Study of p53 gene alteration as a biomarker to evaluate the malignant risk of Lugol-unstained lesion with non-dysplasia in the oesophagus

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Mutations of the p53 gene are detected frequently in oesophageal dysplasia and cancer. It is unclear whether Lugol-unstained lesions (LULs) with non-dysplastic epithelium (NDE) are precursors of oesophageal squamous cell carcinoma (ESCC). To study the genetic alterations of NDE in the multistep process of oesophageal carcinogenesis, we determined the relationship between p53 mutations and LULs-NDE. Videodendoscopy with Lugol staining was performed prospectively in 542 oesophageal cancer-free subjects. Lugol-unstained lesions were detected in 103 subjects (19%). A total of 255 samples, including 152 LULs (NDE, 137; dysplasia, 15) and 103 paired samples of normal staining epithelium, were obtained from 103 subjects. After extraction of DNA and polymerase chain reaction analysis, direct sequencing method was applied to detect mutations of the p53 gene. The p53 mutation was detected in five of 137 samples with LULs-NDE (4%) and in five of 15 samples with dysplasia (33%). A hotspot mutation was found in 20% of LULs-NDE with p53 mutation and in 40% of dysplasia with p53 mutation. In contrast, no p53 mutations were found in 103 paired NDE samples with normal Lugol staining. In biopsy samples from oesophageal cancer-free individuals, the p53 missense mutations containing a hotspot mutation were found in NDE, which was identified as an LUL. These findings suggest that some LULs-NDE may represent the earliest state of oesophageal squamous cell carcinoma in Japanese individuals.

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Oesophageal squamous cell carcinoma (ESCC) is one of the most common carcinoma worldwide, with marked variation in its incidence rate among different countries, distinct geographic areas, and different ethnic groups (Parkin et al, 1988). Among oesophageal cancers in Japanese patients, 95% are squamous cell carcinomas (Registration Committee for Esophageal Cancer). In Western countries and Japan, heavy cigarette smoking and alcohol intake are the risk factors, whereas in the developing countries, exposure to dietary carcinogens and nutritional deficiencies are believed to be the major aetiologic factors (Yang, 1980; Von Rensburg, 1981; Parkin et al, 1988; Yokoyama et al, 1993). However, results from previous studies suggest that malignant transformation of human oesophageal epithelium is a multistage progressive process (Yang, 1980; Yang and Qiu, 1987; Qiu and Yang, 1988; Wang et al, 1990; Bennett et al, 1992; Wang et al, 1993; Greenblatt et al, 1994).

Characterisation of human oesophageal precancerous lesions at the molecular level is of critical importance to our understanding of the aetiology of ESCC and to the identification of useful biomarkers for prevention studies of that disease. Mutation analyses among high-risk Chinese populations have demonstrated that p53 gene mutations occur at an early stage of oesophageal carcinogenesis, both in the setting of basal cell hyperplasia (BCH) and in dysplastic lesions (Bennett et al, 1992; Wang et al, 1993; Gao et al, 1994; Jaskiewicz and De Groot, 1994; Parenti et al, 1995). An early indicator of abnormality in individuals predisposed to ESCC is an increased proliferation of the oesophageal epithelial cells, morphologically manifested as BCH, dysplasia, and cancer in situ. Most of these lesions could be considered as precancerous lesions because of the presence of p53 mutations (Yang, 1980; Yang and Qiu, 1987; Qiu and Yang, 1988; Wang et al, 1990; Mandard et al, 2000). But it is under debate whether BCH is a precancerous lesion for ESCC or not, as no hotspot mutations of the p53 gene were found in BCH samples (Shi et al, 1999).

