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# Injection of LPS Causes Transient Suppression of Biological Clock Genes in Rats

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***Background.*** **The biological clock regulates circadian rhythm and is important for sustaining homeostasis. Here we examined the response of biological clock genes to systemic inflammatory stimulation.**

***Materials and methods.*** **At 08:00 h (= Zeitgeber time [ZT] 01), male Wistar rats (7-wk-old) maintained on a 12:12 h light:dark cycle (light on 07:00–19:00 h) received intravenous injection of 1 mg/kg lipopolysaccharide (LPS group) or 0.3 mL saline (control group). They were then sacrificed every 4 h (09:00 h = ZT 02, 13:00 h = ZT 06, 17:00 h = ZT 10, 21:00 h = ZT 14, 01:00 h = ZT 18, 05:00 h = ZT 22) over a 2-d period, and blood, brain, and liver samples were obtained for analysis ($n = 4$ at each time for each group). The expression levels of clock gene, *rPer2*, and those of clock controlled gene, *rDBP*, were quantified in the suprachiasmatic nucleus by *in situ* hybridization, while those of *rPer1*, *rPer2*, *rDBP*, *rPPARA*, and *rFKBP51* in the liver were determined by quantitative RT-PCR.**

***Results.*** **In the suprachiasmatic nucleus of control rats, *rPer2* and *rDBP* mRNA expression levels showed robust circadian patterns with peak levels at ZT 06 and ZT 10, respectively. LPS significantly suppressed both genes on day 1 but recovery was noted on day 2. Similarly, LPS significantly suppressed *rPer1*, *rPer2*, *rDBP*, *rPPARA*, and *rFKBP51* mRNA expression levels in the liver on day 1 but recovery was noted on day 2, whereas a robust circadian pattern was noted in the control group.**

***Conclusion.*** **Our results indicate that LPS causes transient suppression of the biological clock genes and suggest that the biological clock plays an important role in the response to systemic inflammatory stimulation.** 

***Key Words:*** **rodent; cytokines; inflammation; lipopolysaccharide; molecular biology; clock genes; clock controlled genes; biological clock; biological response; sepsis.**

## INTRODUCTION

The circadian rhythms, which are recognized in all living organisms from yeast to mammals, are daily oscillations in various behavioral, physiological, and biochemical processes. There is ample evidence that the biological clock regulates the circadian rhythm and controls hundreds of functions in the human body, including sleep-wake cycle, body temperature, behavioral pattern, blood pressure, hormone production, digestive secretion, and immune activity [1].

In mammals, the central pacemaker of the biological clock is located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus. The biological clock resides not only in the SCN but also in most peripheral tissues [2]. These "peripheral clocks" are regulated by the "central clock", through both the autonomic nervous system and neuroendocrine system. Recently, the molecular mechanism of the biological clock has been

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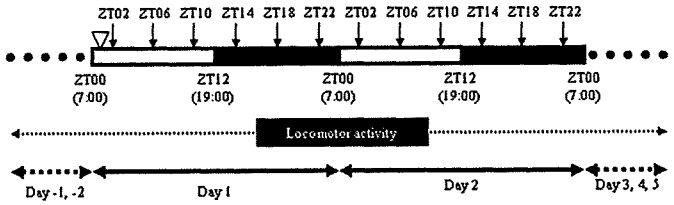



**FIG. 1.** Illustration of experimental protocol. The open bars indicate the light-on phase, and closed bars indicate the light-off phase. White arrowhead indicates the time of LPS (1 mg/kg) or saline (0.3 mL) injection (day 1, ZT 01). Arrows indicate the sampling times of brain, liver, and blood, and four rats were sacrificed at each time point for each group. Locomotor activity was measured from day 2 to day 5.

elucidated. Eight core clock genes, *Clock*, *casein kinase Iε* (*CKIε*), *cryptochrome1* (*Cry1*), *cryptochrome2* (*Cry2*), *period1* (*Per1*), *period2* (*Per2*), *period3* (*Per3*), and *Bmal1* have been identified. The transcriptional feedback loops of clock genes are essential for rhythm generation [3]. On the other hand, we used microarray analysis and liver RNA isolated from *Clock* mutant mice and identified the clock controlled genes (CCG), whose expression is synchronized to the expression of clock genes [4]. These genes are associated with various physiological functions such as cell cycle, lipid metabolism, immune functions, and proteolysis. Based on the above background, it is conceivable that biological clock disorders could be directly associated with various physiological disorders.

The period of free-running oscillation of the clock gene expression is intrinsically close to, but usually not equal to 24 h, and these circadian expressions are entrained by environmental stimuli. The strongest environmental time cue for entraining the clock with the earth's environmental cycle is the light-dark cycle. The role of light as an entrainer has been well investigated. On the other hand, several studies have shown that the expression of clock genes is under the influence of some unusual conditions such as time-imposed restricted feeding [5, 6], glucocorticoid injections [7], and total parenteral nutrition [8, 9]. These results indicate that the biological clock does not only generate circadian rhythmicity but also maintains homeostasis by resetting the phase of the biological clocks in response to environmental changes.

Patients who undergo major surgery or those who suffer from severe sepsis are exposed not only to external but also to internal environmental changes, which include activation of neuroendocrine and cytokine systems. Such patients often display symptoms suggesting a circadian rhythm disorder, such as insomnia or delirium. These suggest that systemic inflammatory stimulus may affect on the biological clock. The aim of this study was to clarify whether the biological clock would respond to systemic inflammatory stimulus. We examined clock gene expression in central and peripheral clocks in rats that were given lipopolysaccharide (LPS).

## MATERIALS AND METHODS

### Animals

Seven-wk-old male SD rats were obtained from Charles River Co., Japan, housed individually, and maintained on a 12/12 h light/dark cycle (light on from 07:00 to 19:00, light off from 19:00 to 07:00). Under the same conditions, eight rats were selected randomly as the behavioral group, and their spontaneous locomotor activity was monitored by SUPERMEX (Muromachi Kikai Co., Ltd., Tokyo, Japan) to analyze the behavioral rhythm [10]. On the eighth day, all rats, including the behavioral group, were divided into two groups at random. The experimental protocol is illustrated in Fig. 1. The control group was administered saline (0.3 mL) through the tail vein at ZT 01 on day 1 (Zeitgeber time 01 = 08:00 h). Zeitgeber time is a standard of time based on the period of a Zeitgeber, which is any external cue that entrains the internal time keeping system of organisms. The strongest Zeitgeber for animals is light. Under 12/12 h light/dark cycle, the time of light-on usually defines ZT 0 and the time of light-off defines ZT 12. Rats of the LPS group received intravenous injection of lipopolysaccharide sodium (1 mg/kg) at the same time. A preliminary study was performed to choose this dose of LPS, which caused an increase of proinflammatory cytokines significantly (data not shown). In the behavioral group, the rhythms were measured for 5 d after administration ($n$ = 4 for each group). Under pentobarbital sodium anesthesia (35 mg/kg), all rats, excluding the behavioral group, were sacrificed at specified times (ZT 02 = 09:00 h, 06 = 13:00 h, 10 = 17:00 h, 14 = 21:00 h, 18 = 01:00 h, and 22 = 05:00 h) over a period of 2 d, and brain, liver, and blood samples were obtained from each animal ($n$ = 4 at each time point for each group). The anesthesia and sampling from ZT 14 to ZT 22 were performed under a dim red light. Blood serum samples were immediately separated from the collecting blood. Brain samples were extracted after fixation with transcardial perfusion through the left ventricle with 4% paraformaldehyde-phosphate-buffered saline. All samples except brain samples were stored at −80°C until analysis. The Committee for Use of Laboratory Animals at Osaka University Medical School approved all experimental procedures described in this report.

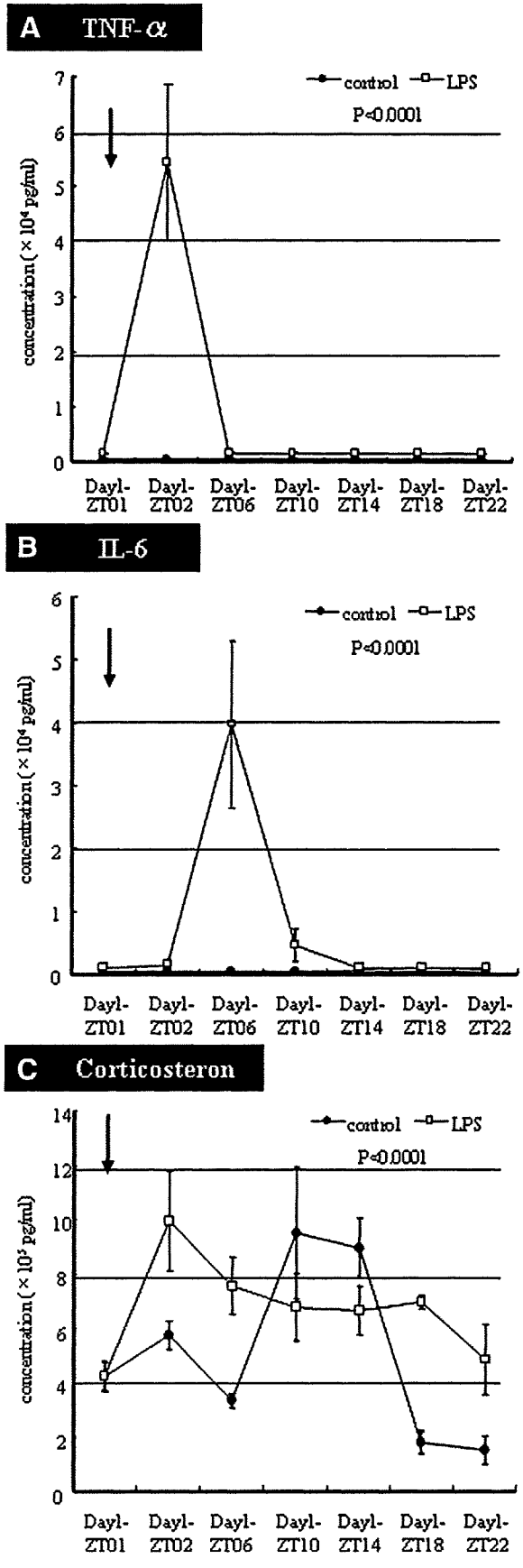
### Measurement of Locomotor Activity

SUPERMEX apparatus surveys the measurement area with multiple lenses of a sensor monitor, which is mounted above the cage and is able to detect the infrared radiation emitted by animals. Locomotor activity was assessed as a single count when the animal moved from one region of the measurement area, which was optically divided by the multiple lenses, to a neighboring region. The animals

