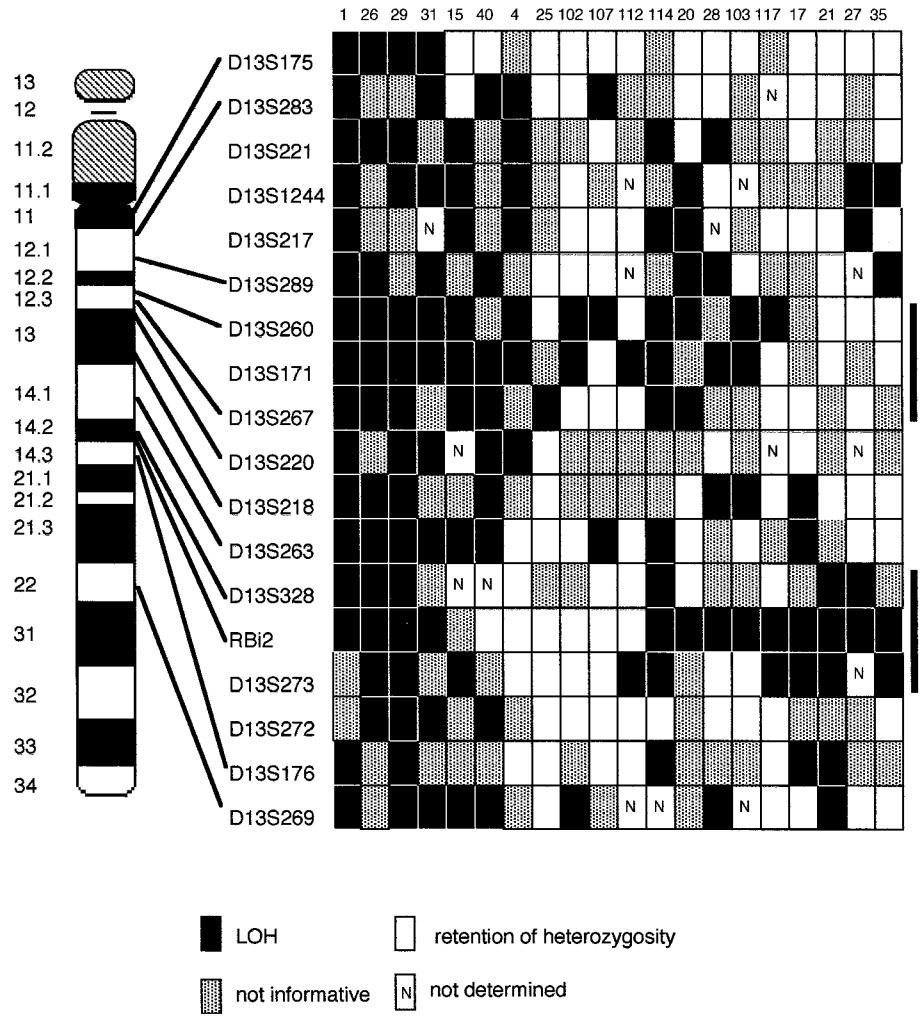

Fig. 2. Schematic representation of deletion mapping on chromosome 13q in 20 ESCs. The location of the 18 microsatellite markers used in this study is indicated to the *right* of an ideogram of 13q. Tumor numbers are shown at the *top*. *Vertical bars* on the *right* indicate the two commonly deleted regions.

Expression of pRB in ESC Cell Lines. Because one of the commonly deleted regions was located close to the *RB1* gene, we examined the expression of pRB in ESC cell lines. Western blotting analysis revealed that among 31 ESC cell lines, 29 cell lines expressed pRB of normal length, but 2 cell lines (KYSE 270 and KYSE 850) showed loss of expression (Fig. 4). We then analyzed allelic status at the *RBi2* locus. Of 31 ESC cell lines, 26 cell lines (83.9%), including

Table 2 *Frequency of LOH at each polymorphic microsatellite marker on chromosome 13q in ESC*

