Table 1. Relationship between clinical characteristics and genotypes of *ADH1B* and *ALDH2* (n = 66)

	Group 1 (n = 13)	Group 2 (n = 37)	Group 3 (n = 8)	Group 4 (n = 8)	P-value
ADH1B	**1/*1*	**1/*2 + *2/*2*	**1/*1*	**1/*2 + *2/*2*	–
ALDH2	**1/*1*	**1/*1*	**1/*2*	**1/*2*	–
Age (years)	50.0 (40.0, 57.0)	56.0 (43.0, 63.0)	55.0 (44.0, 57.0)	54.0 (50.5, 63.5)	0.557
Height (cm)	167.9 ± 5.6	166.0 ± 6.2	168.8 ± 7.0	169.1 ± 5.0	0.409
Weight (kg)	66.9 ± 16.2†	57.3 ± 7.7†	58.8 ± 10.1	59.8 ± 9.3	0.050
Amount of ethanol consumed in the previous 24 h (mL)	120 (110, 180)	100 (80, 160)	120 (105, 140)	150 (95, 205)	0.364
Interval from the last drink (h)	12.0 (8.0, 14.0)	13.0 (10.0, 24.0)	9.5 (8.0, 14.0)	10.5 (7.5, 14.0)	0.285
Number of cigarettes smoked in the previous 24 h (pieces)	20.8 ± 10.2	15.6 ± 13.3	20.6 ± 12.1	20.6 ± 10.2	0.430

†$P < 0.05$ for pairwise comparisons adjusted for multiplicity. Values are means ± standard deviation (SD) or medians (25th percentile, 75th percentile); P-values are for homogeneity among the four groups based on analysis of variance (ANOVA) and the Kruskal–Wallis test, respectively. ADH1B, alcohol dehydrogenase 1B; ALDH2, aldehyde dehydrogenase 2.

N^2-ethylidene-dG in the human body,[15] the measured N^2-Et-dG level was considered as a surrogate marker of the N^2-ethylidene-dG adduct (Fig. 1).

The median N^2-ethylidene-dG levels (Q1, Q3) were 2.14 (0.97, 2.37)/10^7 bases in Group 1, 2.38 (1.18, 2.98)/10^7 bases in Group 2, 5.38 (3.19, 6.52)/10^7 bases in Group 3, and 21.04 (12.75, 34.80)/10^7 bases in Group 4 (Fig. 2).

There was no significant difference in N^2-ethylidene-dG levels between Group 1 and Group 2. On the other hand, N^2-ethylidene-dG levels were significantly higher in Group 4 than in Group 3 ($P < 0.01$).

N^2-ethylidene-dG levels were significantly higher in the *ALDH2*1/*2* group than in the *ALDH2*1/*1* group, regardless of *ADH1B* genotype (*ADH1B*1/*1*, $P < 0.05$; *ADH1B*2* carrier, $P < 0.01$).

Discussion

This study demonstrated for the first time the relationship between the levels of N^2-ethylidene-dG and *ADH1B* and *ALDH2* genotypes in human samples. We found that the level of N^2-ethylidene-dG in the DNA of leukocytes from alcoholics was remarkably elevated in individuals with a combination of *ADH1B*2* and *ALDH2*2* alleles.

It was reported that N^2-ethylidene-dG was unstable.[7] In our *in vitro* experiments in which HL-60 cells (human promyelocytic leukemia cells) were cultured with acetaldehyde contained in medium, the half-life of this adduct was approximately 35 h (Kimiko Hori, Shin'ichiro Miyamoto, Yoshiyuki Yukawa, Manabu Muto, Tsutomu Chiba and Tomonari Matsuda, unpublished data, 2012). However, the levels of N^2-ethylidene-dG in the leukocytes of alcoholics, as assessed in this study, were more abundant than those of N^2-Et-dG (*ALDH2*1/*1*, 0.04 ± 0.03/10^7 bases; *ALDH2*1/*2*, 0.28 ± 0.11/10^7 bases), α-*S*-Me-γ-OH-PdG (*ALDH2*1/*1*, 0.09 ± 0.01/10^7 bases; *ALDH2*1/*2*, 0.20 ± 0.03/10^7 bases), and α-*R*-Me-γ-OH-PdG (*ALDH2*1/*1*, 0.13 ± 0.01/10^7 bases; *ALDH2*1/*2*, 0.25 ± 0.03/10^7 bases) in leukocytes from similar populations reported previously.[13] Therefore, N^2-ethylidene-dG should be a sensitive biomarker of acetaldehyde exposure. Although the mutagenicity of this adduct remains undetermined, the fact that N^2-ethylidene-dG is converted to N^2-Et-dG, α-*S*-Me-γ-OH-PdG and α-*R*-Me-γ-OH-PdG, which are more stable and mutagenic[7–12,26] also shows the biological significance of N^2-ethylidene-dG.

Yokoyama *et al.* [23] reported an absence of differences in blood acetaldehyde concentration between *ALDH2*1/*2* intoxicated alcoholics carrying the *ADH1B*1/*1* and *ADH1B*2* alleles, whereas the slope of the increase in blood acetaldehyde concentration according to increasing concentration of blood ethanol levels was significantly steeper in *ADH1B*2* carriers with *ALDH2*1/*2* than in other combinations of *ADH1B* and *ALDH2* genotypes. Other investigators also reported that the combination of *ADH1B*2* and *ALDH2*1/*2* evoked the most intense alcohol flushing response, which was associated with an acetaldehydemia reaction.[3,27] Therefore, the present data could be interpreted as indicating that the exposure to increased levels of acetaldehyde increases the amount of acetaldehyde-derived DNA adducts, especially in individuals with a combination of the *ADH1B*2* and *ALDH2*2* alleles.

