Copy-Neutral Loss of Heterozygosity at the $p53$ Locus in Carcinogenesis of Esophageal Squamous Cell Carcinomas Associated with $p53$ Mutations

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Abstract

Purpose: LOH at the $p53$ locus has been reported to be associated with esophageal squamous cell carcinogenesis. The aim of this study is to identify potential mechanisms resulting in LOH around the $p53$ locus in its carcinogenesis.

Experimental Design: We investigated 10 esophageal cancer cell lines and 91 surgically resected specimens, examining them for LOH at the $p53$ locus on chromosome 17. We examined the $p53$ gene by using microsatellite analysis, comparative genomic hybridization (CGH), FISH, and single-nucleotide polymorphism–CGH (SNP–CGH).

Results: In an analysis of specimens by microsatellite markers, a close positive correlation was found between $p53$ mutations and LOH at the $p53$ locus ($P<0.01$). Although four cell lines were found to be homozygous for $p53$ mutations, LOH at the $p53$ locus was not detected by CGH. Among two $p53$ mutant cancer cell lines and five $p53$ mutant/LOH cancer specimens analyzed by FISH, both the cell lines and four of the specimens exhibited no obvious copy number loss at the $p53$ locus. SNP–CGH analysis, which allows both determination of DNA copy number and detection of copy-neutral LOH, showed that LOHs without copy number change were caused by whole or large chromosomal alteration.

Conclusions: LOH without copy number change at the $p53$ locus was observed in $p53$ mutant esophageal squamous cell carcinomas. Our data suggest that copy-neutral LOH occurring as a result of chromosomal instability might be the major mechanism for inactivation of the intact allele in esophageal squamous cell carcinogenesis associated with $p53$ mutation. Clin Cancer Res; 17(7); 1731–40. ©2011 AACR.
mutations in the tumor suppressor gene have been reported. Among these are frequent point mutations in the tumor suppressor gene $p53$, which have been found in both primary ESCCs and ESCC cell lines (16). The point mutations found in this gene occur even at an early stage of ESCC and correlate with tumor progression (17), suggesting an important role for these mutations in esophageal squamous cell carcinogenesis. Several of our own reports on ESCC have also shown an association of $p53$ gene alterations with the development of this cancer (18–22). However, the exact mechanism of $p53$ gene inactivation in the development of ESCC is unclear.

The elucidation of the mechanisms causing LOH in ESCC will give us further understanding of esophageal squamous cell carcinogenesis and will also have preventive, diagnostic, and therapeutic implications for this aggressive disease. In this study, we examined the $p53$ gene by using comparative genomic hybridization, FISH, microsatellite analysis, and single-nucleotide polymorphism—comparative genomic hybridization to identify potential mechanisms resulting in LOH around the $p53$ locus, especially focusing on its copy number alterations. We herein provide the first evidence that, in $p53$ mutant esophageal squamous cell carcinoma, there is LOH at the $p53$ locus occurring without copy number change, mainly caused by chromosomal instability.

Materials and Methods

Cell lines and surgical specimens

A total of 101 ESCCs, including 10 cell lines and 91 surgical specimens, were used. Ten ESCC cell lines (TE-1, -2, -3, -5, -8, -10, -12, -13, -14, and -15) were kindly provided by the Cancer Cell Repository, Institute of Development, Aging and Cancer, Tohoku University, Japan. All ESCC cell lines were cultured in RPMI-1640 medium containing 10% FBS. The cancerous and corresponding non-cancerous tissues from surgically resected ESCCs were collected from patients who underwent surgery without preoperative therapy between 1994 and 2006 at the Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University, Japan. All samples were diagnosed as squamous cell carcinomas histologically by means of hematoxylin and eosin staining by pathologists. All tissue specimens were obtained after receiving written, informed consent of patients.

DNA preparation

DNA was extracted as described previously (23, 24). Briefly, the frozen samples were incubated in a lysis buffer (0.01 mol/L Tris-HCl, pH 8.0; 0.1 mol/L EDTA, pH 8.0; 0.5% SDS) containing proteinase K (100 μg/mL) at 37°C for 2 hours. The samples were extracted twice in phenol, then once in phenol/chloroform and once in chloroform. Following ethanol precipitation, the samples were diluted to TE (0.01 mol/L Tris-HCl, pH 8.0; 0.01 mol/L EDTA, pH 8.0) buffer.