Although endoscopic detection for early ESCC is extremely important because of excellent 5-year survival rate (Yoshinaka et al, 1991; Kumagai et al, 1993), two-thirds of oesophageal intraepithelial carcinomas have been overlooked by conventional endoscopy alone (Sugimachi et al, 1989). A simple technique of spraying Lugol solution in the oesophagus is highly sensitive for identifying dysplasia and intraepithelial carcinoma (Mori et al,
MATERIALS AND METHODS

Study design

To investigate whether LULs-NDE were related to the carcinogenesis of oesophageal squamous epithelium or not, the p53 mutational status in LULs-NDE was analysed prospectively. Secondary end points were to elucidate whether BCH is related to oesophageal carcinogenesis through the p53 mutational status and examine malignant potential in multiple LULs. Recruited subjects were composed of oesophageal cancer-free individuals who visited our hospital for a health checkup between April 1999 and March 2001. Subjects were recruited on the basis of the following criteria: male and female individuals, age in the range of 20–80 years, the subjects performance status being ‘zero’ according to Eastern Cooperative Oncology Group (ECOG), and the subjects with no symptoms of dysphagia, abdominal pain, chest and/or back pain, or vomiting were eligible. As LULs can be caused by reflux oesophagitis, subjects with heartburn and those receiving proton pump inhibitor therapy were excluded. Subjects who had active malignant disease, and who had undergone oesophagectomy, or chemoradiotherapy for ESCC, were excluded. After endoscopic observation, subjects who had oesophageal varices, Barrett’s oesophagus, or reflux oesophagitis were also excluded. Although heavy cigarette smoking and alcohol intake are the major risk factors of ESCC, whether the oesophageal precancerous lesions are caused by such daily consumption or not is uncertain. Therefore, the subjects were not selected based on risk factors such as smoking and alcohol drinking. Participants were interviewed using structured questionnaires, which included queries about smoking and drinking status after recruitment. All subjects gave informed consent for participation in the study. The study protocol was approved by the Human Ethics Review Committee of Showa University School of Medicine.

DNA extraction

Ten 2-μm-thick sections were obtained from each archival block of formalin-fixed and paraffin-embedded dysplastic and nondysplastic tissue. One section of each block was stained with haematoxylin and eosin. The percentage of neoplastic cells was estimated by light microscopic evaluation, and the samples containing a minimum of 60% dysplastic cells were chosen. DNA samples were extracted by the ethanol/xylene method from the remaining nine sections (Goelz et al, 1985).

Analysis of the p53 gene

Specimens were mixed with 50 μl of digestion buffer (0.04% proteinase K, 10 mM Tris-HCl at pH 8.0, 1 mM EDTA, and 1% Tween 20) and incubated at 37°C for 18 h. The DNA fragments were analysed for mutations in p53 exons 5, 6, 7, and 8, as described in our previous report (Makino et al, 2000). Primers used for polymerase chain reaction (PCR) amplification of the p53 gene were as follows: for exon 5, 5'-TTCACTTGTTGGCTTGTATT-3' and 5'-CTCTCCAGCCCCAGCTGCTC-3'; for exon 6, 5'-ATTCCT- CACTTATTGCTC-3' and 5'-TCTTCCAGAGAGCGCGAT-3'; for exon 7, 5'-ACAGGTTCTCCCTCAGAGGCGCA-3' and 5'-TGGTGCA- GGGTGCAAGTGCC-3'; for exon 8, 5'-GTAGGACCTGTATTTCC TTAGCGC-3' and 5'-CTTGGCTTCTCCACGCGGTCTTGG-3'. Polymerase chain reaction conditions were set as described in our report (Makino et al, 2000). The PCR products were purified and directly sequenced using a 3100 sequencing machine (Applied Molecular Diagnostics).
Biosystems, Foster City, CA, USA). Peak patterns were analysed using Sequencing Analysis Software (Applied Biosystems, Foster City, CA, USA), and mutations and amino-acid changes were identified (Figure 2). To ensure reproducibility of our data, direct sequencing was performed at least twice in DNA samples.

Statistical analysis
As LULs were found in approximately 20% of 1000 patients undergoing routine endoscopy in our previous experience, sample size was estimated to be 500 patients to collect at least 100 patients with LULs. To avoid bias, the data regarding the detection of p53 mutation in LULs and their paired normal Lugol staining areas were re-identified for genetic and clinicopathologic analyses. These data were then matched after the genetic and clinicopathologic analyses were completed. The significance of differences between the two groups was assessed by the $\chi^2$ test or Wilcoxon rank-sum test. *P*-value of less than 0.05 was considered significant.