**TABLE 1**

**Primers Used in Real Time Reverse Transcription-Polymerase Chain Reaction Analysis**

| Gene | Accession no. | | Primer sequence 5′ to 3′ |
|---|---|---|---|
| *rPer1* | AB092976 | Forward | CCCTCGATGTAACGGCTTG |
| | | Reverse | CCACCCTCTTCCATAGGCT |
| *rPer2* | AB016532 | Forward | GCCAACACAGACGACAATA |
| | | Reverse | GTTGCCTTTCTCCTCACTT |
| *rDBP* | NM_012543 | Forward | TCAGAGGAGGAATTGAAGCC |
| | | Reverse | CCTTGAGTCTTCTTGCGTCT |
| *rPPARA* | NM_013196 | Forward | GGGTCATACTCGCAGGAAAG |
| | | Reverse | TGGCAGCAGTGGAAGAATC |
| *rFKBP51* | XM_342103 | Forward | AGGTGAACCCTCAGAACAA |
| | | Reverse | GCCTTCTTGCTCAATGCTT |
| *rβ-actin* | NM031144 | Forward | GAGGAAGAGGATGCGGCA |
| | | Reverse | CCTAAGGCCAACCGTGAA |




were introduced into an experimental cage and total locomotor activity counts in each 10-min segment were automatically recorded by a personal computer interfaced to the sensor monitor.

### *In Situ* Hybridization of Rat Brain Frozen Sections

The expressions of *rPer2* and *rDBP* genes, which are CCGs known to participate in protein metabolism, were analyzed in brain samples. Brain samples were postfixed and soaked in 0.1 M phosphate-buffered saline containing sucrose. They were then embedded in Tissue-Tek OTC compound (Sakura Finetechnical Co., Tokyo, Japan) and slowly frozen on dry ice. Serial coronal brain sections of 8 μm thickness were prepared with a cryostat (HM 505 E; MICROM, Walldorf, Germany) for examination of the entire SCN region, and kept at −80°C until use. Digoxigenin-labeled RNA probes were generated from *rPer2* cDNA fragment (bases: 1-5453; Gene Bank accession number AB016532) and rDBP cDNA fragment (bases: 1-801; Gene Bank accession number NM_012543) using a DIG RNA labeling kit (Boehringer-Mannheim, Mannheim, Germany). *In situ* hybridization and detection of the probes were carried out as described previously [11, 12]. Densitometric analysis of the hybridization intensity was performed using Mac Scope version 2.5 (Mitani Corp., Fukui, Japan).

### Quantitative RT-PCR Analysis

In addition to *rPer1*, *rPer2*, we analyzed previously the expression levels of various clock genes including *rDBP* (albumin D-site binding protein), *rPPARA* (peroxisome proliferator-activated receptor α), and *rFKBP* (FK506-binding proteins), which are CCGs known to participate in protein metabolism, lipid metabolism, and immune function, respectively, using liver tissue samples [4, 13]. Total RNA was extracted from liver samples obtained in the present study, and cDNA was generated with AMV Reverse Transcriptase (Promega, Madison, WI) using Oligo $(DT)_{15}$ primer. PCR amplification was carried out using Light Cycler (Roche Diagnostics, Mannheim, Germany) [14]. Primers were synthesized by Nihon Gene Research Laboratories (Sendai, Japan) (see Table 1 for a list of primers used). PCR conditions were set up as follows; an initial denaturing step at 95°C for 10 min, and 40 cycles at 95°C for 15 s, 60°C for 10 s, and 72°C for 20 s. Quantification data were analyzed using the Light Cycler™ analysis software. A standard curve was constructed with serial dilutions of cDNA obtained from one of the samples. The expression of each gene in each sample was normalized against β-actin, a housekeeping gene. Reproducibility and dose dependency for quantification using this method were confirmed in a preliminary study using sequentially diluted samples (data not shown).

### Enzyme-Linked Immunosorbent Assay

Blood serum samples were immediately separated and stored at −80°C until use. Inflammatory cytokines (tumor necrosis factor-alpha [TNF-α] and interleukin-6 [IL-6]) and corticosterone were measured by enzyme-linked immunosorbent assay using commercially available kits (TNF-α and IL-6; BioSource, Camarillo, CA, plasma corticosterone; Buehlmann Laboratories, Allschwil, Switzerland and Immunotech-Beckman Coulter Company, Marseille, France).

**FIG. 2.** Changes in plasma concentrations of TNF-α (A), IL-6 (B), and corticosterone (C) after LPS (1 mg/kg) or saline (0.3 mL) injection. Arrows indicate the time of injection (day 1, ZT 01). Data are expressed as mean ± SEM of four rats for the control group and four rats of the LPS group. The *P* value is for comparison of data of the LPS and control groups.

### Statistical Analysis

All data were expressed as mean ± SEM. Differences between groups were examined for statistical significance using one-way and two-way factorial ANOVA analyses. A $P$ value less than 0.05 denoted the presence of a statistically significant difference.

## RESULTS

Intravenous injection of 1 mg/kg LPS increased plasma cytokine levels (Fig. 2). Plasma TNF-$\alpha$ concentration was increased markedly at ZT 02 on day 1. The peak concentration of IL-6 occurred 4 h after that of TNF-$\alpha$. In the control group, no such increases in plasma concentrations of these cytokines were noted. On the other hand, corticosterone concentration increased following LPS injection, and the 24-h rhythm was completely disturbed. The above changes were significantly different between the two groups ($P < 0.01$, each). Figure 3 showed the clock gene expression levels in the SCN. The peak expression levels of *rPer2* and *rDBP* in the control group were observed at ZT 06 and ZT 10, respectively. In the LPS group, both genes were suppressed slightly throughout the day of injection but their levels almost recovered on day 2. The peak expression levels occurred 8 h after those of the control group (at ZT 14 and ZT 18). The above changes in *rPer2* and *rDBP* gene expression levels were significantly different between the two groups ($P < 0.01$, each).

We also analyzed the mRNA expressions of *rPer1* and *rPer2* clock genes in the liver (Fig. 4). The peak expression levels of *rPer1* and *rPer2* in the control group were observed at ZT 14. The expression levels of both genes showed a significant daily fluctuation in the control group, although *rPer1* expression was clearly induced at ZT 02 in saline-injected rats. In the LPS group, the expression levels of both genes were suppressed on day 1 but the levels recovered on day 2. There were significant differences in *rPer1* and *rPer2* expressions between the two groups ($P < 0.01$, each).

The peak mRNA expression levels of *rDBP*, *rPPARA*, and *rFKBP* in the control group were observed around ZT 10 or ZT 14 (Fig. 5). The expression levels of all three genes showed daily fluctuations in the control group, although clear inductions of *rPPARA* and *rFKBP* expressions were noted at ZT 02 on day 1, similar to *rPer1* expression. On the other hand, LPS suppressed the mRNA expression levels of all three genes on day1, although the levels returned to normal on day 2, similar to the expression of clock genes. There were significant differences between the control and LPS groups with regard to *rDBP*, *rPPARA*, and *rFKBP* gene expression ($P < 0.01$, each).

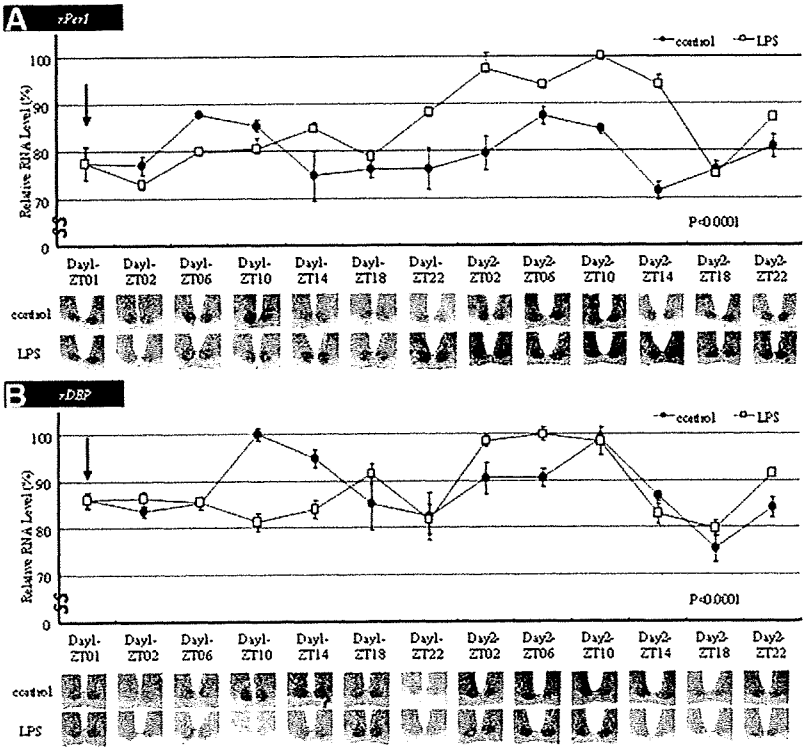



**FIG. 3.** Circadian profiles of *rPer2* (A) and *rDBP* (B) mRNA expression levels in the SCN after LPS (1 mg/kg) or saline (0.3 mL) injection. Data are representative results of *in situ* hybridization and quantitative results of signal densities in the SCN. The peak density was defined as 100%. Arrows indicate the time of injection (day 1, ZT 01). Data are expressed as mean ± SEM of four rats for the control group and four rats of the LPS group. The $P$ value is for comparison of data of the LPS and control groups. (Color version of figure is available online.)