In the present study, we measured DNA adducts quantitatively in the leukocytes of Japanese alcoholics and showed that the levels of N^2-ethylidene-dG in individuals with *ALDH2*1/*2* were higher than those detected in individuals with *ALDH2*1/*1*. Furthermore, individuals with a combined genotype of *ADH1B*2* and *ALDH2*1/*2* exhibited the highest levels of N^2-ethylidene-dG. Recently, Weng *et al.* reported that DNA damage (as assessed using an alkaline comet assay) was significantly increased in elderly habitual drinkers with the *ALDH2*2* allele. Furthermore, habitual drinkers with a

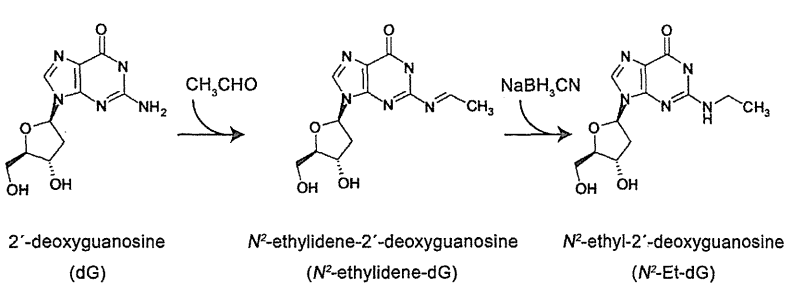

Fig. 1. Formation of acetaldehyde-derived DNA adducts. Acetaldehyde binds to deoxyguanosine (dG) and forms the unstable DNA adduct, N^2-ethylidene-2′-deoxyguanosine (N^2-ethylidene-dG). After addition of $NaBH_3CN$, N^2-ethylidene-dG is converted to the stable adduct, N^2-ethyl-2′-deoxyguanosine (N^2-Et-dG).

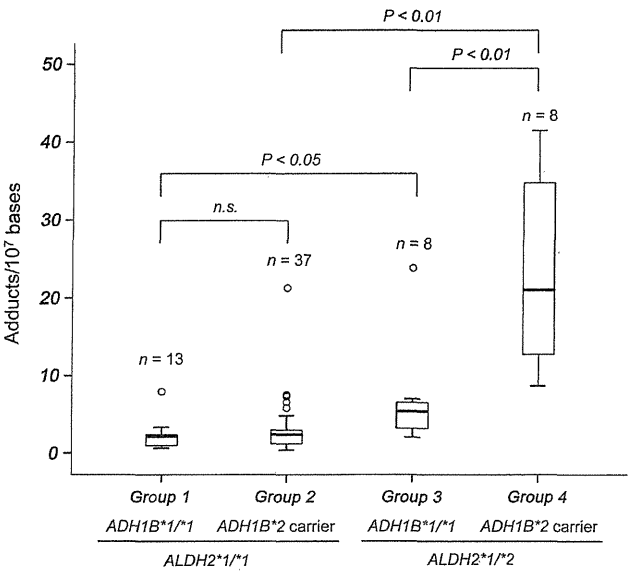

Fig. 2. N^2-ethylidene-2′-deoxyguanosine (N^2-ethylidene-dG) levels according to combinations of the *ADH1B* and *ALDH2* genotypes. N^2-ethylidene-dG levels were significantly higher in the blood DNA of alcoholics with the combination of *ADH1B*2* and *ALDH2*1/*2* compared with alcoholics with the combination of *ADH1B*1/*1* and *ALDH2*1/*2* ($P < 0.01$). N^2-ethylidene-dG levels were significantly higher in the blood DNA of alcoholics with *ALDH2*1/*2* compared with those with *ALDH2*1/*1*, regardless of *ADH1B* genotype (*ADH1B*1/*1*, $P < 0.05$; *ADH1B*2* carriers, $P < 0.01$). Boxes represent 25th to 75th percentile values (interquartile range [IQR]). The band in the box represents the median. Outer lines represent the highest datum below 1.5 IQR above the 75th percentile and the lowest datum above 1.5 IQR below the 25th percentile. Small circles represent outliers. The Mann–Whitney test was used to compare N^2-ethylidene-dG levels among genotype groups. Holm's method was used to adjust the *P*-values for multiplicity.

combined genotype of *ADH1B*2/*2* and *ALDH2*1/*2* displayed significantly higher levels of DNA damage.[28] These results are concordant with the present data. Thus, these results indicate that acetaldehyde-induced DNA damage is closely associated with the combination of *ADH1B*2* and *ALDH2*1/*2*.

If acetaldehyde-derived DNA adducts are associated with esophageal carcinogenesis, the risk of disease would be highest among the subjects carrying *ADH1B*2* plus *ALDH2*1/*2*. However, a recent meta-analysis showed that *ADH1B*1/*1* plus *ALDH2*1/*2* was associated with the highest risk of esophageal cancer among heavy drinkers (odds ratio, 12.45; 95% CI, 2.9–53.46) compared with carriers of *ADH1B*2* plus *ALDH2*1/*1*.[29] In individuals with *ALDH2*1/*2*, this risk was higher in *ADH1B*1/*1* compared with *ADH1B*2* carriers. These results suggest that N^2-ethylidene-dG adduct levels in leukocytes may not be an adequate biomarker of the risk of esophageal cancer. In addition to the acetaldehyde that circulates systemically, which is produced in the liver from orally ingested ethanol, acetaldehyde produced locally by ADH7[30,31] and CYP2E1[32] in the mucosa of the upper aerodigestive tract and by ADHs of oral microbes[33–35] has been considered to play an important role in the carcinogenicity of the upper aerodigestive tract. Thus, the relationship between locally produced acetaldehyde-derived DNA damage and the risk of esophageal cancer warrants investigation.

