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

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Running head: Copy-Neutral Loss of Heterozygosity

Acronyms: loss of heterozygosity (LOH), comparative genomic hybridization (CGH), fluorescence in situ hybridization (FISH), single-nucleotide polymorphism-CGH (SNP-CGH), uniparental disomy (UPD), esophageal squamous cell carcinoma (ESCC), retention of heterozygosity (ROH)

Key words: carcinogenesis, tumor suppressor gene, esophageal squamous cell carcinoma, acquired uniparental disomy, chromosomal instability

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Statement of Translational Relevance

Esophageal squamous cell carcinoma is highly aggressive, and, until recently, it has almost always been associated with a dismal prognosis. The elucidation of the mechanisms causing loss of heterozygosity in esophageal squamous cell carcinoma 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 using comparative genomic hybridization, fluorescence in situ hybridization, microsatellite analysis, and single-nucleotide polymorphism-comparative genomic hybridization to identify potential mechanisms resulting in loss of heterozygosity 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 loss of heterozygosity at the \( p53 \) locus occurring without copy number change, mainly caused by chromosomal instability.
Abstract

Purpose: Loss of heterozygosity (LOH) at the \textit{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 \textit{p53} locus in its carcinogenesis.

Experimental Design: We investigated ten esophageal cancer cell lines and 91 surgically-resected specimens, examining them for LOH at the \textit{p53} locus on chromosome 17. We examined the \textit{p53} gene using microsatellite analysis, comparative genomic hybridization (CGH), fluorescence in situ hybridization (FISH), and single-nucleotide polymorphism-CGH (SNP-CGH).

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

Conclusions: LOH without copy number change at the \textit{p53} locus was observed in \textit{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.
Introduction

The inactivation of tumor suppressor genes causes the disruption of critical events in the control of cell proliferation, leading to the development of malignant clones (1). During tumorigenesis, loss of tumor suppressor gene function is generally thought to occur in two steps, the first being mutation in one allele – a generally silent mutation that may be inherited – followed by the somatic loss or inactivation of the second allele, or loss of heterozygosity (LOH). Although LOH is a critical step in tumorigenesis, for most tumor suppressor genes, the underlying mechanisms of LOH in cancer have been poorly understood until very recently. Several investigations have focused on conventional mapping of LOH occurring in cancer, commonly using genome-wide technologies like comparative genomic hybridization (CGH), fluorescence in situ hybridization (FISH), or LOH analysis using microsatellite markers. CGH and FISH can identify regions with altered DNA copy number, while copy number changes cannot be detected by conventional analysis with microsatellite markers (2).

Uniparental disomy (UPD) arises when an individual inherits two copies of a particular chromosome from the same parent, either maternal or paternal, and no copies of this chromosome from the other parent. UPD has occasionally been documented in pediatric cancers associated with inherited syndromes, such as Beckwith-Wiedemann syndrome (3). In these pediatric tumors, UPD was confirmed using DNA from both parents. It is more
difficult, however, to demonstrate acquired UPD by conventional strategies in adult cancer types, where parental DNA samples are usually not available. Recently, analysis using high-resolution, single-nucleotide polymorphism (SNP) oligonucleotide genomic microarrays has permitted the detection of copy number and copy number-neutral changes in the same experiment (2). It has been reported that acquired UPD is frequently encountered in hematological malignancies (4-7). In some studies acquired UPD has also been observed in solid tumors (8-10), indicating that UPD can occur not only in familial diseases but also in acquired sporadic tumors. However, our knowledge about acquired UPD regions in sporadic tumors is still very limited.

Esophageal cancer is highly aggressive, and, until recently, it has almost always been associated with a dismal prognosis. Treatment and management have evolved in recent years, with dramatic advances in diagnostic techniques, the implementation of radical esophagectomy with extensive lymphadenectomy, and the development of chemo-radiotherapy (11, 12). Consequently, the prognosis for those with this cancer has improved (13). Nonetheless, early detection, as well as prevention, could still provide the best chance for avoiding death due to this aggressive cancer (14, 15). Better understanding of the molecular mechanism of carcinogenesis should lead to improved screening and treatment of esophageal cancer.