Locus symbol	LOH/informative cases (%)
D13S175	5/48 (10.4)
D13S283	8/34 (23.5)
D13S221	10/39 (25.6)
D13S1244	12/43 (27.9)
D13S217	10/39 (25.6)
D13S289	12/34 (35.3)
D13S260	18/41 (43.9)
D13S171	17/44 (38.6)
D13S267	17/39 (43.6)
D13S220	10/23 (43.5)
D13S218	10/43 (23.3)
D13S263	18/46 (39.1)
D13S328	12/41 (29.3)
RBi2	17/49 (34.7)
D13S273	11/38 (28.9)
D13S272	7/40 (17.5)
D13S176	10/28 (35.7)
D13S269	12/34 (35.3)
13q	34/60 (56.7)

Table 3 *Relationship between LOH on chromosome 13q and clinicopathological findings (tumor-node-metastasis classification)*

	LOH	Retention	*P*
Location of the lesions			NS[a]
Cervical esophagus	2	2	
The upper thoracic portion	3	4	
The mid-thoracic portion	20	12	
The lower thoracic portion	9	8	
pT-primary tumor			NS
T_1	3	4	
T_2	14	8	
T_3	13	11	
T_4	4	3	
pN-regional lymph nodes			0.0062
N_0	7	15	
N_1	27	11	
pM-distant metastasis			NS
M_0	23	20	
M_{1a}	5	1	
M_{1b}	6	5	
G histopathological grading			NS
G_1	5	6	
G_2	19	16	
G_3	7	4	
G_4	1	0	
GX	2	0	
Stage grouping			NS
Stage I	2	3	
Stage IIa	4	10	
Stage IIb	7	3	
Stage III	10	4	
Stage IV	11	6	

[a] NS, not significant.

Table 4 *LOH and detailed classification of lymph node metastasis*[a]

	chromosome 13q			*D13S171*		
	LOH	Retention	*P*	LOH	Retention	*P*
Lymph node status			0.0053			0.0441
Involved	28	12		15	15	
Not involved	6	14		2	12	
n factors[b]			0.0431			NS
n0	6	14		2	12	
n1	2	0		0	2	
n2	10	4		6	5	
n3	8	3		3	3	
n4	8	5		6	5	

[a] LOH and detailed classification of lymph node analysis according to Ref. 36.
[b] Degree of metastasis to the lymph node. In short, metastasis located at a distance from the primary tumor is associated with an increases in the number of n factors.

Table 5 *BRCA2 polymorphisms observed in ESC*

All samples were obtained from Asian patients.

Exon	Nucleotide no.	Nucleotide change and allele number (frequency)
2	203	GCA(Ala)48(0.60)/ACA(Thr)32(0.40)
10	1342	CAT(His)22(0.29)/AAT(Asn)54(0.71)
10	1365	TCA(Ser)72(0.92)/TCG(Ser) 6(0.08)
11	2457	CAT(His)73(0.94)/CAC(His) 5(0.06)
11	3624	AAA(Lys)49(0.64)/AAG(Lys)27(0.36)
14	7470	TCA(Ser)39(0.59)/TCG(Ser)27(0.41)
27	10462	ATT(Ile)74(0.97)/GTT(Val) 2(0.03)

KYSE 270 and KYSE 850, showed homozygosity, and only 5 cell lines (16.1%) were heterozygous. In the case of KYSE 850, normal DNA was available, and LOH was confirmed.

DISCUSSION

In this study, we have identified two commonly deleted regions on 13q in ESC, one of which showed a significant association with lymph node metastasis. Of the 18 polymorphic markers tested, LOH at *D13S171* on chromosome 13q12–13 was very frequent (38.6%) and was significantly correlated with lymph node metastasis. Because this marker flanks the *BRCA2* tumor suppressor gene, we examined point mutation in 40 tumors and examined mRNA expression in 31 cell lines.