RESULTS

Characteristics of subjects
Out of 542 subjects, LULs were found in 103 (19%). The mean age was 62 years, ranging from 25 to 80 years, and the male to female ratio was 63/50. Of the 103 subjects, 35 (34%) and 31 (30%) had a daily habit of cigarette smoking and alcohol drinking, respectively. No significant difference in the frequency of daily cigarette smoking ($P = 0.213$) or alcohol ($P = 0.107$) consumption was seen between the subjects with LULs and those without.

Histologic and clinicopathologic findings
The samples of LULs consisted of 137 NDE and 15 dysplastic samples, whereas no dysplastic samples were detected in the normal Lugol staining samples (Table 1). Whereas the histologic finding in all samples of LULs-NDE was oesophagitis, 78% of the 103 normal Lugol staining epithelium samples were oesophagitis ($P < 0.0001$). The histologic grade of dysplastic samples was
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ESCCs were located in the same portion (Registration Committee for Esophageal Cancer). The characteristics of LULs-NDE were minute size (<5 mm in diameter), oval shape, and location in the middle third of the oesophagus.

### Table 1  Histologic findings of biopsy samples from 103 oesophageal cancer-free patients

|                          | Lugol-unstained lesions | Normal Lugol staining epithelium | P-value |
|--------------------------|-------------------------|----------------------------------|---------|
| Number of samples        | 152                     | 103                              |         |
| Histologic findings      |                         |                                  |         |
| Dysplasia                | 15                      | 0                                | <0.0001 |
| Oesophagitis             | 137                     | 80                               |         |
| Normal epithelium        | 0                       | 23                               |         |
| Basal cell hyperplasia   | Present                 | 3                                | 0.986   |
| Absent                   | 149                     | 101                              |         |

### Table 2  Clinicopathologic characteristics and presence of p53 mutation of LUL-NDE and dysplasia

|                          | LUL-NDE (n = 137) | Dysplasia (n = 15) | P-value |
|--------------------------|------------------|-------------------|---------|
| Mean size (mm)           | 4                | 9                 | 0.032   |
| Range (mm)               | 1–6              | 5–20              |         |
| Shape of LUL             |                  |                   | <0.0001 |
| Oval                     | 108              | 5                 |         |
| Irregular                | 29               | 10                |         |
| Location                 |                  |                   | 0.441   |
| Upper third              | 19               | 1                 |         |
| Middle third             | 90               | 9                 |         |
| Lower third              | 28               | 5                 |         |
| P53 mutation             |                  |                   | <0.0001 |
| Present                  | 5                | 5                 |         |
| Absent                   | 132              | 10                |         |
| Hotspot mutation (10 samples with p53 mutation) | | | 0.490 |
| Present                  | 1                | 2                 |         |
| Absent                   | 4                | 3                 |         |

LUL-NDE = Lugol-unstained lesion with non-dysplastic epithelium; location = location of the oesophagus.

### Table 3  Mutation of the p53 gene in patients with Lugol-unstained lesions

| Case | Age/sex | Histology | Size (mm) | p53 | Ex | Codon | BC | AAC |
|------|---------|-----------|-----------|-----|----|-------|----|-----|
| 1    | 52/M    | itis      | 3         | P   | 7  | 242   | TGC→TCC | K→S |
| 2    | 53/M    | sev, dys. | 6         | P   | 6  | 218   | GTG→GAG | V→E |
| 3    | 78/M    | mod, dys. | 0         | P   | 6  | 192   | CAG→TAG | *  |
| 4    | 71/M    | mild, dys.| 8         | P   | 5  | 175   | CGC→CAC | R→H |
| 5    | 53/F    | mild, dys.| 6         | P   | 5  | 175   | CGC→GCC | R→G |
| 6    | 63/M    | sev, dys. | 13        | P   | 5  | 184   | GAT→AAT | D→N |
| 7    | 68/F    | itis      | 3         | P   | 7  | 241   | TCC→TAC | S→Y |
| 8    | 75/M    | itis      | 4         | P   | 8  | 273   | CGT→TGT | R→C |
| 9    | 60/F    | itis      | 4         | P   | 7  | 239   | AAC→GAC | N→D |