PCR direct sequencing of the $p53$ gene

As previously described (23, 24), using with genomic DNA extracted from cell lines and tissue samples, a 275-bp fragment containing exon 6, a 439-bp fragment containing exon 7, and a 445-bp fragment containing exons 8 and 9 of the $p53$ gene were amplified by PCR (Nippon Gene). The PCR primers for the amplification of a 406-bp fragment containing exon 5 of $p53$ were as follows: exon 5 forward, TGC AGG AGG TGC TTA CAC ATG; exon 5 reverse, TCC ACT CCG ATA AGA TGC TG. Mutations in $p53$ were detected by PCR direct sequencing of all PCR products by using each forward and reverse primer with the dideoxy nucleotide chain-termination method (Big-dye sequencing kit; Applied Biosystems) and then were sequenced with the ABI Prism 310 Genetic Analyzer (Applied Biosystems).

LOH analysis with microsatellite markers

LOH was analyzed by using microsatellite markers by DNA sequencing. The PCR reactions and running
conditions with the Perkin-Elmer Genetic Analyzer 310 have been described previously (25, 26); 2 microsatellite instability markers–17S796 and D17S1353, which are close to the 5' and 3' end of the p53 gene, respectively, were used. The highest peaks in the curve cluster of the PCR product electrophoresis profiles from the cancerous tissues and corresponding noncancer tissue were compared. However, when the 2 alleles overlapped either partially or totally, the case was not informative for LOH estimation. When the peak of cancer tissue decreased by more than 30% in comparison with its normal counterpart, it was defined as LOH.

**Whole genomic CGH analysis**

Copy number analysis of the p53 locus was performed by whole genome CGH array. A tiling array was designed with a mean probe density of 1 probe/1,169 bp, 50-mer length, covering whole chromosomal regions, including chromosome 17. Hybridizations were performed in the NimbleGen Service Laboratory as described previously (27). We compared genomic DNA from 4 ESCC cell lines (TE-5 and 8–p53 mutant; TE-2 and -15–p53 wild type) to that of reference human genomic DNA (Promega). Genomic DNA from 2 ESCC specimens (case 2–p53 mutant/LOH; case 6–p53 wild type) were also analyzed by using it with genomic DNA from normal esophageal tissue from the same case as a reference.

**FISH**

For further analysis of p53 copy number, we applied FISH as described previously (28). A p53 probe and a centromere control for chromosome 17 were designed, allowing simultaneous determination of the number of p53 gene and chromosome 17 copies (GSP Lab, Inc.). We analyzed 4 ESCC cell lines (TE-5 and -8–p53 mutant; TE-2 and -15–p53 wild type) and 6 ESCC specimens (cases 1, 2, 3, 4, and 5–p53 mutant/LOH; case 6–p53 wild type). Hybridization signals were scored in at least 100 intact, nonoverlapping.
randomly selected nuclei. The numbers of p53 gene (red) and centromere signals (green) were recorded for each cell. The ratio of p53 signals to chromosome 17 centromere signals per nucleus was calculated. Cells were considered deleted if the number of centromere signals was more than twice the number of p53 signals (29).

**SNP–CGH analysis**

Four surgically resected ESCC specimens (cases 1, 2, and 3—p53 mutant/LOH; case 6—p53 wild type) and their corresponding noncancerous tissues were genotyped by using 1,140,419 autosomal SNPs (HumanOmni1-Quad BeadChip; Illumina Inc.) and copy number variation was analyzed with GenomeStudio V2009.1 (Illumina Inc.) as described previously (30). Two transformed parameters, the log-normalized intensity ratio (log R ratio) and B allele frequency, were plotted along the entire genome for all SNPs on the array in the single sample analysis mode.

**Results**

**p53 mutation and LOH**

Of the 10 ESCC cell lines, 2 transversions (TE-5 and -8) and 2 transitions (TE-1 and -10) causing amino acid changes were recognized by direct sequencing of genomic DNA (Fig. 1A). We also found that wild-type signals were completely substituted by mutant signals in all 4 p53 mutant ESCC cell lines, suggesting they all carried homozygous p53 mutations.