Regarding *ADH1B*, the *ADH1B*1* allele increases the risk of cancer in the esophagus[29,36] and in the head and neck region;[36] conversely, Kanda *et al.* reported that *ADH1B*2/*2* increases the risk of pancreatic cancer in heavy drinkers and that the combination of the *ADH1B*2/*2* and *ALDH2*2* alleles is associated with the highest carcinogenic risk.[37] Therefore, the carcinogenic risk associated with the *ADH1B* genotype may exhibit opposite trends in different organs. In contrast, the relationship between the *ALDH2*2* allele and increased carcinogenic risk has been reported in a variety of organs (e.g., esophagus,[29,36] head and neck region,[36] stomach, lung,[38] and pancreas[39]), whereas there are no reports of the association of *ALDH2*1/*1* with elevated carcinogenic risk. Thus, the carcinogenetic effect of *ALDH2*1/*2* is apparent. In this study, N^2-ethylidene-dG levels in individuals with *ALDH2*1/*2* were significantly higher than those detected in individuals with *ALDH2*1/*1*, regardless of *ADH1B* genotype.

In conclusion, the combination of *ADH1B*2* and *ALDH2*1/*2* strongly enhanced DNA damage in the leukocytes of alcoholics. Although further investigations are needed to clarify the involvement of exposure to acetaldehyde in the mechanism of esophageal carcinogenesis associated with alcohol drinking, the results of this study are expected to provide a new perspective on the carcinogenicity of the acetaldehyde associated with alcoholic beverages, from the aspect of DNA damage.

Acknowledgments

This work was supported in part by management expenses grants from the Government of Japan to the National Cancer Center (grant number 101106).

Disclosure statement

The authors have no conflict of interest.

References

1 Hongo M, Nagasaki Y, Shoji T. Epidemiology of esophageal cancer: Orient to Occident. Effects of chronology, geography and ethnicity. *J Gastroenterol Hepatol* 2009; **24**: 729–35.
2 Secretan B, Straif K, Baan R *et al.* A review of human carcinogens—Part E: tobacco, areca nut, alcohol, coal smoke, and salted fish. *Lancet Oncol* 2009; **10**: 1033–4.
3 Matsuo K, Wakai K, Hirose K, Ito H, Saito T, Tajima K. *Alcohol dehydrogenase 2* His^{47}Arg polymorphism influences drinking habit independently of *aldehyde dehydrogenase 2* Glu^{487}Lys polymorphism: analysis of 2,299 Japanese subjects. *Cancer Epidemiol Biomarkers Prev* 2006; **15**: 1009–13.
4 Higuchi S, Matsushita S, Murayama M, Takagi S, Hayashida M. Alcohol and aldehyde dehydrogenase polymorphisms and the risk for alcoholism. *Am J Psychiatry* 1995; **152**: 1219–21.
5 Fang JL, Vaca CE. Development of a ^{32}P-postlabelling method for the analysis of adducts arising through the reaction of acetaldehyde with 2′-deoxyguanosine-3′-monophosphate and DNA. *Carcinogenesis* 1995; **16**: 2177–85.
6 Fang JL, Vaca CE. Detection of DNA adducts of acetaldehyde in peripheral white blood cells of alcohol abusers. *Carcinogenesis* 1997; **18**: 627–32.
7 Wang M, McIntee EJ, Cheng G, Shi Y, Villalta PW, Hecht SS. Identification of DNA adducts of acetaldehyde. *Chem Res Toxicol* 2000; **13**: 1149–57.
8 Hecht SS, McIntee EJ, Wang M. New DNA adducts of crotonaldehyde and acetaldehyde. *Toxicology* 2001; **166**: 31–6.
9 Terashima I, Matsuda T, Fang TW *et al.* Miscoding potential of the N^2-ethyl-2′-deoxyguanosine DNA adduct by the exonuclease-free Klenow fragment of *Escherichia coli* DNA polymerase I. *Biochemistry* 2001; **40**: 4106–14.
10 Matsuda T, Terashima I, Matsumoto Y, Yabushita H, Matsui S, Shibutani S. Effective utilization of N^2-ethyl-2′-deoxyguanosine triphosphate during DNA synthesis catalyzed by mammalian replicative DNA polymerases. *Biochemistry* 1999; **38**: 929–35.
11 Upton DC, Wang X, Blans P, Perrino FW, Fishbein JC, Akman SA. Replication of N^2-ethyldeoxyguanosine DNA adducts in the human embryonic kidney cell line 293. *Chem Res Toxicol* 2006; **19**: 960–7.
12 Brooks PJ, Theruvathu JA. DNA adducts from acetaldehyde: implications for alcohol-related carcinogenesis. *Alcohol* 2005; **35**: 187–93.