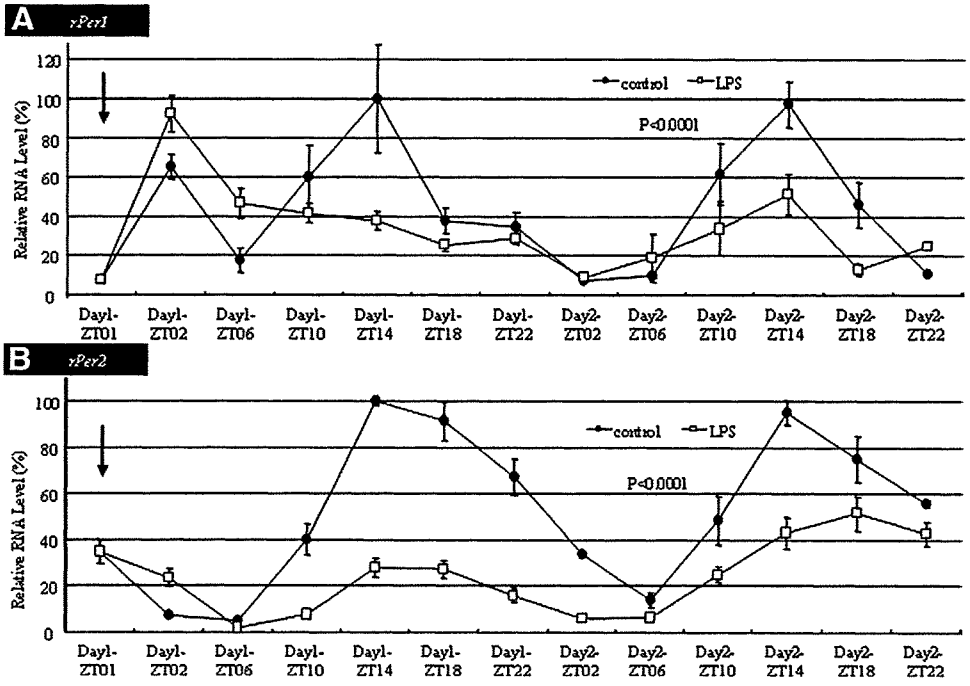



**FIG. 4.** Circadian profiles of *rPer1* (A) and *rPer2* (B) mRNA expression levels in the liver after LPS (1 mg/kg) or saline (0.3 mL) injection. Peak density was defined as 100%. Arrows indicate the time of injection (day 1, ZT 01). Data are expressed as mean ± SEM of four rats for the control group and four rats of the LPS group. The *P* value is for comparison of data of the LPS and control groups.

The locomotor activity was recorded over a 7-d period. The total count of movements during 4-h period was plotted in Fig. 6. Saline-injected control rats showed characteristic rhythmic activity with low daytime and high nighttime activities. The normal circadian rhythm was impaired following LPS injection. Compared with saline-injected rats, LPS caused a significant depression of nighttime activity on days 1 and 2. The normal activity rhythm, which did not exhibit a phase shift, was recognized on day 3 after LPS administration.

## DISCUSSION

This is the first report that shows altered oscillation profile of biological clock gene expression in response to a systemic inflammatory stimulus. Not only the central clock in SCN, but also the peripheral clock in the liver was affected by immune challenge of bacterial LPS. LPS injection significantly suppressed the expression levels of clock genes in both the SCN and liver on day 1. However, this suppression was transient, and normalization of the expression levels was noted on the next day.

Several studies have examined the effects of LPS injection on circadian rhythm. Marpegan *et al.* [15] reported that LPS injection in mice resulted in phase shift of locomotor activity, suggesting that activation of the immune system is an entraining signal for the murine biological clock. However, the investigators did not measure clock gene expression.

Takahashi *et al.* [16] demonstrated that injection of LPS (50 μg/kg) did not affect *mPer1* and *mPer2* expression levels in the SCN of mice. The discrepancy between their results and those of the present study may be explained by differences in the measurement time, the dose of LPS injected, and experimental animal species. They measured mPer1 and mPer2 mRNA levels at 1, 3, and 24 h after LPS injection, but not 8 h after injection, a time point where a significant clock gene induction was seen in our study. In addition, LPS injection induced a significant increase in plasma concentrations of inflammatory cytokines (IL-6 and TNF-α) and activation of the hypothalamic-pituitary-adrenal-axis in our study, whereas such changes were not described the above study. Givalois *et al.* reported that plasma concentrations of IL-6 and IL-1β were significant higher in animals given 1 mg/kg of LPS than those given 50 μg/kg of LPS [17]. Thus, it is possible that systemic inflammatory stimulus was not enough in Takahashi's study.

In the liver, LPS produced early suppression of *rPer1* and *rPer2* mRNA levels, with subsequent suppression of mRNA expression of CCGs including *rDBP*, *rPPARA*, and *rFKBP51*. The albumin D-site binding protein (DBP) is the founding member of the proliferator-activated receptor (PAR) family of bZip transcription factors. DBP binds to the consensus DNA recognition

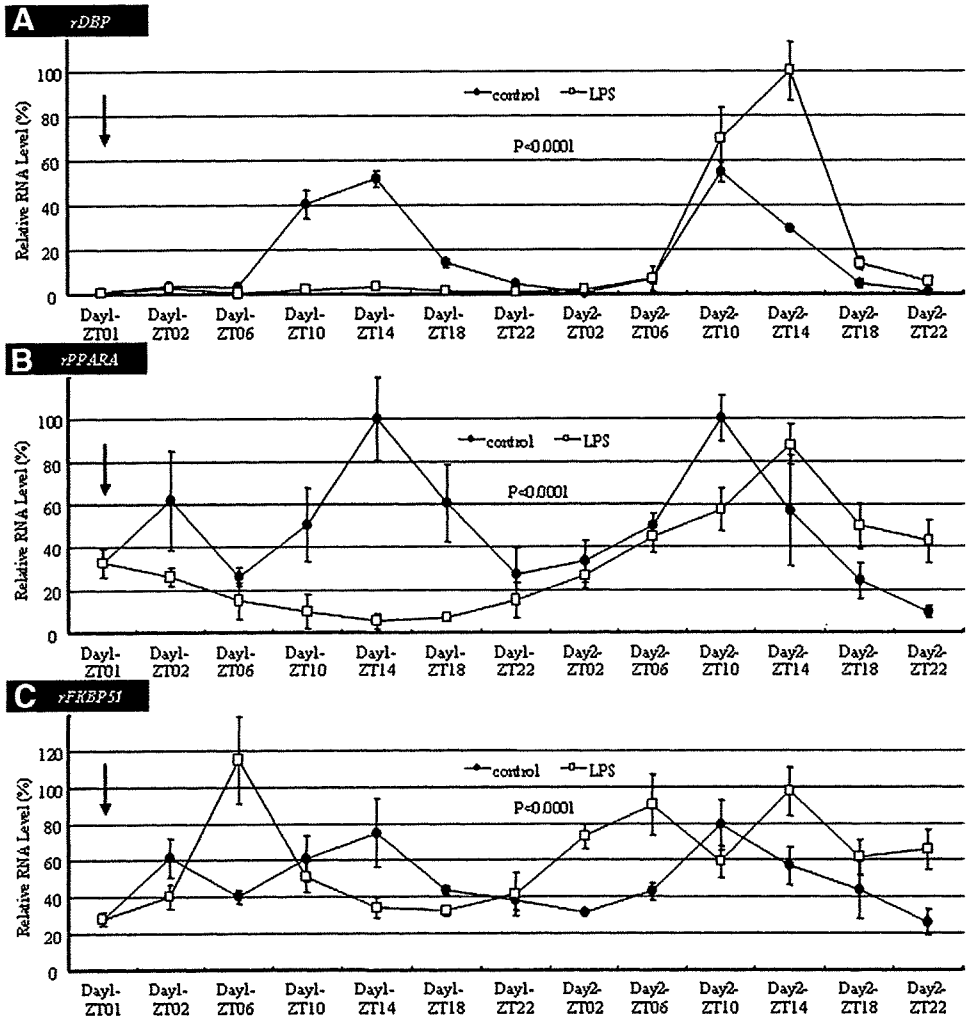



**FIG. 5.** Circadian profiles of *rDBP* (A), *rPPARA* (B), and *rFKBP51* (C) mRNA expression levels in the liver after LPS (1 mg/kg) or saline (0.3 mL) injection. The peak density was defined as 100%. Arrows indicate the time of injection (day 1, ZT 01). Data are expressed as mean ± SEM of four rats for the control group and four rats of the LPS group. The *P* value is for comparison of data of the LPS and control groups.

sequence on the promoters and activates the transcription of several genes, including the albumin gene [18] and the cytochrome P450 cholesterol 7$\alpha$ hydroxylase (CYP7) gene [19, 20], which is the rate limiting enzyme in the conversion of cholesterol to bile acid. Suppression of DBP expression may lead to decreased syn-