itis = oesophagitis; dys. = dysplasia; sev = severely; mod = moderately; P = presence of a p53 mutation; A = absence of a p53 mutation; EX = exon; BC = base change; AAC = amino-acid change; * = stop codon.
This study is aimed to evaluate whether LULs-NDE are related to the carcinogenesis of oesophageal squamous epithelium or not, and p53 mutational status in LULs-NDE is analysed on the basis of molecular events in the progressive process of carcinoma. As p53 mutation is a well-known sequence in carcinoma, this biomarker was determined to identify the precancerous lesions in the study. The unique observation in this prospective study is that missense mutations of the p53 gene were found in LULs-NDE, although no p53 mutations were found in paired normally Lugol-stained non-dysplastic epithelium in subjects with LULs-NDE. The results strongly suggest that some of the LULs-NDE can progress to dysplastic lesions through p53 alterations and support the hypothesis that some of 'Lugol-unstained non-dysplastic areas' in Japanese individuals without reflux esophagitis play an important role in oesophageal carcinogenesis.

Mutations of the p53 tumour suppressor gene are the most common genetic abnormalities in solid human cancers (Nigro et al, 1989; Hollstein et al, 1990; Lane, 1992; Vogelstein et al, 2000; Vousden and Lu, 2002; Oliver et al, 2004). Missense mutations are found in 78% of the 6177 somatic p53 mutations in exons 5 – 8 (Hussain and Harris, 1999), suggesting a correlation between the degree of evolutionary diversity and the structural or functional importance of individual amino-acid residues (Greenblatt et al, 1994). The change of protein structure or function caused by the individual amino-acid residues in LULs-NDE might be early molecular events in carcinogenesis. In contrast, p53 gene mutations have been proposed to be concentrated in six hotspots (Hainaut et al, 1997; Hussain and Harris, 1999; Vikhanskaia et al, 2005). Based on the updated p53 Gene Mutation Database containing 5961 mutations, codons 175, 245, 248, 249, 273, and 282 have been identified as mutation hotspots in all cancers, and the incidence of the hotspot mutations is specific molecular alterations in solid human cancers (Hainaut et al, 1997). A hotspot can identify a relationship between the mutation, protein structure and function, and carcinogenesis (Hainaut et al, 1994). We also identified the histologic findings of BCH in LULs-NDE and the paired normal Lugol-stained lesions in symptom-free patients and that p53 mutations can be found in BCH and dysplastic samples, whereas no hotspot mutations are contained in these mutations (Shi et al, 1999). We also identified the histologic findings of BCH in LULs-NDE and the paired normal Lugol-stained area according to histologic criteria used in the Chinese group (Dawsey et al, 1994), whereas prevalence of BCH was low in our Japanese subjects and no p53 mutations were found. We do not believe that the role of BCH is related to oesophageal carcinogenesis in the Japanese population. In contrast, we did not suggest that the daily cigarette or alcohol consumption was directly related to the occurrence of LULs-NDE in this study despite high risk factors in patients with ESCC.

Using Lugol solution spraying methods, as the normal squamous epithelium contains glycogen that interacts with the

Table 4 Relationship between presence of p53 mutation and BCH or squamous atypia in 137 LULs-NDE

| p53 mutation Present (n = 5) | p53 mutation Absent (n = 132) | P-value |
|-----------------------------|-------------------------------|---------|
| Squamous atypia Present (n = 22) | 1 | 21 | 0.807 |
| Absent (n = 115) | 4 | 111 | |
| BCH Present (n = 3) | 0 | 3 | 0.733 |
| Absent (n = 134) | 5 | 129 | |

LULs-NDE = Lugol-unstained lesions with non-dysplastic epithelium; BCH = basal cell hyperplasia.