doi: 10.1111/j.1349-7006.2012.02360.x

13 Matsuda T, Yabushita H, Kanaly RA, Shibutani S, Yokoyama A. Increased DNA damage in ALDH2-deficient alcoholics. *Chem Res Toxicol* 2006; **19**: 1374–8.
14 Matsuda T, Matsumoto A, Uchida M *et al.* Increased formation of hepatic N^2-ethylidene-2′-deoxyguanosine DNA adducts in *aldehyde dehydrogenase* 2-knockout mice treated with ethanol. *Carcinogenesis* 2007; **28**: 2363–6.
15 Wang M, Yu N, Chen L, Villalta PW, Hochalter JB, Hecht SS. Identification of an acetaldehyde adduct in human liver DNA and quantitation as N^2-ethyldeoxyguanosine. *Chem Res Toxicol* 2006; **19**: 319–24.
16 Chen L, Wang M, Villalta PW *et al.* Quantitation of an acetaldehyde adduct in human leukocyte DNA and the effect of smoking cessation. *Chem Res Toxicol* 2007; **20**: 108–13.
17 Balbo S, Hashibe M, Gundy S *et al.* N^2-ethyldeoxyguanosine as a potential biomarker for assessing effects of alcohol consumption on DNA. *Cancer Epidemiol Biomarkers Prev* 2008; **17**: 3026–32.
18 Singh R, Sandhu J, Kaur B *et al.* Evaluation of the DNA damaging potential of cannabis cigarette smoke by the determination of acetaldehyde derived N^2-ethyl-2′-deoxyguanosine adducts. *Chem Res Toxicol* 2009; **22**: 1181–8.
19 Hecht SS, Seow A, Wang M *et al.* Elevated levels of volatile organic carcinogen and toxicant biomarkers in Chinese women who regularly cook at home. *Cancer Epidemiol Biomarkers Prev* 2010; **19**: 1185–92.
20 Nagayoshi H, Matsumoto A, Nishi R, Kawamoto T, Ichiba M, Matsuda T. Increased formation of gastric N^2-ethylidene-2′-deoxyguanosine DNA adducts in aldehyde dehydrogenase-2 knockout mice treated with ethanol. *Mutat Res* 2009; **673**: 74–7.
21 IARC. *Monographs on the Evaluation of Carcinogenic Risks to Humans: Re-evaluation of Some Organic Chemicals, Hydrazine and Hydrogen Peroxide*, Vol. 71. Lyon, France: International Agency for Research on Cancer, 2009; 319–35.
22 IARC. *Monographs on the Evaluation of Carcinogenic Risks to Humans: Tobacco Smoke and Involuntary Smoking*, Vol. 83. Lyon, France: International Agency for Research on Cancer, 2004; 51–119.
23 Yokoyama A, Tsutsumi E, Imazeki H, Suwa Y, Nakamura C, Yokoyama T. Polymorphisms of alcohol dehydrogenase-1B and aldehyde dehydrogenase-2 and the blood and salivary ethanol and acetaldehyde concentrations of Japanese alcoholic men. *Alcohol Clin Exp Res* 2010; **34**: 1246–56.
24 Harada S, Zhang S. New strategy for detection of ALDH2 mutant. *Alcohol Alcohol Suppl* 1993; **1A**: 11–3.
25 Xu YL, Carr LG, Bosron WF, Li TK, Edenberg HJ. Genotyping of human alcohol dehydrogenases at the ADH2 and ADH3 loci following DNA sequence amplification. *Genomics* 1988; **2**: 209–14.
26 Garcia CC, Angeli JP, Freitas FP *et al.* [$^{13}C_2$]-Acetaldehyde promotes unequivocal formation of 1,N^2-propano-2′-deoxyguanosine in human cells. *J Am Chem Soc* 2011; **133**: 9140–3.
27 Takeshita T, Mao XQ, Morimoto K. The contribution of polymorphism in the alcohol dehydrogenase beta subunit to alcohol sensitivity in a Japanese population. *Hum Genet* 1996; **97**: 409–13.
28 Weng H, Weng Z, Lu Y, Nakayama K, Morimoto K. Effects of alcohol-drinking behaviour and *ADH1B* and *ALDH2* polymorphisms on basal DNA damage in human mononuclear cells as determined by the comet assay. *Mutat Res* 2010; **701**: 132–6.
29 Yang SJ, Yokoyama A, Yokoyama T *et al.* Relationship between genetic polymorphisms of ALDH2 and ADH1B and esophageal cancer risk: a meta-analysis. *World J Gastroenterol* 2010; **16**: 4210–20.
30 Dong YJ, Peng TK, Yin SJ. Expression and activities of class IV alcohol dehydrogenase and class III aldehyde dehydrogenase in human mouth. *Alcohol* 1996; **13**: 257–62.
31 Yin SJ, Chou FJ, Chao SF *et al.* Alcohol and aldehyde dehydrogenases in human esophagus: comparison with the stomach enzyme activities. *Alcohol Clin Exp Res* 1993; **17**: 376–81.
32 Lieber CS. Microsomal ethanol-oxidizing system (MEOS): the first 30 years (1968–1998)—a review. *Alcohol Clin Exp Res* 1999; **23**: 991–1007.
33 Muto M, Hitomi Y, Ohtsu A *et al.* Acetaldehyde production by non-pathogenic *Neisseria* in human oral microflora: implications for carcinogenesis in upper aerodigestive tract. *Int J Cancer* 2000; **88**: 342–50.
34 Kurkivuori J, Salaspuro V, Kaihovaara P *et al.* Acetaldehyde production from ethanol by oral streptococci. *Oral Oncol* 2007; **43**: 181–6.
35 Tillonen J, Homann N, Rautio M, Jousimies-Somer H, Salaspuro M. Role of yeasts in the salivary acetaldehyde production from ethanol among risk groups for ethanol-associated oral cavity cancer. *Alcohol Clin Exp Res* 1999; **23**: 1409–15.
36 Yokoyama A, Omori T. Genetic polymorphisms of alcohol and aldehyde dehydrogenases and risk for esophageal and head and neck cancers. *Alcohol* 2005; **35**: 175–85.
37 Kanda J, Matsuo K, Suzuki T *et al.* Impact of alcohol consumption with polymorphisms in alcohol-metabolizing enzymes on pancreatic cancer risk in Japanese. *Cancer Sci* 2009; **100**: 296–302.
38 Yokoyama A, Muramatsu T, Ohmori T *et al.* Alcohol-related cancers and aldehyde dehydrogenase-2 in Japanese alcoholics. *Carcinogenesis* 1998; **19**: 1383–7.
39 Miyasaka K, Hosoya H, Tanaka Y *et al.* Association of aldehyde dehydrogenase 2 gene polymorphism with pancreatic cancer but not colon cancer. *Geriatr Gerontol Int* 2010; **10**(Suppl 1): S120–6.