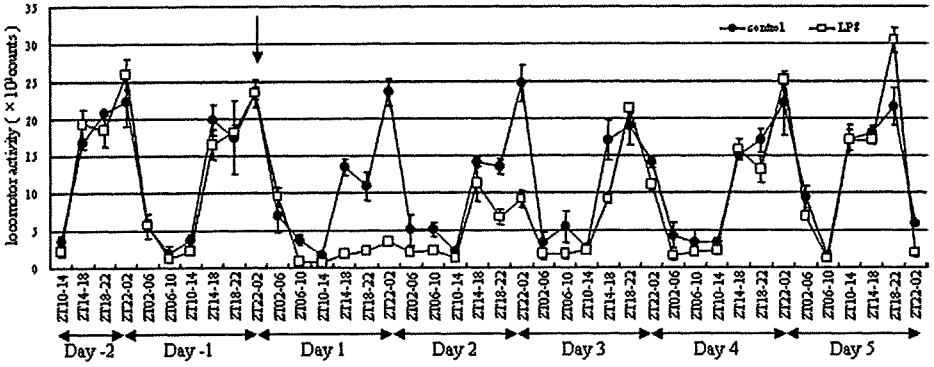



**FIG. 6.** Changes in locomotor activity after LPS (1 mg/kg) or saline (0.3 mL) injection. The locomotor activity assessed every 10 min as movement of animals detected by infrared radiation was recorded over a 7-d period. The total count of movements during 4-h period was plotted. Arrows indicate the time of injection (day 1, ZT 01). Data are expressed as mean ± SEM of four rats for the control group and four rats of the LPS group. The *P* value is for comparison of data of the LPS and control groups.

thesis of both albumin and bile acid. PPARA is a member of the nuclear receptor superfamily of ligand-activated transcription factors, and regulates the gene expression levels of several enzymes associated with the peroxisomal $\beta$-oxidation pathway such as acyl-CoA synthetase, acyl-CoA oxidase, and acyl-CoA dehydrogenase [21–23]. The FK506-binding proteins (FKBPs) constitute a class of intracellular receptors that bind the immunosuppressive drug FK506 and rapamysin [24]. Numerous family members have been identified, including FKBP-12, -13, -25, -51, and -52. It is the FKBP-FK506 complex that binds to and inhibits calcineurin, a serine-threonine phosphatase, which is a key signal transducing molecule leading to the transcription of IL-2, a lymphokine required for T-cell activation [25]. Although we have not examined such changes at protein level, it is supposed that LPS injection may result in functional changes.

Hypermetabolism is well known as one of the characteristic metabolic responses to inflammatory stimulus induced by infection, surgery, and trauma. It is also known that there is a period in which energy expenditure does not increase but rather decreases immediately after the inflammatory stimulus. This hypometabolic period, or the "ebb phase", is followed by hypermetabolic period, i.e., the "flow phase" [26]. The suppression of clock gene and CCG expression, which was noted immediately after LPS injection, may correspond to the "ebb phase". In other words, the biological clock may play an important role in the early phase reaction to severe stress.

It is not clear whether the response of gene expression was caused by the direct action of LPS or by indirect action through other factors. Marpegan *et al.* [15] demonstrated that the phase delaying effect of LPS on locomotor activity was blocked by preadministration of sulfasalazine, a specific NF-$\kappa$B inhibitor. On the other hand, Ohdo *et al.* [27] demonstrated that injection of interferon-$\gamma$ in mice caused suppression of the rhythm of locomotor activity and clock gene expression in the liver and SCN, and that such suppression was mediated by interferon-$\gamma$ receptor in the SCN. Furthermore, Lundkvist *et al.* [28] demonstrated that rhythmic spontaneous action potential firing from SCN neurons was reduced by TNF-$\alpha$ *in vitro*, and induced disruption of circadian rhythm. LPS does not cross the blood-brain barrier in rats but it can bind to specific receptors in the cerebral vascular endothelium, leading to the release of various proinflammatory cytokines such as nitric oxide, IL- 1$\beta$ [29]. On the other hand, LPS-induced cytokines may also enter the brain via the organum vasculosum laminae terminalis where the blood–brain barrier is "leaky" due to the presence of a fenestrated endothelium [30]. Thus, the mechanism through which LPS alters the circadian system to suppress the expression levels of clock genes might be an indirect action through inflammatory cytokines such as IL- 6, TNF-$\alpha$, and IFN-$\gamma$.

In the present study, LPS significantly suppressed locomotor activity on day 1, a response known as "sickness behavior". Similar studies have been reported and their results are in agreement with our observations [31, 32]. In these studies, intraperitoneal injection of a high dose of LPS resulted initially in depression of locomotor activity and induction of hyperthermia, which were followed by a late phase of high body temperature. The suppression in locomotor activity seems to be associated with the suppression of *rPer2* mRNA expression in SCN. However, whether suppression of *rPer2* mRNA expression is responsible for the "sickness behavior" remains unknown at present. On the other hand, based on the recovery of locomotor activity and *rPer2* mRNA expression rhythm as early as day 2, LPS seems to have no significant effect on the phase of the biological clock.

In conclusion, we demonstrated in the present study that a systemic inflammatory stimulus (LPS) acutely suppressed the biological clock both in SCN and liver. Consequently, this resulted in the suppression of several clock-controlled genes that are associated with metabolism and immune response. The mechanism of the biological response to LPS has been studied classically from the viewpoint of neuroendocrine pathway and currently from the viewpoint of cytokine network. To our knowledge, there are no studies that examined the response of CNS and liver clock genes to LPS injection. Integration of biological clock research into the field of biological response to systemic inflammatory stimulation should enhance our understanding of the mechanisms of biological response as well as the development of a new concept on systemic inflammatory response.

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DISEASES OF THE ESOPHAGUS

Original article

# Evaluation of clinical significance of $^{18}$F-fluorodeoxyglucose positron emission tomography in superficial squamous cell carcinomas of the thoracic esophagus

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*SUMMARY.* **$^{18}$F-fluorodeoxyglucose (FDG) positron emission tomography (PET) is used for pre-treatment staging and evaluation of response to pre-operative therapy in advanced thoracic esophageal cancers. To evaluate the clinical significance of PET diagnosis of superficial thoracic esophageal cancers, FDG-PET was conducted preoperatively in 41 patients with such cancers without pre-operative therapy. We compared the PET diagnosis with clinicopathological findings with respect to both the primary tumor and lymph node (LN) metastasis. Of the 41 superficial thoracic esophageal cancers, 21 (51.2%) were PET positive for primary tumors. Although tumor length and histological type did not correlate with FDG uptake by primary tumors, non-flat (elevated or depressed) tumors showed significantly stronger FDG uptake than flat ones. Of 28 tumors infiltrating the deep submucosal layer, 19 (67.9%) were PET positive, while only two (15.4%) of 13 tumors infiltrating only the mucosa or shallow submucosal layer were PET positive. MANOVA identified FDG uptake as the only independent risk factor for deep submucosal invasion (odds ratio, 7.407; $P = 0.0279$). In 13 patients with pathological LN metastasis, although no LN metastasis was detected by FDG-PET, FDG uptake by the primary tumors was the only risk factor for LN metastasis ($P = 0.0318$). PET-negative tumors tended to reflect longer disease-free survival than PET-positive tumors, although this was not significant. FDG-PET is useful for detecting tumors infiltrating the middle or deep submucosal layer (sm2/sm3), and for predicting LN metastasis in patients with superficial thoracic esophageal cancers. FDG-PET is helpful for decision-making regarding treatment of such patients.**

*KEY WORDS:* **FDG-PET, lymph node metastasis, submucosal layer, superficial squamous cell carcinoma.**

## INTRODUCTION

The prognosis of patients with thoracic esophageal cancers is poor,[1-3] partly because the cancer is usually at an advanced stage with lymph node metastasis or hematogenous metastasis at the time of diagnosis. Recently, more frequent endoscopic examinations using the Lugol dye method have led to the identification of a larger number of esophageal cancers defined as superficial tumors limited to the mucosal or submucosal layer.[4] For patients with superficial esophageal cancers, chemoradiotherapy (CRT) and endoscopic mucosal resection (EMR) are other treatment options in addition to surgery[5-8] and selection of the most suitable treatment depends upon precise clinical staging, including the depth of the primary tumor, lymph node involvement and distant metastasis. For instance, when tumors infiltrate only the mucosa or shallow submucosal layer and have no lymph node metastasis, they are candidates for EMR. On the other hand, when tumors infiltrate the deep submucosal layer or are associated with lymph node metastasis, they may be contraindicated for EMR and CRT or surgery may be a more suitable therapeutic option.

Recently, $^{18}$F-fluorodeoxyglucose-positron emission tomography (FDG-PET) has been introduced as a novel diagnostic modality for advanced esophageal cancers. Previous studies have described the usefulness of FDG-PET for evaluating the response to neoadjuvant chemotherapy and chemoradiotherapy, in addition to the staging of advanced esophageal cancers before treatment.[9-12] Since January 2000 in our institution all patients with superficial as well

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as those with advanced esophageal cancers have undergone FDG-PET. We also have used FDG-PET for both pre-treatment staging for decision-making on the therapeutic strategy and for evaluation of the response to neoadjuvant chemotherapy for esophageal cancers. However, until now, there have been no reports that assessed the usefulness of a FDG-PET diagnosis for superficial esophageal cancers and it is not clear whether FDG-PET is helpful for decision-making regarding the treatment of superficial esophageal cancers. The present study was designed to evaluate the clinical significance of FDG-PET for superficial thoracic esophageal cancers.