Recent analyses of breast cancer, ovarian cancer, hepatocellular carcinoma, and head and neck carcinoma have revealed that somatic mutation of the *BRCA2* gene is very rare, despite the high frequencies of LOH around the *BRCA2* locus (19–22, 33). We also detected no mutation of the *BRCA2* gene instead of a frequent LOH at the *BRCA2* locus. In this study, we screened point mutations intensively through all coding exons and exon-intron junctions of the *BRCA2* gene by PCR-SSCP, whereas PCR products were examined with two different assays, conventional SSCP and low pH SSCP (29). The high sensitivity of our methods was demonstrated by the fact that we detected seven polymorphisms with single-base substitutions. Therefore, it is

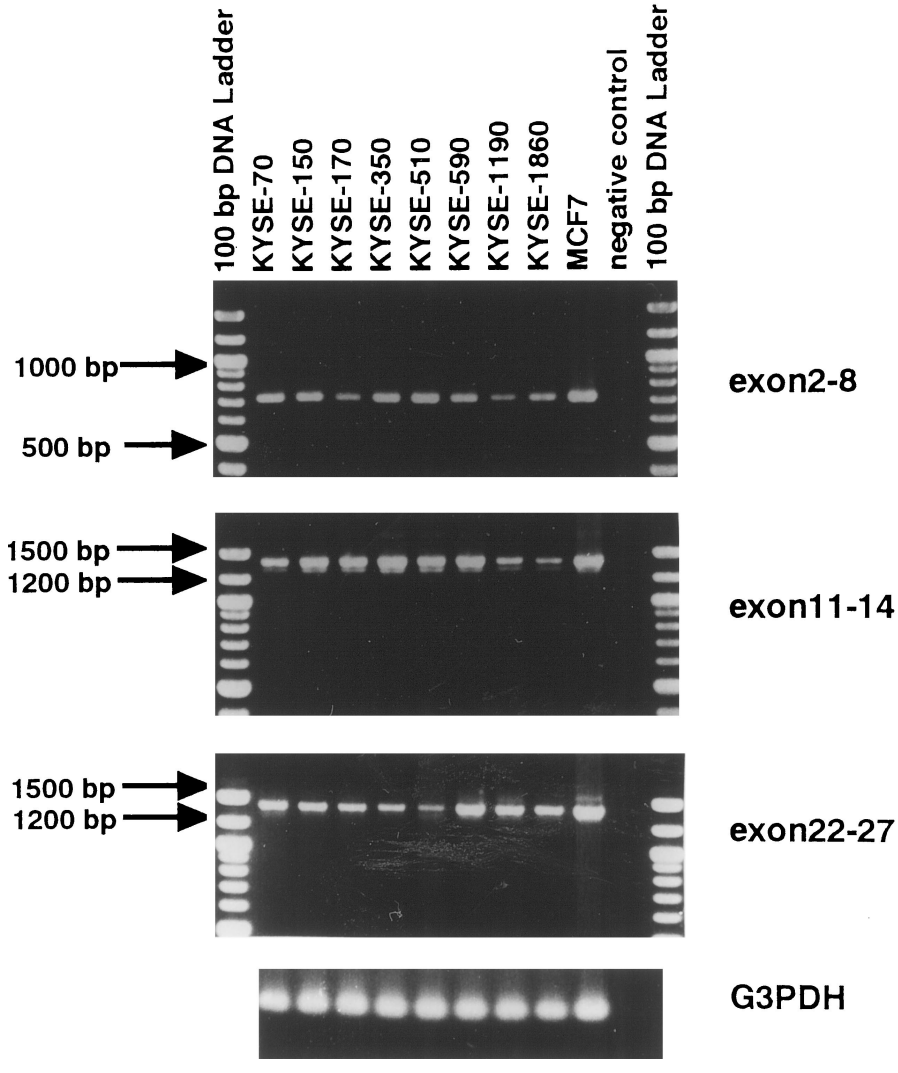

Fig. 3. Representative example of RT-PCR of BRCA2 expression in ESC cell lines. The location of the primers used is indicated on the *right*. MCF7 was used as a positive control. Normal transcripts were detected in ESC cell lines at all regions.