OPEN ACCESS Freely available online

PLoS one

Tissue Damage in the Canine Normal Esophagus by Photoactivation with Talaporfin Sodium (Laserphyrin): A Preclinical Study

Takahiro Horimatsu*, Manabu Muto, Yusuke Yoda, Tomonori Yano, Yasumasa Ezoe, Shinichi Miyamoto, Tsutomu Chiba

Department of Gastroenterology and Hepatology, Graduate School of Medicine Kyoto University, Kyoto, Japan

Abstract

Background: Treatment failure at the primary site after chemoradiotherapy is a major problem in achieving a complete response. Photodynamic therapy (PDT) with porfimer sodium (Photofrin®) has some problems such as the requirement for shielding from light for several weeks and a high incidence of skin phototoxicity. PDT with talaporfin sodium (Laserphyrin) is less toxic and is expected to have a better effect compared with Photofrin PDT. However, Laserphyrin PDT is not approved for use in the esophagus. In this preclinical study, we investigated tissue damage of the canine normal esophagus caused by photoactivation with Laserphyrin.

Methodology/Principal Findings: Diode laser irradiation was performed at 60 min after administration. An area 5 cm oral to the esophagogastric junction was irradiated at 25 J/cm^2, 50 J/cm^2, and 100 J/cm^2 using a three-step escalation. The irradiated areas were evaluated endoscopically on postirradiation days 1 and 7, and were subjected to histological examination after autopsy. The areas injured by photoactivation were 52 mm^2, 498 mm^2, and 831 mm^2 after irradiation at 25 J/cm^2, 50 J/cm^2, and 100 J/cm^2, respectively. Tissue injury was observed in the muscle layer or even deeper at any irradiation level and became more severe as the irradiation dose increased. At 100 J/cm^2 both inflammatory changes and necrosis were seen histologically in extra-adventitial tissue.

Conclusions/Significance: To minimize injury of the normal esophagus by photoactivation with Laserphyrin, diode laser irradiation at 25 J/cm^2 appears to be safe. For human application, it would be desirable to investigate the optimal laser dose starting from this level.

Citation: Horimatsu T, Muto M, Yoda Y, Yano T, Ezoe Y, et al. (2012) Tissue Damage in the Canine Normal Esophagus by Photoactivation with Talaporfin Sodium (Laserphyrin): A Preclinical Study. PLoS ONE 7(6): e38308. doi:10.1371/journal.pone.0038308

Editor: Eric Y. Chuang, National Taiwan University, Taiwan

Received December 20, 2011; **Accepted** May 7, 2012; **Published** June 13, 2012

Funding: This study was supported in part by grants-in-aid for cancer research from the Ministry of Health (H21-Rinshokenkyu-Ippan-018), Labor, and Welfare of Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: thorimat@kuhp.kyoto-u.ac.jp

Introduction

Photodynamic therapy (PDT) is a local endoscopic treatment using a photochemical reaction induced by an oncotropic photosensitizer and a laser [1–3]. PDT is useful for treating superficial esophageal cancer, preventing the development of adenocarcinoma from high-grade dysplasia in the Barrett's esophagus, and alleviating stenosis caused by advanced esophageal cancer [4–6].

We have reported on the benefits of PDT as a salvage treatment for local residues and recurrence after definitive chemoradiotherapy (CRT) for esophageal cancer. Salvage PDT has excellent treatment outcomes for local residues and recurrence after CRT with a 59.5–62.0% complete response rate and a 5-year overall survival of 36.1% without severe adverse events [7,8]. By contrast, salvage surgery after definitive CRT is associated with high postoperative mortality (>10%) [9,10]. Accordingly, in carefully selected patients without metastasis, salvage PDT after definitive CRT is a potential curative treatment option that may improve quality of life and prolong survival.

PDT with porfimer sodium (Photofrin®), a hematoporphyrin derivative and the first clinically approved photosensitizer, has clinical disadvantages such as the requirement for shielding from light for 4–6 weeks and a high incidence (25%) of skin toxicity because of photosensitivity [11,12]. PDT with mono-L-aspartyl chlorin e6 (NPe6, talaporfin sodium, Laserphyrin®), a second-generation photosensitizer, has advantages such as: 1) a shorter period of light shielding of about 2 weeks, 2) 10% incidence of skin toxicity because of reduced photosensitivity, and 3) expected treatment effect in the deep-lying tissue areas with a laser at 664 nm instead of at 630 nm as used with Photofrin PDT [13,14].

The first two advantages are meaningful for patients because of the shorter period of shielding and lower incidence of toxicity, but the third is associated with opposite effects, i.e., an antitumor effect and an increased risk of an adverse event such as perforation, especially in the esophagus.