Figure 3 (A) Histologic findings of squamous atypia in a Lugol-unstained lesion with p53 mutation. The region with squamous atypia was a small portion in contact with the basal cell layer. In the region, the nucleus was slightly enlarged, whereas pleomorphism and hyperchromasia were not seen. According to histological criteria of the Chinese group, the findings of slightly mononuclear enlargement having neither pleomorphism nor hyperchromasia were insufficient for diagnosis of dysplasia, and was decided as inflammation containing atypia. (B) Histologic findings of no squamous atypia in a Lugol-unstained lesion with p53 mutation. Of the five Lugol-unstained lesions with non-dysplastic epithelium (LULs-NDE) containing p53 mutation, squamous atypia was not found in four LULs-NDE.

DISCUSSION

This study is aimed to evaluate whether LULs-NDE are related to the carcinogenesis of oesophageal squamous epithelium or not, and p53 mutational status in LULs-NDE is analysed on the basis of molecular events in the progressive process of carcinoma. As p53 mutation is a well-known sequence in carcinoma, this biomarker was determined to identify the precancerous lesions in the study. The unique observation in this prospective study is that missense mutations of the p53 gene were found in LULs-NDE, although no p53 mutations were found in paired normally Lugol-stained non-dysplastic epithelium in subjects with LULs-NDE. The results strongly suggest that some of the LULs-NDE can progress to dysplastic lesions through p53 alterations and support the hypothesis that some of 'Lugol-unstained non-dysplastic areas' in Japanese individuals without reflux esophagitis play an important role in oesophageal carcinogenesis.

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Using Lugol solution spraying methods, as the normal squamous epithelium contains glycogen that interacts with the
iodine of Lugol’s solution, normal epithelium of the oesophagus becomes uniformly greenish brown (Sugimach et al., 1991; Katagiri et al., 2004). Dysplastic and inflammatory epithelia of the oesophagus are not stained, as the region showing dysplasia and oesophageitis has a reduced or no glycogen content (Sugimach et al., 1991). Therefore, these minute lesions that were not identifiable by conventional endoscopic observation become visible when Lugol’s solution is used. There is a high possibility that inflammation having a reduction in glycogen content is related to the initiation of oesophageal carcinogenesis because no squamous atypia and no \( p53 \) mutations are found in normal Lugol staining areas with sufficient glycogen content. Squamous atypia would be transitional lesions from oesophageitis to dysplasia.

Although the prevalence of multiple LULs was low in oesophageal cancer-free subjects (0.9%), dysplasia occurred frequently in subjects with multiple LULs (60%). Muto et al. (2002) reported that multiple LULs were found in 27% of head and neck cancer patients, and secondary ESCCs were found in 72% of such cancer patients with multiple LULs. They provided essential information about field cancerisation and malignant potential with respect to multiple LULs. The field cancerisation phenomena proposed that multiple squamous cell carcinomas occurred either simultaneously with the primary lesion (synchronous) or after a period of time (metachronous) in the oesophagus and the head and neck region. There is a possibility that widespread epithelial oncogenic alterations were found in patients with multiple LULs. In case 2, the same mutation at codon 218 was found in both LUL-NDE and dysplastic lesion, whereas \( p53 \) mutation was not detected in background normal Lugol staining epithelium. The \( p53 \) mutational status, in this case, reflects the phenomena of field cancerisation, which can be considered as high malignant potential.

The \( p53 \) missense mutations containing a hotspot mutation were found in LULs-NDE in oesophageal cancer-free individuals without reflux oesophagitis. The finding suggests that LUL-NDE is an initial lesion for oesophageal carcinogenesis, and that the role of BCH is less clear for oesophageal carcinogenesis in Japanese individuals. The characteristic findings of high-risk population of oesophageal carcinoma were evaluated by genetic analyses, because it appeared that we emphasise the importance of both endoscopic detection of LUL-NDE and molecular diagnosis. We concluded that the understanding of aetiology in human oesophageal precursor at the molecular level could provide essential information about the identification of useful biomarkers for prevention studies.

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LUXUs = Lugol-unstained lesions.

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