Of the 91 surgically resected specimens with ESCC investigated in this study, p53 gene mutations in exons...
5–9 were found in 50 patients, and 2 patients had double mutations. The frequency of p53 gene mutations in ESCC was therefore 54.9% in our study. Of the 52 mutations, 14 (26.9%) were located in exon 5, 13 (25.0%) in exon 6, 14 (26.9%) in exon 7, 9 (17.3%) in exon 8, and 2 (3.8%) in exon 9. Among the 52 mutations identified, transversions were predominant (22 of 52, 42.3%), followed by transitions (15 of 52, 28.8%) and frameshifts (15 of 52, 28.8%).

LOH was found in 47 out of 79 informative cases (59.5%), based on analysis with 2 microsatellite markers of the p53 locus (Fig. 2A). A close positive correlation was recognized between p53 hot spot mutations and LOH at the p53 locus (P < 0.01, Fisher’s exact test; Fig. 1B).

LOH at the p53 locus and copy number change  
We first performed CGH with a representative case carrying a p53 mutation/LOH (case 2) and a control case with p53 wild type/ROH (retention of heterozygosity; case 6) to test whether copy number loss was seen at the p53 locus. We found no obvious genetic loss at the p53 locus in both cases (data from case 2 in Fig. 2A). CGH was further applied to 2 p53 mutant ESCC cell lines and 2 p53 wild-type ESCC cell lines, which also showed no genetic loss at the p53 locus in all 4 cell lines (Fig. 1C).

Next, FISH was performed to analyze copy number change in individual cancer cells. As shown in Figure 1D, no obvious copy number loss was detected at the p53 locus by FISH in p53 mutant ESCC cell lines. Copy number evaluation determined by FISH is summarized in Table 1. In this analysis, cells were considered deleted if the ratio of p53 signals to chromosome 17 centromere signals per nucleus was less than 0.5. Cells deleting the p53 locus were observed in only 1 ESCC sample (case 2) among the p53 mutant ESCCs (including 2 ESCC cell lines and 5 surgically resected ESCC specimens) tested. On the contrary, both the cell lines and 4 of the specimens with p53 mutation exhibited no obvious copy number loss at the p53 locus.

SNP–CGH analysis  
Finally, we performed SNP–CGH analysis to clarify potential mechanisms of disruption of the intact allele in p53 mutant ESCCs. With regard to chromosome 17, data from 1 p53 wild-type/ROH ESCC specimen (case 6 in Fig. 2B) and all noncancerous tissue samples (data not shown) showed no deflection in the log R ratio, and the heterozygotes were clustered around +0.5 in the B allele frequency. Strikingly, for 2 p53 mutation/LOH ESCC specimens (cases 2 and 3 in Fig. 2B), there was no deflection in the log R ratio and the heterozygous state split into 2 clusters in the B allele frequency for the entire chromosome 17. The data from case 1 also showed no deflection in the log R ratio, and the heterozygous state split into 2 clusters in the B allele frequency in a large portion of chromosome 17 containing the p53 locus. Additionally, there was an increased deflection in the log R ratio, and a larger split between 2 clusters in the B allele frequency in the rest of the chromosome (Fig. 2B).

We further analyzed all chromosomes and compared the alterations between the p53 wild-type/ROH and p53 mutation/LOH ESCC specimens. All 3 p53 mutant/LOH ESCC cases showed drastic chromosomal alterations in multiple chromosomes, similar to those seen in chromosome 17 (case 1 is representative; Fig. 3), in contrast to the p53 wild-type/ROH ESCC case, which showed no deflection in the log R ratio, and the heterozygotes were clustered around +0.5 in the B allele frequency in all chromosomes (case 4 in Fig. 4).

Discussion  
Esophageal cancers are classified into 2 histologic types: ESCC and adenocarcinoma. The incidences of these types
Figure 3. SNP–CGH analysis in all chromosomes in a p53 mutation/LOH ESCC clinical sample (case 1). Drastic chromosomal alterations were seen in multiple chromosomes, including chromosome 17.
Figure 4. SNP–CGH analysis in all chromosomes of a p53 wild-type/ROH ESCC clinical sample (case 6). No chromosomal alterations were observed.
show remarkable variations in geographic distribution, which means that each area has particular environmental risk factors for esophageal carcinogenesis. Cigarette smoking and alcohol consumption are considered to be significant risk factors for the development of ESCC (31, 32). To elucidate the mechanisms of carcinogenesis, therefore, it should be a useful strategy to investigate the direct evidence showing a causal relationship of exposure to these environmental risk factors with the genetic abnormalities observed in ESCC.