Applying Laserphyrin PDT as a salvage treatment of the deep-lying tissue seems to be an advantage for eliminating residual tumors. However, Laserphyrin PDT is approved only in Japan and only for superficial lung cancer. No investigation has reported on the safety of Laserphyrin PDT in esophageal cancer and the normal esophagus. Investigating the antitumor effects of photoactivation is also difficult because of the difficulty in developing esophageal cancer in large animal models. However, before we can apply Laserphyrin PDT to human esophageal disease, we must know whether it is safe as a treatment in the esophageal wall.

To investigate the relationship between the irradiation dose and tissue injury in the normal esophagus, we conducted a preclinical study of Laserphyrin PDT using a canine model.

Results

General Conditions

In the 25 J/cm^2 irradiation group, one dog vomited 50 ml fluid, and all dogs in the 50 J/cm^2 and 100 J/cm^2 irradiation groups vomited; average volumes were 33 ml and 113 ml, respectively. There was a tendency for vomiting to become more severe as the irradiation dose increased.

Appetite loss was not observed in any dog in the 25 J/cm^2 irradiation group but was apparent in all dogs in the 50 J/cm^2 and 100 J/cm^2 irradiation groups. A loss of body weight was observed on day 7 and was related to the irradiation dose: 8.3% in the 25 J/cm^2 group, 11.2% in the 50 J/cm^2 group, and 14.8% in the 100 J/cm^2 group.

Laboratory Data (Figure 1 and Table 1)

In the 25 J/cm^2 group, the white blood cell (WBC) counts were 13.1, 12.3, and 12.7×10^3/mm^3 before irradiation and on days 1 and 7 after irradiation, respectively; these values did not differ significantly. In the 50 J/cm^2 group, the WBC count increased from 10.7 to 17.9 on day 1 but returned to 12.4×10^3/mm^3 on day 7; the value on day 7 did not differ from that before irradiation. In the 100 J/cm^2 group, the WBC count increased from 12.4 to 25.9×10^3/mm^3 on day 1 and remained at 23.1×10^3/mm^3 on day 7.

In the 25 J/cm^2 group, C-reactive protein (CRP) concentration increased from 1.2 to 12.3 mg/dl on day 1 and decreased to 5.5 mg/dl on day 7. In the 50 J/cm^2 group, it increased from 3.2 to 48.6 on day 1 and decreased to 22.7 on day 7. In the 100 J/cm^2 group, C-reactive protein concentration increased from 2.2 to 61.8 mg/dl on day 1 and remained high at 56.9 mg/dl on day 7.

In the 25 J/cm^2 group, serum lactate dehydrogenase (LDH) level increased from 38.3 to 56.0 IU/L on day 1 and decreased to 45 IU/L on day 7. In the 50 J/cm^2 group, serum LDH level increased from 49.7 to 84 IU/L on day 1 and decreased to 64.7 IU/L on day 7. In the 100 J/cm^2 group, serum LDH level increased from 40.3 to 103 IU/L on day 1 and remained high at 117.7 IU/L on day 7.

Compared with the baseline values, the WBC count and CRP and LDH concentrations increased with irradiation dose. The WBC and CRP and LDH concentrations returned to the baseline levels in the 25 J/cm^2 group but remained high or increased further in the 100 J group.

The Laserphyrin concentration before irradiation was 20–30 μg/ml in all dogs, except for a value of 62.3 μg/ml in one dog in the 50 J/cm^2 group.

Endoscopic Findings (Figures 2 and 3)

In the 25 J/cm^2 group, a reddish color change was observed at the site of irradiation of the mucosa on day 1 in two dogs, and no change was detected in one dog. On day 7, ulceration was observed at the site of irradiation in the sites that showed redness on day 1, but only redness and erosion were recognized in the other dog.

In the 50 J/cm^2 group, redness and erosion were observed at the site of irradiation on day 1. Ulceration was observed on day 7 in all dogs, and purple color changes were observed at the ulcer base in two dogs.

In the 100 J/cm^2 group, redness and erosion were observed at the site of irradiation in all dogs on day 1, and a blackish-purple color change was observed in one dog. On day 7, ulceration, redness, and erosion appeared in all dogs, and blackish-purple color changes occurred at the ulcer base in two dogs.

Macroscopic Findings of the Autopsy (Figure 4 and Table 1)

In the 25 J/cm^2 group, mucosal redness and erosion were observed in all dogs, and shallow ulceration was observed in two. No damage was observed at the outside of the esophageal wall in any case.

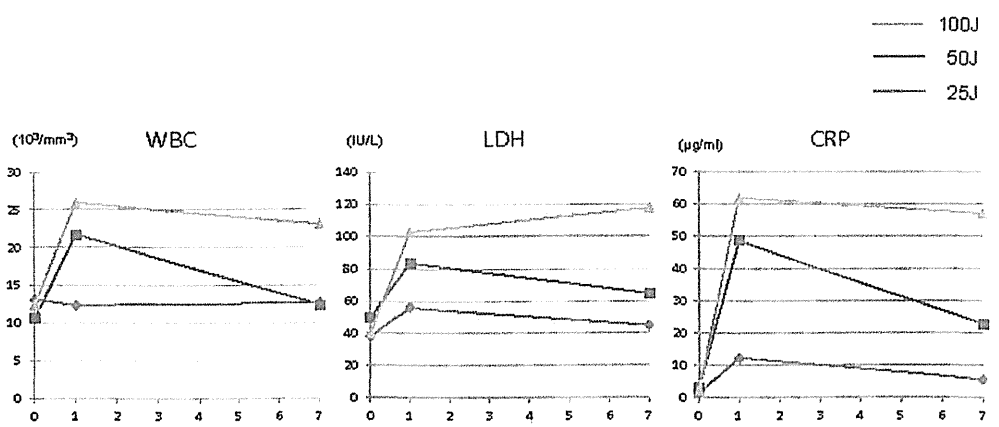

Figure 1. Laboratory data before and after irradiation. Compared with the baseline values, WBC count and CRP and LDH concentrations were increased with increasing radiation dose. WBC count and CRP and LDH concentrations returned to the baseline values on day 7 in the 25 J/cm^2 group but remained high, especially in the 100 J/cm^2 group.
doi:10.1371/journal.pone.0038308.g001

Table 1. The injured areas and serum concentration of Laserphyrin.