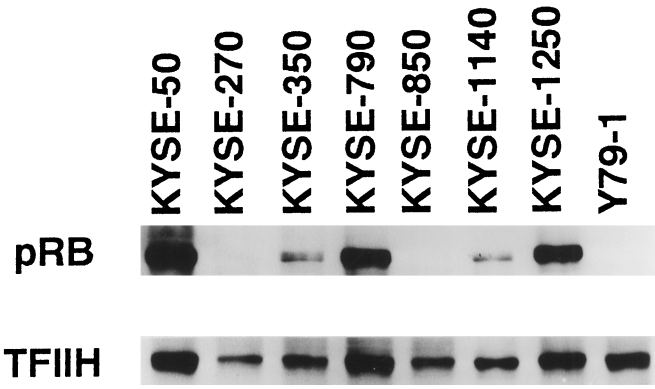

Fig. 4. Expression of pRB in ESC cell lines. All but two ESC cell lines expressed pRB. KYSE 270 and KYSE 850 showed a loss of pRB expression. TFIIH was used as a control of nuclear protein. Y79-1 was a retinoblastoma cell line used as a negative control.

unlikely that we overlooked frequent tumor-specific mutations. Whereas some of our ESC cell lines showed loss of expression of $p16^{INK4a}$ and *FHIT* with CpG island methylation (31, 32), RT-PCR analysis showed no aberrant transcript of the *BRCA2* gene, suggesting that transcriptional block by the CpG island methylation might not be involved in the *BRCA2* gene in ESC. Although this study did not confirm BRCA2 protein expression, without tumor-specific mutations and aberrant expression in this region, the *BRCA2* gene might not be a target of allelic loss, and unidentified tumor suppressor gene(s) might be responsible for lymph node metastasis in ESC.

Another commonly deleted region was located on 13q14, where LOH at the *RBi2* locus within the *RB1* gene was frequent (34.7%). Because the *RB1* gene was the target for allelic loss on 13q and loss of protein expression was frequent in retinoblastoma, osteosarcoma, lung cancer, and breast cancer (14, 17, 18), we examined the protein expression of the *RB1* gene in ESC cell lines. However, we found loss of the pRB in only 2 of 31 ESC cell lines (6.5%), although these cell lines revealed a high frequency of homozygosity at the *RBi2* marker (83.9%). Because heterozygosity at the *RBi2* locus was 86% in our cancer patients, it seems that the majority of homozygous cell lines resulted from LOH. Therefore, the remaining allele may not be inactivated by mutations. These results suggest that the *RB1* gene might not be the target of 13q loss in ESC. Previous studies have revealed that the expression of pRB is inversely correlated with that of $p16^{INK4a}$ protein in various human cancer cells derived from the esophagus, lung, liver, pancreas, mesothelium, and colon (34). We have also previously revealed that among 30 of 31 ESC cell lines described above, 28 show a loss of $P16^{INK4a}$ gene expression (31). This further supports the idea that inactivation of the *RB1* gene might be infrequent in ESC and that a target gene on 13q might be a gene other than the *RB1* gene.

Lymph node metastasis was shown to be one of the most important prognostic factors in ESC (1, 35). Shibagaki *et al.* (11) have reported that LOH on 13q is significantly associated with lymph node metastasis and poor prognosis. This study further confirmed a significant correlation between lymph node metastasis and 13q loss, which suggests the possibility that lymph node metastasis could be predicted by examining LOH on 13q in the primary tumor. Furthermore, recent progress in the treatment of ESC has made it possible to endoscopically resect superficial-type ESCs. Thus, a more accurate diagnosis about lymph node metastasis may be required in such a case (2). However, the usual approach using computed tomography or endoscopic ultrasonography has a limited ability to detect micrometastasis in a lymph node. More accurate diagnosis may be possible by using this simple, PCR-based molecular biological approach.

In summary, our detailed deletion mapping on 13q in ESC identified two distinct, commonly deleted regions around the *BRCA2* gene and the *RB1* gene. Although LOH on *D13S171* flanking the *BRCA2* gene was frequent and was significantly correlated with lymph node metastasis, tumor-specific mutations of the *BRCA2* gene were not detected, suggesting that the unidentified tumor suppressor gene(s) flanked by *D13S171* on 13q12–13 might contribute to lymph node involvement in ESC.

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