## MATERIALS AND METHODS

### Patients

From April 2000 to December 2004, 199 patients with thoracic esophageal cancers underwent surgery at the Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University. Among them, 168 patients underwent FDG-PET before treatment. Of the latter patients, 162 were found to have squamous cell carcinomas and six had adenocarcinomas. All patients received endoscopic examinations and upper gastrointestinal series. All patients received helical computed tomography (CT) of the neck, chest and upper abdomen as continuous 5-mm thick slices. All patients were given a 120–150 mL intravenous injection of iodinated contrast material. Lymph nodes measuring more than 10 mm diameter at the longest were diagnosed as metastasis positive by two radiologists working independently. Of 168 patients, 65 received pre-operative chemotherapy and 22 received pre-operative chemoradiotherapy followed by surgery. All 42 patients with cT1 tumors were enrolled in this study, with the exception of one patient who received preoperative chemotherapy after identification of lymph node metastasis by PET. In the 41 cases with superficial esophageal cancers, all visible lesions were classified according to the Japanese classification as type I, type IIa, type IIb, type IIc and type III.[13] In this study, we regarded type IIa, IIb and IIc as flat types and types I and III as non-flat types. All 41 patients underwent resection of the thoracic esophagus with two- or three-field lymphadenectomy via right thoracotomy. During the same period, of the 42 patients who had superficial thoracic esophageal cancers, 35 underwent EMR and seven received CRT.

### Whole body FDG-PET

All patients fasted for at least 4 h before an intravenous administration of approximately 370 MBq of FDG. The serum glucose level of each patient was measured just before the FDG injection and was confirmed to be less than 150 mg/dL. Simultaneous emission–transmission PET scans were acquired 1 h after the FDG injection (transmission source: 68 Ge-68 Ga line source). The FDG-PET was performed with a dedicated PET scanner (HEADTOME/SET 2400 W, Shimazu Co., Kyoto, Japan), which has 32 rings of bismuth germanate detectors and simultaneously produces 63 slices of 3.125 mm in thickness along a 20-cm longitudinal field. The intrinsic resolution was 3.7 mm full width at half-maximum, and the sensitivity of the device was 7.3 cp/Bq/cm$^3$. The scans required four bed positions to cover the area from the neck to the pelvis, each with an acquisition time of 10 min, resulting in a total scanning time of 40 min.

The images were reconstructed with an iterative median root prior reconstruction algorithm (mask size $3 \times 3$, $\beta$ 0.3, subsets 24, iteration 1). Trans-axial, coronal and sagittal images sets were displayed on a monitor screen of a high-resolution computer as a linear gray scale. In semi-quantitative analysis, regions of interests were selected semi-automatically as the most intense area of FDG accumulation for the primary tumor in the PET image, and the maximal standardized uptake value ($SUV_{max}$) was calculated according to the following formula: PET count of the most intense point × calibration factor (MBq/kg)/injection dose (MBq)/bodyweight (kg). The FDG uptake of the tumor is visible when the $SUV_{max}$ is above approximately 2.5. Thus, cases with $SUV_{max}$ in the primary tumor or lymph nodes of $\geq 2.5$ were judged as PET positive.

### Histopathological examination

Serial 4-mm thick tissue sections of the primary tumors and lymph nodes were cut from surgical specimens fixed with 10% buffered formalin, embedded in paraffin and stained with hematoxylin and eosin using routine methods. Tumor depth, tumor extension and lymph node metastasis were examined independently by two pathologists. Infiltration of superficial esophageal cancers was sub-classified into six categories according to the following criteria (Fig. 1): m1, intraepithelial carcinoma; m3, tumor extremely close to or infiltrating the lamina muscularis mucosa; m2, tumors intermediate between m1 and m3; sm1, tumor invasion to the shallow strata (medial third) of the submucosal layer; sm2, tumor invasion into the middle strata (middle third) of the submucosal layer; sm3, massive tumor invasion into the deep strata (outer third) of the submucosal layer.[5,10,14-16]

### Follow-up studies

After discharge from hospital, patients were seen every month for the first 2 years, and every 2 months

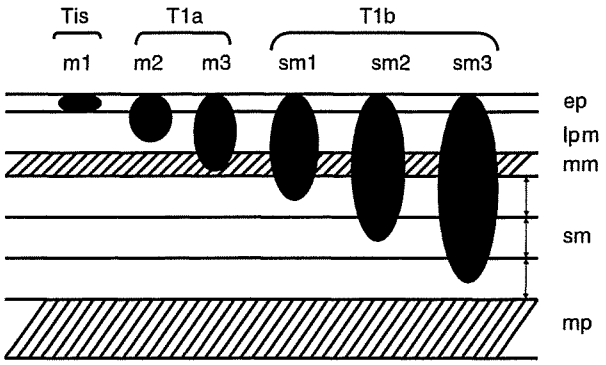


**Fig. 1** Pathologic sub-classification of superficial esophageal cancer. ep, epithelium; lpm, lamina propria mucosa; m1, intraepithelial carcinoma; m2, tumors intermediate between m1 and m2; m3, tumor extremely close to or infiltrating the lamina muscularis mucosa; mm, muscularis mucosa; sm, submucosa; mp, muscularis propria; sm1, tumor invasion to the shallow strata (medial third) of the submucosal layer; sm2, tumor invasion into the middle strata (middle third) of the submucosal layer; sm3, tumor invasion into the deep strata (outer third) of the submucosal layer.

thereafter. CT of the neck, thorax and upper abdomen was performed every 4 months for the first 2 years and every 6 months thereafter, and an upper gastrointestinal endoscopy was performed annually. All data were collected, entered prospectively into a database and updated at regular intervals. The median follow-up period of all 41 patients was 32.1 months (range, 7.6–76.3). Complete follow-up information until death or June 2006 was available for all patients.

### Statistical analysis and ethical considerations

Disease-free survival was calculated from the date of operation to reccurrence or to the last known date of follow-up. Actual survival was calculated by the Kaplan–Meier method and statistically evaluated by the log–rank test. For statistical evaluations, Student's *t*-test, Mann–Whitney's *U*-test, the $\chi^2$ test and logistic regression analysis were used. A *P* value of less than 0.05 was accepted as statistically significant. The above analyses were carried out using the StatView J 5.0 software statistical package (Abacus Concepts, Berkeley, CA).

## RESULTS

### FDG uptake by primary tumor and metastatic lymph nodes

Of the primary tumors, almost all cT2–cT4 tumors were detected by FDG-PET except for three cT2 tumors, while only 22 (52.4%) of 42 cT1 tumors were detected. With respect to lymph node metastasis, the frequency of PET-positive results was 2.4% (1/42) for cT1 tumors, 40.9% (18/44) for cT2 tumors, 57.6% (34/59) for cT3 tumors and 60.9% (14/23) for cT4 tumors, respectively.

### FDG uptake by the primary tumor in 41 superficial thoracic esophageal cancers

Among the 42 patients with cT1 tumors, one was diagnosed as having lymph node metastasis by FDG-PET and received pre-operative chemotherapy. This case was excluded from the following examinations. Table 1 summarizes the relationship between FDG uptake by the primary tumor and the clinical findings in 41 patients with superficial esophageal cancers. All 41 patients were found to have squamous cell carcinomas. With respect to the location of the primary tumor, the frequencies of PET-positive results in the upper, middle and lower thoracic esophageal cancers were 75.0% (6/8), 40.9% (9/22) and 54.5% (6/11), respectively. With respect to the gross type of the primary tumors, non-flat tumors (elevated or depressed types) showed significantly more PET-positive cases than flat tumors (73.7% *vs* 31.8%). Of

**Table 1** Relationship between $^{18}$F-fluorodeoxyglucose (FDG) uptake by the primary tumor and clinicopathological findings in 41 superficial esophageal cancers

| Superficial esophageal cancers | | ($n = 41$) | FDG uptake by the primary tumor | | *P* value |
|---|---|---|---|---|---|
| | | | Positive | Negative | |
| Age of patient | | 63.3 ± 8.9 | 64.0 ± 7.4 | 62.4 ± 10.3 | 0.2695 |
| Sex of patient | Male | 36 | 19 | 17 | 0.5922 |
| | Female | 5 | 2 | 3 | |
| Location of tumor | Upper | 8 | 6 | 2 | 0.2472 |
| | Middle | 22 | 9 | 13 | |
| | Lower | 11 | 6 | 5 | |
| Macroscopic type | Flat type | 22 | 7 | 15 | 0.0075 |
| | Non-flat type | 19 | 14 | 5 | |
| Histopathological type | Well scc | 8 | 5 | 3 | 0.7726 |
| | Mod scc | 25 | 12 | 13 | |
| | Por scc | 8 | 4 | 4 | |
| Length of tumor | < 30 mm | 21 | 12 | 9 | 0.4369 |
| | ≥ 30 mm | 20 | 9 | 11 | |
| Lymph node status | pN(–) | 28 | 11 | 17 | 0.0249 |
| | pN(+) | 13 | 10 | 3 | |

**Table 2** Relationship between $^{18}$F-fluorodeoxyglucose (FDG) uptake by the primary tumor and tumor depth in 41 superficial esophageal cancers

| Superficial esophageal cancers | ($n = 41$) | FDG uptake by the primary tumor | | $P$ value |
|---|---|---|---|---|
| | | Positive | Negative | |
| Depth of tumor invasion | m3 (7) | 1 | 6 | 0.0010 |
| | sm1 (6) | 1 | 5 | |
| | sm2 (3) | 1 | 2 | |
| | sm3 (25) | 18 | 7 | |
| Total | (41) | 21 | 20 | |

m3, tumor extremely close to or infiltrating the lamina muscularis mucosa; sm1, tumor invasion to the shallow strata (medial third) of the submucosal layer; sm2, tumor invasion into the middle strata (middle third) of the submucosal layer; sm3, tumor invasion into the deep strata (outer third) of the submucosal layer.