In Japan, the incidence of ESCC is markedly high compared with that of esophageal adenocarcinoma. We have reported that cigarette smoking and alcohol consumption by the Japanese people are associated with \textit{p53} abnormalities in subjects with ESCC (19, 20). Mutational analysis of tumors also provides clues to the exogenous and endogenous mutagenesis mechanisms because mutations reflect specific types of DNA damage. In particular, the mutation spectrum of the \textit{p53} gene has been used as a tool in predicting the role of carcinogenic factors in specific types of cancer (33). The most frequent mutation in ESCC among Japanese is reported to be a G:C to T:A transversion (24). G:C to T:A transversions have been found to occur preferentially at defined codons known to be sites of adduct formation for the metabolites of benzo[a]pyrene, a major tobacco carcinogen (34). Therefore, it has been suggested that a point mutation induced by environmental risk factors might be the “first hit” in the \textit{p53} gene.

LOH is a possible event for the “second hit” in \textit{p53} in \textit{p53} mutant cancer. Using high-resolution fluorescence microsatellite analysis, LOH in ESCC was reported to be observed at a high frequency in multiple microsatellite markers (35), suggesting that LOH plays a role in esophageal squamous cell carcinogenesis. In this study, a close positive correlation was found between \textit{p53} hot spot mutations and LOH at the \textit{p53} locus. We also found that wild-type signals were completely substituted by mutant signals in all 4 \textit{p53} mutant ESCC cell lines (TE-1, 5, -8, and -10). This indicates that all 4 ESCC cell lines carried homozygous \textit{p53} mutations, implying that the signals theoretically resulted from a mutation plus an LOH event. These data suggest that “two hits” in the \textit{p53} tumor suppressor gene, consisting of a \textit{p53} mutation on one allele and LOH through inactivation of the other allele, might be the dominant event in carcinogenesis.

The question was how LOH is generated in esophageal squamous cell carcinogenesis. We performed CGH with a representative ESCC sample carrying a \textit{p53} mutation/LOH and 2 \textit{p53} mutant ESCC cell lines to test whether copy number loss was seen at the \textit{p53} locus, but no obvious genetic loss was found (Figs. 1C and 2A). These data suggest the occurrence of LOH without copy number change. Next, FISH was performed to analyze copy number change in individual cancer cells. We found that many ESCCs with \textit{p53} mutations had no obvious copy number loss at the \textit{p53} locus (Table 1). Taking into consideration that acquired UPD genotypes and karyotypes appear normal when examined by conventional cytogenetic analysis, CGH, or FISH, we infer that the majority of LOH events at the \textit{p53} locus in \textit{p53} mutant ESCCs result from acquired UPD.

We performed SNP–CGH analysis to clarify the potential mechanisms of disruption of the intact allele in \textit{p53} mutant ESCC. The development of high-density SNP genotyping technology for genomic profiling represents a further advance, because simultaneous measurement of both signal intensity variations and changes in allelic composition makes it possible to detect both copy number changes and copy-neutral LOH events (30). This is particularly important, because copy-neutral LOH is receiving greater attention as a mechanism of possible tumor initiation (4–10).

Data from the \textit{p53} wild-type/ROH ESCC specimen (case 6 in Fig. 2B) and all normal samples (data not shown)
indicated no chromosomal alterations. Strikingly, there was no deflection in the log \( \beta \) ratio, and the heterozygous state split into 2 clusters in the B allele frequency for the entire chromosome 17 in 2 \( p53 \) mutation/LOH ESCC specimens (cases 2 and 3 in Fig. 2B). Typically, chromosomal deletion with duplication, mitotic recombination, and mitotic gene conversion are possible mechanisms of copy number–neutral LOH in cancers (ref. 10; Fig. 5). The results indicate that the majority of cancer cells in these 2 cases have alterations affecting the entire length of chromosome 17 such as, perhaps, whole chromosome deletion with duplication. The SNP–CGH data from case 1 suggest that the large chromosomal deletion including the \( p53 \) locus (i.e., possibly unbalanced translocation) was associated with LOH. Combined with the results from FISH, the duplication of the \( p53 \) mutant allele was also suggestive in case 1.