Numerous molecular alterations associated with the genesis of esophageal squamous cell carcinoma (ESCC) 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 using CGH, FISH, microsatellite analysis, and SNP-CGH 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 ESCC, 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% fetal bovine serum. The cancerous and corresponding noncancerous 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 M Tris-HCl, pH 8.0, 0.1 M EDTA, pH 8.0, 0.5% SDS) containing proteinase K (100 µg/ml) at 37°C for 2 h. The samples were extracted twice in phenol, then once in phenol/chloroform and once in chloroform. Following ethanol precipitation, the samples were diluted in TE (0.01 M Tris-HCl, pH 8.0, 0.01 M 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, Japan). 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 CGG ATA AGA TGC TG. Mutations in p53 were detected by PCR direct sequencing of all PCR products using each forward and reverse primer with the dideoxynucleotide chain-termination method (Bigdye sequencing kit; Applied Biosystems, USA) and then were sequenced with the ABI Prism 310 Genetic Analyzer (Applied Biosystems).

**LOH analysis with microsatellite markers**

LOH was analyzed 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 MSI 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 to 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/1169 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 four ESCC cell lines (TE-5, -8 – p53 mutant; TE-2, -15 – p53 wild type) to that of reference human genomic DNA (Promega, USA). Genomic DNA from two ESCC specimens (case #2 – p53 mutant/LOH; case #6 – p53 wild type) were also analyzed using 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 (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., Japan). We analyzed four
ESCC cell lines (TE-5, -8 – p53 mutant; TE-2, -15 – p53 wild type) and six ESCC specimens (cases #1, 2, 3, 4, 5 – p53 mutant/LOH, case #6 – p53 wild type). Hybridization signals were scored in at least 100 intact, non-overlapping, 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, 3 – p53 mutant/LOH, case #6 – p53 wild type) and their corresponding noncancerous tissues were genotyped using 1,140,419 autosomal SNPs (HumanOmni1-Quad BeadChip, Illumina Inc., USA) 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, two transversions (TE-5, -8) and two transitions (TE-1, -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 four *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 two patients had double mutations. The frequency of *p53* gene mutations in ESCC was therefore 54.9% in our study. Fourteen of the 52 mutations (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 (22/52, 42.3%), followed by transitions (15/52, 28.8%) and frameshifts (15/52, 28.8%).

LOH was found in 47 out of 79 informative cases (59.5%), based on analysis with two 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 two p53 mutant ESCC cell lines, as well as two p53 wild type ESCC cell lines, which also demonstrated no genetic loss at the p53 locus in all four cell lines (Fig. 1C).

Next, FISH was performed to analyze copy number change in individual cancer cells. As shown in Fig. 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 < 0.5. Cells deleting the p53 locus were observed in only one ESCC sample (case #2) among the p53 mutant ESCCs (including 2 ESCC cell lines and 5 surgically-resected ESCC specimens) tested. On the other hand, both of the cell lines and four 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 one 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 two p53 mutation/LOH ESCC specimens (case #2 and #3 in Fig. 2B), there was no deflection in the log R ratio and the heterozygous state split into two 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 two 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 two 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 three p53 mutant/LOH ESCC cases demonstrated drastic chromosomal alterations in multiple chromosomes, similar to those seen in chromosome 17 (case #1 is representative; Fig. 4), 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. 5).
Discussion

Esophageal cancers are classified into two histological types; ESCC and adenocarcinoma. The incidences of these types 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). In order 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 *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 *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 p53 gene.

LOH is a possible event for the “second hit” in p53 in 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 p53 hot spot mutations and LOH at the p53 locus. We also found that wild type signals were completely substituted by mutant signals in all four p53 mutant ESCC cell lines (TE-1, 5, -8, and -10). This indicates that all four ESCC cell lines carried homozygous p53 mutations, implying that the signals theoretically resulted from a mutation plus an LOH event. These data suggest that “two hits” in the p53 tumor suppressor gene, consisting of a 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 p53 mutation/LOH and two p53 mutant ESCC cell lines to test whether copy number loss was seen at the p53 locus, but no obvious genetic loss was found (Fig. 2A, 1C). 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
*p53* mutations had no obvious copy number loss at the *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 *p53* locus in *p53* mutant ESCCs result from acquired UPD.

We performed SNP-CGH analysis to clarify the potential mechanisms of disruption of the intact allele in *p53* mutant ESCC. The development of high-density SNP genotyping technology for genomic profiling represents a further advance, since 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, since copy-neutral LOH is receiving greater attention as a mechanism of possible tumor initiation (4-10).

Data from the *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 R ratio, and the heterozygous state split into two clusters in the B allele frequency for the entire chromosome 17 in two *p53* mutation/LOH ESCC specimens (case #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 (10) (Fig. 3). The results indicate that the majority of cancer cells in these two 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, since Peiffer et al. 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 (Figure 2), whereas copy number loss was found by FISH (Table 1). Based on 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 three cases with \(p53\) mutant/LOH (case #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 three p53 mutant/LOH ESCC cases indeed demonstrated drastic chromosomal alterations in multiple chromosomes (case #1 is representative; Fig. 4), 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).