**Table 3** ANOVA and MANOVA of risk factors for sm2 or sm3 invasion

| Variables | ANOVA | | MANOVA | | |
|---|---|---|---|---|---|
| | Odds ratio | $P$ value | Odds ratio | 95% CI | $P$ value |
| Location of the primary tumor<br>Upper versus middle/lower | 1.571 | 0.6145 | | N.D. | |
| Histopathological type<br>Poor versus not poor | 4.200 | 0.2040 | | N.D. | |
| Macroscopic type<br>Non-flat versus flat type | 8.000 | 0.0159 | 4.760 | 0.771–29.375 | 0.0928 |
| Length of the primary tumor<br>≥ 30 mm versus < 30 mm | 1.083 | 0.9058 | | N.D. | |
| FDG uptake by primary tumor<br>Positive versus negative | 10.99 | 0.0059 | 7.407 | 1.242–43.478 | 0.0279 |

FDG, 18F-fluorodeoxyglucose, CI, confidence interval; N.D. not done; sm2, tumor invasion into the middle strata (middle third) of the submucosal layer; sm3, tumor invasion into the deep strata (outer third) of the submucosal layer.

19 non-flat tumors there were no significant differences in FDG uptake between the elevated (0-I type) and the depressed type (0-III type). Neither tumor differentiation nor tumor length correlated with FDG uptake.

With regard to tumor invasion in superficial esophageal cancers, seven of the 41 were m3 tumors, six were sm1, three were sm2 and 25 were sm3 (Table 2). With regard to the relationship between FDG uptake and tumor depth, 19 (67.9%) of 28 sm2/sm3 tumors were PET positive, while only two (15.4%) of 13 m3/sm1 tumors were PET positive. Thus, the sensitivity and specificity rates for predicting sm2/sm3 invasion by FDG uptake were 67.9% and 55.0%, respectively. Thus, there was a significant tendency for FDG-PET to detect tumors infiltrating the deep submucosal layer ($P = 0.0010$, Table 2).

In ANOVA, among the various clinicopathological variables tested, the non-flat tumors and positive FDG uptake of primary tumors were statistically significant risk factors for sm2/sm3 invasion (Table 3). In MANOVA using these two factors, positive FDG uptake of the primary tumors was the only significant independent risk factor for sm2/sm3 invasion (Table 3).

## Lymph node metastasis by PET diagnosis

In 42 patients with superficial esophageal cancers at initial diagnosis, only one showed a positive FDG uptake for lymph node metastasis and received pre-operative chemotherapy followed by surgery. Of the remaining 41 superficial esophageal cancers without pre-operative therapy, 13 showed pathological lymph node metastasis, but none of these could be detected by FDG-PET. With respect to predictors of lymph node metastasis, ANOVA revealed that positive FDG uptake by the primary tumors was the only statistically significant risk factor for lymph node metastasis. A borderline risk factor for predicting lymph node metastasis in ANOVA was sm2/sm3 invasion (Table 4).

## Disease-free survival of 41 patients with superficial thoracic esophageal cancer

The m3/sm1 tumors showed better prognosis than the sm2/sm3 tumors (3-year disease free survival rate; 90.9% *vs* 74.3%, Fig. 2a). Similarly, pN0 tumors showed better prognosis than pN1 tumors (3-year disease free survival rate; 87.5% *vs* 63.5%, Fig. 2b). However, these differences were not statistically significant. In an analysis of survival based on FDG uptake by primary tumors, PET-negative tumors tended to show longer disease-free survival than PET-positive tumors (3-year disease free survival; 87.5% *vs* 73.0%), although this difference was not statistically significant.

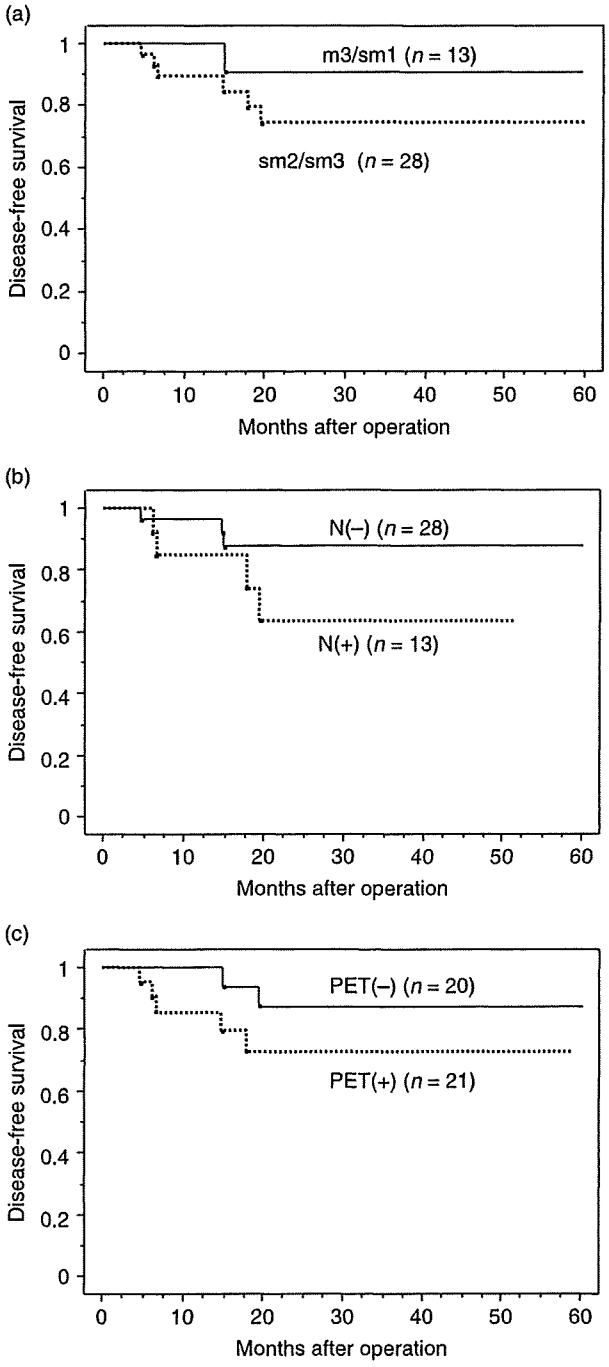


**Fig. 2** Disease-free survival after esophagectomy of 41 patients with superficial thoracic esophageal cancer according to the depth of tumor invasion, (a) pathological lymph node involvement (b) pathological lymph node involvement and $^{18}$F-fluorodeoxyglucose (FDG) uptake by the primary tumor (c) N(–), no lymph node metastasis; N(+), lymph node metastasis. PET(–), negative FDG uptake by the primary tumor; PET(+), positive FDG uptake by the primary tumor.

## DISCUSSION

Previous studies demonstrated that only 0% to 8.7% of mucosal tumors showed lymph node metastasis while the frequency of lymph node metastasis in submucosal tumors ranged from 38.9 to 53.3%.[5,14,15,17-19]

**Table 4** ANOVA of risk factors for lymph node metastasis

| Variables | Odds ratio | *P* value |
|---|---|---|
| Location of the primary tumor | | |
| Upper versus middle/lower | 3.000 | 0.1785 |
| Histopathological type | | |
| Poorly versus not poorly | 1.533 | 0.6063 |
| Clinical N(+) | | |
| cN(+) versus cN(–) | 1.680 | 0.4022 |
| Macroscopic type | | |
| Non-flat versus flat | 3.460 | 0.1054 |
| The depth of tumor invasion | | |
| sm2/sm3 versus m3/sm1 | 8.250 | 0.0578 |
| Length of the primary tumor | | |
| ≥ 30 mm versus < 30 mm | 2.309 | 0.2455 |
| Lymphatic invasion | | |
| Positive versus negative | 1.213 | 0.7816 |
| FDG uptake by primary tumor | | |
| Positive versus negative | 5.155 | 0.0318 |

FDG, $^{18}$F-fluorodeoxyglucose; m3, tumor extremely close to or infiltrating the lamina muscularis mucosa; mm, muscularis mucosa; sm, submucosa; mp, muscularis propria; sm1, tumor invasion to the shallow strata (medial third) of the submucosal layer; sm2, tumor invasion into the middle strata (middle third) of the submucosal layer; sm3, tumor invasion into the deep strata (outer third) of the submucosal layer.

Furthermore, when submucosal tumors were subclassified into three categories (sm1, sm2 and sm3), only 11.1–26.5% of the sm1 tumors showed lymph node metastasis, whereas the incidence of lymph node metastasis in sm2/sm3 tumors was at least 41.9–53.0%, being equivalent to that of advanced esophageal cancers.[5,14,15] Thus, sm2 and sm3 tumors need to be treated using a protocol identical to that used for advanced esophageal cancers, such as esophagectomy with lymphadenectomy or chemoradiotherapy. In our study, 19 (90.5%) of 21 PET positive tumors showed sm2 or sm3 invasion. Consequently, FDG-PET could distinguish tumors infiltrating the deep submucosal layer (sm2/sm3 tumors) from superficial esophageal cancers. Previous reports revealed that non-flat (elevated or depressed) tumors of the macroscopic type had a close correlation with deep submucosal invasion.[5] In our series, ANOVA indicated that a non-flat tumor was a significant risk factor for sm2 or sm3 invasion, whereas MANOVA did not. Part of the reason for this may be that some patients with flat superficial esophageal cancers received EMR and were excluded from this study. In fact, those with flat tumors enrolled in this study tended to have more invasive tumors than those for which EMR was applied.

Recently, in Japan where gastric cancers are more common than in the West, superficial esophageal cancers have been more frequently identified as in our series because endoscopic examinations are more frequently performed and the indication of EMR has been extended to include a radical resection procedure for mucosal tumors.[5] Furthermore, a recent study demonstrated that EMR is a feasible and safe procedure for patients with tumors that are slightly

invading the submucosa if they have no lymph node metastasis.[20] In the present study, FDG uptake by the primary tumors was a good indicator for lymph node metastasis in patients with superficial esophageal cancers. Thus, in such cancers, PET-positive tumors can be excluded from the indication for an EMR procedure.