In this study, we used genomic DNA extracted from both normal and ESCC specimens without applying microdissection, because Peiffer and colleagues have reported that this SNP–CGH assay had sufficient sensitivity in mixed tumor-normal samples to detect single-copy changes in tumor samples contaminated by as much as 50% normal tissue (30). However, the findings from case 2 were considered to lack consistency; no evidence of \( p53 \) copy number loss was obtained by CGH or SNP–CGH (Fig. 2), whereas copy number loss was found by FISH (Table 1). On the basis of these results, it is possible that the results from CGH and SNP–CGH might be affected by contamination with normal cells, as only cancer cells were evaluated by FISH. Nevertheless, it is clear that 3 cases with \( p53 \) mutant/LOH (cases 1, 2, and 3) showed chromosomal alterations and that the \( p53 \) wild-type/ROH case (case 1) did not, based on the results from SNP–CGH.

Whole chromosome deletion, which has been considered to be caused by inappropriate chromosomal segregation at mitosis, was found to cause a subset of LOH in ESCC. Interestingly, multiplication of the remaining homologous chromosome was observed in most ESCC cases, and was also considered to be caused by inappropriate chromosomal segregation. Considering that 3 \( p53 \) mutant/LOH ESCC cases indeed showed drastic chromosomal alterations in multiple chromosomes (case 1 is representative; Fig. 3), the cells with \( p53 \) mutation might have in common a defect in the regulation of chromosomal segregation, leading to the occurrence of LOH. It has been reported that the transcriptional induction of \( p53 \) by mitotic checkpoint activation is essential in protecting cells from developing abnormal levels of chromosome ploidy caused by mitotic failure (36, 37). Thus, defects in mitotic spindle and other checkpoints in esophageal cancer cells hit by \( p53 \) mutation at one allele might cause chromosomal instability and lead to malignant transformation.

It is also probable that duplication of an inactivated mutant allele is beneficial in the selection process through total knockout of the \( p53 \) tumor suppressor gene. Recent evidence from studies of myeloid leukemias indicates that acquired UPD probably represents a mechanism for making an oncogenic gene homozygote (activated oncogene or inactivated tumor suppressor gene) without suffering lethal effects from haplo-insufficient genes located within the lost region (38–40).

On the basis of the results from CGH, amplifications were recognized in chromosome 17 in the \( p53 \) wild-type cell lines, TE-2 and TE-15 (Fig. 1C). In a previous report, amplification was frequently observed in ESCC by CGH analysis (41). Furthermore, region 17q has been reported to exhibit amplification in more than 65% of ESCC samples (42), which is compatible with our results using CGH. We assume that these chromosomal abnormalities may occur in a \( p53 \)-independent manner.

In conclusion, LOH without copy number change at the \( p53 \) locus was observed in \( p53 \) mutant ESCCs. This suggests that copy-neutral LOH occurring by chromosomal instability might constitute one of the major mechanisms for inactivation of the intact allele in esophageal squamous cell carcinogenesis associated with \( p53 \) mutation. Whether \( p53 \) mutations truly affect chromosomal instability in esophageal carcinogenesis requires further experimental investigation. To the best of our knowledge, this is the first report concerning copy-neutral LOH occurring around the tumor suppressor gene in ESCC. In this study, however, the number of subjects was insufficient to analyze the clinical significance of copy-neutral LOH in ESCC. We hope that the LOH status at the \( p53 \) locus might prove to be valuable for the clinical management of ESCC if confirmed in larger studies in the future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

This work was supported in part by a Grant-in-Aid from the Ministry of Education, Culture, Sport, Science and Technology of Japan.

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Received August 5, 2010; revised December 2, 2010; accepted December 15, 2010; published OnlineFirst February 15, 2011.

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\textit{Clin Cancer Res} 2011;17:1731-1740. Published OnlineFirst February 15, 2011.

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