Irradiation energy (J/cm^2)	Animal No.	Injured area (mean±SD, mm2)	Laserphyrin conc. (mean±SD, mm2)
25	1	52.3±47.9	24.0±0.67
	2		
	3		
50	4	498.3±430.7	24.6±22.18
	5		
	6		
100	7	831.0±691.7	23.0±9.37
	8		
	9		

The areas of tissue injury were 52.3±47.9 mm^2, 498.3±430.7 mm^2, and 831.0±691.7 mm^2 after irradiation at 25 J/cm^2, 50 J/cm^2, and 100 J/cm^2, respectively. These areas tended to be more extensive as the laser dose increased. The Laserphyrin concentration before irradiation ranged from 20 to 30 μg/ml in all groups.
doi:10.1371/journal.pone.0038308.t001

In the 50 J/cm^2 group, ulceration with peripheral redness and erosion were observed in all dogs, and necrosis occurred in one. Adhesion of the esophageal adventitia to the aorta was recognized in all dogs.

In the 100 J/cm^2 group, ulceration with peripheral redness and erosion were observed in all dogs, and necrosis appeared in two. Adhesion of the esophageal adventitia to the lungs was observed in all dogs, and adhesion to the aorta was detected in two dogs. The areas of tissue injury were 52±48 mm^2, 498±431 mm^2, and 831±692 mm^2 after irradiation at 25 J/cm^2, 50 J/cm^2, and 100 J/cm^2, respectively.

Pathological Findings (Table 2)

In the 25 J/cm^2 group, only erosion was observed at the site of irradiation in one dog, and ulceration was recognized in the other two dogs. Cellular infiltration was observed in the mucosa, submucosa, muscle layer, and adventitia in the 25 J/cm^2 group and within and outside of the adventitia in the 50 J/cm^2 and 100 J/cm^2 groups. Fibrosis and hemorrhage were observed in the submucosa, muscle layer, and adventitia in the 25 J/cm^2 group and within and outside of the adventitia in the 50 J/cm^2 and 100 J/cm^2 groups. Necrosis was observed in the submucosa and muscle layer in the 25 J/cm^2 group and within and outside of the

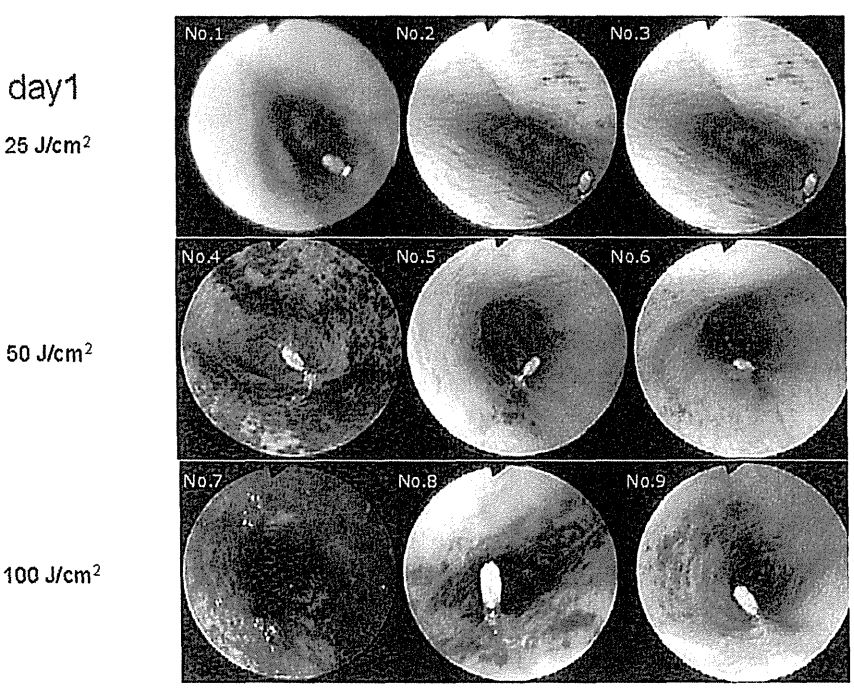

Figure 2. Endoscopic findings in the esophagus on postirradiation day 1. In all dogs except one in the 25 J/cm^2 group, the mucosa at the site of irradiation (the area at 9 o'clock) showed reddish color changes on day 1 (A–C). Redness and ischemic color changes were observed at the site of irradiation in all dogs in the 50 J/cm^2 and 100 J/cm^2 groups (D–I).
doi:10.1371/journal.pone.0038308.g002