In the present study we examined the correlation between FDG uptake by the primary tumors and clinicopathological factors, and found that the FDG uptake correlated with the gross type and depth of tumor invasion. However, there was no correlation between tumor length and the FDG uptake. The reason for this negative result may be intraepithelial spread, which, in superficial esophageal cancers, often shows beyond the range of the main tumor. Because tumor length measurement in our study included intraepithelial spread, tumor length did not always reflect tumor volume and consequently may not correlate with FDG uptake. With respect to the histopathological type, as previously reported, PET sensitivity tended to be lower for well differentiated carcinomas compared with poorly differentiated ones.[21] In our study, however, there was no correlation between the degree of differentiation and FDG uptake in superficial esophageal cancers.

In the diagnosis of lymph node metastasis, the incidences of positive FDG uptake were 40.9% in cT2, 57.6% in cT3 and 60.9% in cT4, although we could not calculate the exact sensitivity because neo-adjuvant chemo- or chemoradiotherapy was applied for almost all patients with PET-positive lymph nodes. However, the sensitivity in this study for lymph node metastasis in cT2–cT4 tumors may be equivalent to that of previous studies, because the estimated specificity for the diagnosis of lymph node metastasis is quite high, as reported previously (median: 90.0%).[22–26] On the other hand, only one case (2.4%) of 42 cT1 tumors was PET positive for lymph node metastasis although 31.7% (13/41) of the superficial esophageal cancers showed pathological lymph node metastasis. This result may arise partly because the size of lymph node metastasis of superficial esophageal cancers is smaller than that of advanced esophageal cancers. Komori *et al.*[27] showed that there was a significant correlation between the area of the largest cancer nest among all metastatic lymph nodes and the depth of tumor invasion, and that in 62% of T1 tumors, the largest cancer nest of the lymph node metastasis was less than 25 $mm^2$. On the other hand, previous studies demonstrated that the smallest lymph node metastasis that was detected by FDG-PET was 6–8 mm in size.[28,29] In our study, the average size of 21 lymph node metastasis in 13 pN(+) patients that could not be detected by FDG-PET was 5.5 mm (range, 2.2–14.5 mm).

In ANOVA, cN(+), according to CT diagnosis, was unfortunately not a statistically significant risk factor for lymph node metastasis. This may result from the high incidence of false negatives for lymph node metastasis. In our study, nine (69.3%) of 13 superficial esophageal cancers with lymph node metastasis were diagnosed as cN(–) by CT. Endoscopic ultrasonography (EUS) is considered to be superior to CT for evaluating regional lymph node metastasis.[30,31] The diagnostic accuracy of EUS for lymph node involvement is reported to be approximately 80%.[30–34] To our regret, we could not perform an EUS study in this series because EUS is not routinely performed in the staging of esophageal cancers in our institute and it is performed for only restricted cases, but assessing lymph node metastasis using both EUS and CT may reduce the number of false negatives for lymph node metastasis in superficial thoracic esophageal cancers. Indeed, EUS is probably the best modality for detecting lymph node metastasis in superficial esophageal cancers, but it requires skill, and it is an invasive methods in certain senses. We consider that FDG-PET is more objective and less invasive than EUS. Previous studies showed lymphatic invasion to be a good predictor for lymph node metastasis in superficial esophageal cancers.[16,19,20] In our series, lymphatic invasion did not correlate with lymph node metastasis, whereas FDG uptake by the primary tumors was a significant risk factor for predicting lymph node metastasis in superficial esophageal cancers. Lymphatic invasion is diagnosed based on a pathological examination after surgical resection, such as EMR and esophagectomy, while FDG-PET is a non-invasive diagnostic modality used before treatment. Thus, FDG uptake by the primary tumors may be a clinically more useful factor for predicting lymph node metastasis than lymphatic invasion.

In conclusion, FDG-PET is useful for detecting tumors infiltrating the middle or deep submucosal layer (sm2/sm3), and for predicting lymph node metastasis in patients with superficial thoracic esophageal cancer. FDG-PET is helpful for making a decision about treatment for superficial thoracic esophageal cancers.

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DISEASES OF THE ESOPHAGUS

Original article

# Long-term and short-term evaluation of esophageal reconstruction using the colon or the jejunum in esophageal cancer patients after gastrectomy

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*SUMMARY.* **For esophageal cancer patients, the gastric tube is the first choice as an esophageal substitute, with the colon or the jejunum being used when the stomach cannot be used. We retrospectively compared these two methods from the viewpoint of peri-operative complications and long-term bodyweight alteration. From 1998 to 2005 53 patients who had undergone subtotal esophagectomy due to thoracic esophageal cancers were given reconstruction with the colon (28 cases) or the jejunum (25 cases). Both intestines were reconstructed via the subcutaneous route and were anastomosed to the internal mammalian artery and vein for a supercharged blood supply. There was no difference in operating time and blood loss. Compared with the colon reconstruction group, the hospital stay of the jejunum reconstruction group was significantly shorter (65 days *vs* 45 days, $P = 0.0120$) and the incidence of anastomotic leakage tended to be less (13 cases, 46% *vs* 6 cases, 24%, $P = 0.1507$), while other operative morbidity did not differ between the two groups. Bodyweight loss, which is a serious postoperative sequela after esophagectomy, was less in the jejunum group than in the colon group, showing a significant difference at 12 months after surgery. Our retrospective study revealed the jejunum to be superior to the colon for the reconstruction after esophagectomy along with gastrectomy, with respect to anastomotic leakage and bodyweight loss. The next step will be to conduct a prospective large cohort study.**

*KEY WORDS*: **esophageal cancer, gastrectomy, reconstruction, supercharged anastomosis.**

## INTRODUCTION

As an esophageal replacement for the reconstruction after subtotal esophagectomy, the first choice is the gastric tube. However, the stomach sometimes cannot be used due to a prior gastrectomy or the coincidence of gastric disorder, including gastric cancer and esophageal cancer invasion into the stomach. According to Japanese reports, reconstruction using organs other than the stomach accounted for 10–15% of all esophageal cancer patients undergoing surgical treatment.[1] The postoperative survival rate of esophageal cancers with a history of gastrectomy is not less than for cases without such a history,[2] therefore surgical treatment is recommended for these patients. However, esophageal reconstruction other than with the gastric tube involves a complicated surgical procedure and shows higher operative morbidity and mortality rate than gastric tube reconstruction.[3,4]

Either the jejunum or the colon can be employed as a substitute for the gastric tube. In our institution, the right colon including the terminal ileum had been used as the first choice. The most serious complication, although rare, was necrosis of the pedicled colon due to insufficient blood supply. This disappeared after we began using a supercharged blood supply by anastomosing the ileocecal artery and vein to the internal mammalian artery and vein.[5] The pedicled jejunum is limited with respect to extension length due to poor connection of marginal vessels, and it was therefore used for lower anastomosis, for example, intra-thoracic anastomosis after partial resection of the lower esophagus. We introduced a supercharge technique to the jejunal reconstruction, anastomosis of internal mammalian vessels with jejunal vessels, which allows us to obtain a sufficient length of the pedicled jejunum

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with sufficient blood flow for cervical anastomosis.[6] In our institution, supercharge anastomosis was introduced for colon reconstruction in 1998 and then widely applied to jejunum reconstruction after 2000. Either the colon or the jejunum was used between 2000 and 2003, but after 2004, the jejunum has been the first choice. This conversion occurred over an extended period as this operation is not frequently performed and the benefit of jejunum reconstruction was not well recognized. At present most surgeons in our institution consider jejunal reconstruction to be superior to that using the colon on several points. We therefore performed this retrospective study to compare the peri-operative and long-term results of these two procedures.

## PATIENTS AND METHODS

### Patients

From 1998 to 2005 a total of 412 patients underwent subtotal esophagectomy with reconstruction due to thoracic esophageal cancer in our department. Fifty-seven (11%) of them had reconstruction with organs other than the stomach. Fifty-three patients, excluding one who died within one month due to liver failure with anastomotic leakage after colon reconstruction, and three who died within 6 months due to esophageal cancer, were enrolled in this retrospective study. The reconstruction had been done with the pedicled intestine using either the right colon, including the terminal ileum (colon group, 28 patients, 53%) or the jejunum (jejunum group, 25 patients, 47%).

The significant difference between the two groups was the period of surgery. The first jejunal reconstruction was performed at the end of 1999. Until then, the colon was the first choice when the stomach could not be used. After 2004, the jejunum has been chosen as the primary reconstruction organ. Thirty-eight patients (72%) underwent gastrectomy before esophagectomy, while the stomach was simultaneously removed with the esophagus in the other cases (15 patients, 28%) (Table 1). The former cases involved distal gastrectomy with Billroth-I (20 patients) or Billroth-II (15 patients) reconstruction or total gastrectomy with Roux-en-Y reconstruction (three patients). The reasons for not being able to use the stomach included gastric cancer (27 patients, 51%) or gastroduodenal peptic ulcer (21 patients, 40%) or involvement of the stomach due to esophageal cancer (five patients, 9%). All

**Table 1** Background of esophageal cancer patients given esophageal reconstruction using the colon or the jejunum

| | Esophageal reconstruction | | | |
|---|---|---|---|---|
| | Colon | Jejunum | Total | *P*-value* |
| Gender | | | | |
| Male | 26 | 23 | 49 | |
| Female | 2 | 2 | 4 | > 0.9999 |
| Age | | | | |
| Average | 63.5 + 7.2 | 66.5 + 7.8 | | 0.1370 |
| Cancer stage† | | | | |
| 0 | 2 | 2 | 4 | |
| I | 5 | 2 | 7 | |
| II | 7 | 10 | 17 | |
| III | 10 | 5 | 15 | |
| IV | 4 | 6 | 10 | 0.7541 |
| Adjuvant therapy‡ | | | | |
| None | 17 | 15 | 32 | |
| Preoperative | 9 | 8 | 17 | |
| Postoperative | 2 | 2 | 4 | 0.9741 |
| Operation period | | | | |
| –1999 | 9 | 1 | 10 | |
| 2000–03 | 17 | 16 | 33 | |
| 2004– | 2 | 8 | 10 | 0.0019 |
| Period of gastrectomy | | | | |
| Synchronous | 5 | 10 | 15 | |
| Metachronous | 23 | 15 | 38 | 0.1257 |
| Reason for gastrectomy | | | | |
| Gastric cancer | 11 | 16 | 27 | |
| Peptic ulcer | 15 | 6 | 21 | |
| ESCC involvement | 2 | 3 | 5 | 0.0894 |
| Total | 28 | 25 | 53 | |