Based on the results from CGH, amplifications were recognized in chromosome 17 in the p53 wild type cell lines, TE-2 and TE-15 (Figure 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 or not 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.

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Figure legends

**Fig. 1.** *p53* mutation, LOH, and *p53* locus copy number analysis in ESCC. (A) Direct sequencing analysis of the *p53* gene in ESCC cell lines. All mutations (yellow arrows) found in ESCC cell lines were homozygous changes. (B) Correlation between *p53* mutations and LOH at the *p53* locus in ESCC clinical samples. A close positive correlation was found between them (p<0.01). (C) CGH analysis with *p53* mutant (TE-5, -8) and wild type (TE-2, -15) ESCC cell lines. There was no genetic loss at the *p53* locus in all ESCC cell lines. (D) FISH analysis with *p53* mutant ESCC cell lines (TE-5, -8). Neither cell line had obvious copy number loss at the *p53* locus.

**Fig. 2.** Chromosome alterations causing LOH in ESCC. (A) Comparison of LOH analysis with microsatellite markers and CGH analysis at the *p53* locus with a *p53* mutant/LOH ESCC clinical sample (case #2). CGH analysis revealed no obvious genetic loss at the *p53* locus, although obvious loss of heterozygosity was observed in LOH analysis with microsatellite markers. (B) SNP-CGH analysis of chromosome 17 in ESCC clinical samples (case #1, 2, 3 – *p53* mutant/LOH; case #6 – *p53* wild type). Data from case #6 showed no deflection in the log R ratio, and the heterozygotes were clustered around +0.5 in the B allele frequency. On the other hand, there was no deflection in the log R ratio, and the heterozygous state split into two clusters in the B allele frequency, in most or all of chromosome 17 in cases #1, #2, and #3.
Fig. 3. A model for the possible mechanisms of LOH. Chromosomal deletion with duplication, mitotic recombination, and mitotic gene conversion are logically all possible mechanisms of copy-number-neutral LOH in cancers (10).

Fig. 4. 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.

Fig. 5. SNP-CGH analysis in all chromosomes of a p53 wild type/ROH ESCC clinical sample (case #6). No chromosomal alterations were observed.
Table 1. Copy number evaluation determined by FISH with ESCC cell lines and clinical samples.

<table>
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<th>copy number</th>
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<td></td>
<td></td>
<td></td>
<td>CEN17q</td>
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<tr>
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<tr>
<td>case</td>
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<td>2.5</td>
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<td>#6 wild type ROH</td>
<td>2.8</td>
<td>3.8</td>
<td>3.8 (1.4)</td>
</tr>
</tbody>
</table>

NOTE: Values are mean copy number counted in consecutive 100 cells. Parentheses indicate p53 locus/CEN17q ratio.

Abbreviations: LOH, loss of heterozygosity; ROH, retention of heterozygosity.
Fig. 1

A

TE-5 exon8
GTG → TTG
Val → Leu

TE-1 exon8
GTG → ATG
Val → Met

Transversion (2/10)

TE-8 exon7
ATG → ATT
Met → Ile

TE-10 exon7
TGC → TAC
Cys → Tyr

Transition (2/10)

B

Mutation frequency

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<td>Wild Type</td>
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<tr>
<td>Mutant</td>
<td>16 (20.3%)</td>
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<td>LOH Wild Type</td>
<td>11 (13.9%)</td>
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<tr>
<td>Mutant</td>
<td>31 (39.2%)</td>
</tr>
</tbody>
</table>

C

p53 locus

TE-8: p53 mutant

TE-5: p53 mutant

TE-15: p53 wild type

TE-2: p53 wild type

D

TE-5: p53 mutant

p53: TexRed, CEN17q: FITC

TE-8: p53 mutant

p53: TexRed, CEN17q: FITC
A

17S1812

p53 mutant/LOH case #2

81% loss in cancer

55% loss in cancer

17S796

p53 mutant/LOH case #2

B

p53 mutant/LOH case #1

p53 mutant/LOH case #2

p53 mutant/LOH case #3

p53 wt/ROH case #6
*p53 mutation

1. chromosome loss

2. partial deletion

3. chromosome loss with duplication

4. mitotic gene conversion

5. mitotic recombination

chromosome 17 pair
p53 mutant/LOH

case #1
p53 wild type/ROH

case #6
Copy-neutral Loss of Heterozygosity at the p53 Locus in Carcinogenesis of Esophageal Squamous Cell Carcinomas Associated with p53 Mutations

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