*Fisher's exact test for gender and period of gastrectomy, $\chi^2$ test for adjuvant therapy and reason for gastrectomy, Student's *t*-test for age and Mann–Whitney *U*-test for cStage and operation period.
†TNM classification.
‡Adjuvant therapy includes 11 cases of chemotherapy and 10 cases of chemoradiotherapy.
ESCC, esophageal squamous cell carcinoma.

patients with peptic ulcers (17 out of 27 patients with gastric cancers but none with gastric involvement due to esophageal cancer) underwent prior distal gastrectomy before esophagectomy. The interval from the gastrectomy to esophagectomy in these patients averaged 22 years (4–37 years). Since gastrectomy due to peptic ulcers has been decreasing recently, the jejunum group tended to include more patients with gastric cancer and synchronous gastrectomy than the colon group. During this period our strategy for each stage of esophageal cancer has basically remained unchanged, and the distribution of the clinical tumor stage, according to TNM classification[7] and the mode of adjuvant therapy was not different between the two groups. Other factors, including age, sex, tumor location and histological type, were similar in both groups.

### Surgical procedure

At first, all patients underwent a right thoracotomy and thoracic node resection as a standard procedure.[8,9] Next, upper abdominal lymph node dissection, including the peri-gastric and celiac nodes was performed, though most of the nodes had already been removed for patients who had had a gastric cancer operation. For 15 synchronous gastrectomy patients, the whole stomach was removed. For 35 patients after distal gastrectomy, a gastric remnant was preserved in 15 of 22 patients in the colon group, while it was removed in all 13 patients in the jejunum group. Cervical node dissection, i.e. three-field lymph node dissection, was performed for the upper esophageal tumor and those with upper mediastinal node metastasis, as we have previously reported.[9]

In the colon reconstruction group, the ileum and the right colon were mobilized from the retroperitoneum. The ileocecal artery and vein were resected from its beginning and the ileum was resected at the feeding lesion of this vessel. On resection of the right colic artery and vein, the ileum and the right colon were lifted using the middle colic artery and vein as a pedicle. In the jejunum reconstruction group, after resection of the second and the third jejunal artery and vein, the jejunum was lifted upward using the fourth jejunum vessels as a pedicle. For lifting via the subcutaneous route, the intestine was anastomosed at the anal end of the esophagus, mostly by circular stapling (22 mm or 25 mm diameter) or, rarely, by hand sewing (the Albert–Lembert method). There was no difference in the sewing procedure for the colon and jejunum groups. In the right anterior thorax, supercharge anastomosis of either the ileocecal or the second jejunal vessels to the internal mammalian vessels was performed under a microscope, as previously reported.[2,5]

Going back to the abdominal field, in the colon group, the anal end of the pedicled colon was anastomosed to the remnant stomach when it was preserved, or to the jejunum when it was removed, or to the duodenum in synchronous total gastrectomy. Finally, the residual ileum and the colon were anastomosed. In the jejunum group, Roux-en-Y reconstruction was performed for all patients. Among three patients given prior total gastrectomy, reconstruction for one was done by interposition of the colon and for two by shifting the Roux-en-Y structure in an upward direction by resecting the jejunal vessels.

Mechanical ventilation was removed the following day after surgery, unless there was a pulmonary complication. Postoperative pulmonary complication was defined as occurring when mechanical ventilation was continued for more than 5 days or a temporary thoracostomy had to be done. Anastomotic leakage was diagnosed by clinical symptoms or esophagography. A major leakage was defined as a condition that could not be cured within 30 days. Other complications included recurrent nerve paralysis, arrhythmia and wound infection, although they were not life threatening nor did they have a significant effect on the length of stay in hospital.

### Surveillance of bodyweight change

The physical condition of the patient after surgery was checked every month for the first six months, then every two months until one year and every 3 months thereafter. The patients' bodyweight data before and after surgery was retrospectively obtained from their charts. Tumor recurrence was surveyed every 3 months by a physical examination and serum tumor markers, every 6 months by computed tomography scan and abdominal ultrasonography, and every year by fiberscopy, until tumor recurrence was evident. When tumor recurrence was diagnosed, bodyweight data was collected until 6 months before the recurrence and abandoned thereafter. The median follow-up period of all 53 patients was 4.8 years and the average was longer in the colon group than in the jejunum group (5.7 years *vs* 3.8 years, $P = 0.0055$).

### Statistical analysis

Differences in the continuous values, including age, operative time, blood loss, bodyweight loss and hospital stay, were evaluated by Student's *t*-test. The association between two non-continuous parameters was evaluated by the Mann–Whitney's *U*-test, $\chi^2$ test and Fisher's exact test. Overall survival rates were calculated by the Kaplan–Meier method and evaluated by the log-rank test. A $P$ value of less than 0.05 was accepted as statistically significant.

## RESULTS

### Operative outcome

There was no significant difference in operative time, blood loss, or lymph node dissection between the two groups (Table 2). With respect to post-operative complications, anastomotic leakage tended to be observed more often in the colon group than in the jejunum group, though the difference was not statistically significant (13 cases *vs* six cases, $P = 0.1507$), while pulmonary and other complications, including recurrent nerve paralysis, were observed to the same extent in both groups. Major leakage, taking more than 30 days to heal before oral food intake could be started, tended to be more frequent in the colon group than in the jejunum group (8/13 cases [62%] *vs* 2/7 cases [29%]). Three cases in the colon group required re-operation, i.e. a musclo-cutaneous patch by pedicled major pectoral muscle, due to prolonged leakage. Anastomotic leakage was the most important factor delaying hospital discharge; the postoperative hospital stay was significantly longer for those with anastomotic leakage than those without it (85 days *vs* 38 days, $P < 0.0001$). There was no difference in the postoperative hospital stay between the colon and the jejunum group without anastomotic leakage (40 days *vs* 36 days), while it tended to be longer in the colon group than in the jejunum group when anastomotic leakage had occurred (94 days *vs* 65 days). In sum, postoperative hospital stay was significantly longer for the colon group than the jejunum group (65 days *vs* 45 days, $P = 0.0120$). With respect to survival, the overall 5-year survival rate was 45.8% in this series and there was no significant difference between the colon and the jejunum groups (Fig. 1).

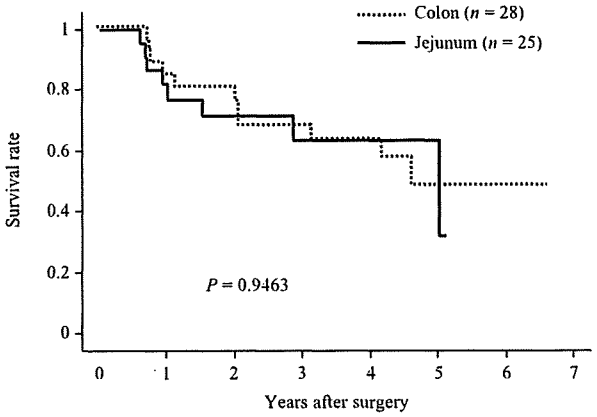


**Fig. 1** Post-operative survival rate of esophageal cancer patients with colon or jejunum reconstruction. Fifty-three esophageal cancer patients could not have stomach reconstruction due to metachronous or synchronous gastrectomy. Their survival curves were plotted by the Kaplan Meier method for colon reconstruction (dotted line) and jejunum reconstruction (continuous line) groups. The difference between two curves was evaluated by log–rank test.

### Bodyweight alteration

Since there was no significant difference in preoperative bodyweight between the colon and the jejunum groups (BMI 20.38 *vs* 20.05, $P = 0.345$), the percent bodyweight loss was used for the comparison (Fig. 2). During the hospital stay, the bodyweight loss was more significant in the colon group than in the jejunum group (–9.6% *vs* –6.7%, $P = 0.0422$) (Table 2). After discharge from the hospital, there was no difference until 6 months. After 6 months, bodyweight gain was observed in the jejunum group while it continued to decline in the colon group, leading to a significant difference at 12 months after surgery (–8.2% *vs* –14.3%, $P = 0.0315$) (Fig. 2a). After 18 months, the difference of

**Table 2** Operative outcome of esophagectomy with reconstruction using the colon or the jejunum

| | Esophageal reconstruction | | |
|---|---|---|---|
| | Colon | Jejunum | *P*-value* |
| Operative time (min) | 638 ± 102 | 666 ± 133 | 0.4005 |
| Blood loss | 1103 ± 531 | 1185 ± 589 | 0.5977 |
| Lymph node dissection | | | |
| Two field | 18 | 15 | |
| Three field | 10 | 10 | 0.7832 |
| Post-operative complications | | | |
| Anastomotic leakage | 13 | 6 | 0.1507 |
| Pulmonary | 2 | 2 | > 0.9999 |
| Others | 5 | 3 | 0.7078 |
| Hospital stay (days) | | | |
| All cases | 65 ± 38 | 45 ± 23 | 0.0120 |
| With leakage | 94 ± 38 | 65 ± 18 | 0.1706 |
| Body weight loss in hospital (%) | –9.6 ± 3.7 | –6.7 ± 6.6 | 0.0422 |

*Student's *t*-test for operative time, blood loss, hospital stay and bodyweight loss. Fisher's exact test for lymph node dissection and each postoperative complication.