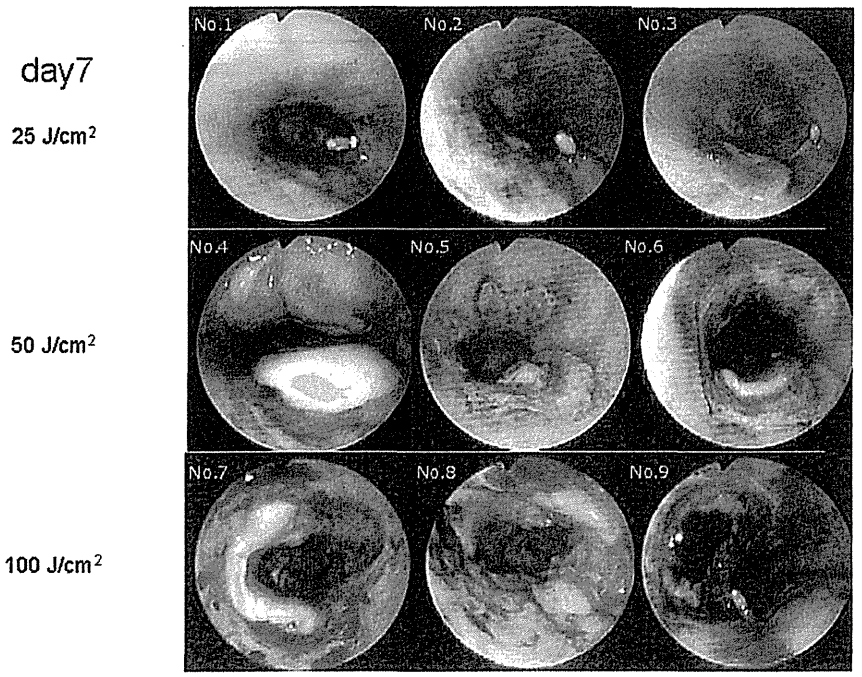

Figure 3. Endoscopic findings in the esophagus on postirradiation day 7. Redness and slight bleeding were observed at the site of irradiation in all dogs on day 7. Ulceration was observed in the 25 J/cm^2 group (A–C) but was more extensive in both the 50 J/cm^2 and 100 J/cm^2 groups (D–I).
doi:10.1371/journal.pone.0038308.g003

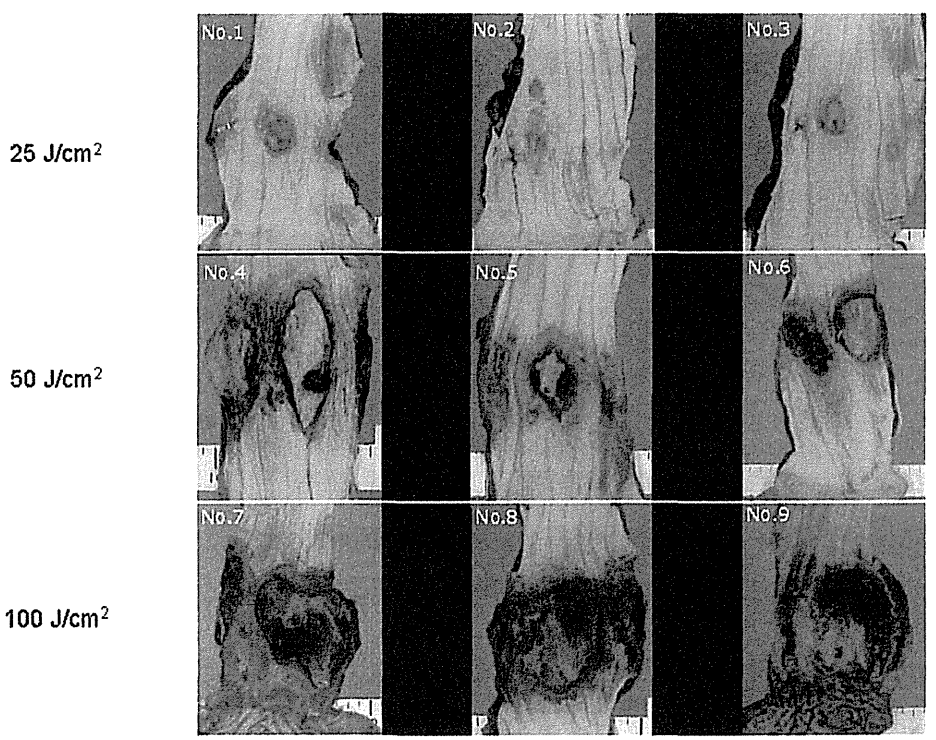

Figure 4. Macroscopic findings in the esophagus. Mucosal redness and ulceration were observed in all dogs in the 25 J/cm^2 group (A–C). The areas of redness, erosion, and ulceration were more extensive in the 50 J/cm^2 and 100 J/cm^2 groups (D–I).
doi:10.1371/journal.pone.0038308.g004

Table 2. Pathological findings in the injury areas.

Laser irradiation		25 J/cm2			50 J/cm2			100 J/cm2		
Animal number		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Cellular infiltration	Mucosa	+	+	+	+	2+	+	-	-	+
	Submucosa	2+	2+	2+	2+	2+	3+	-	3+	2+
	Muscle layer	+	+	+	2+	2+	3+	2+	2+	2+
	Adventitia	2+	+	+	2+	2+	3+	3+	3+	2+
	Out of Adventitia	-	-	-	2+	+	2+	2+	2+	2+
Fibrosis	Mucosa	NE	NE	NE	NE	NE	NE	NE	NE	NE
	Submucosa	+	+	+	2+	2+	+	-	-	+
	Muscle layer	2+	2+	2+	3+	2+	2+	2+	2+	2+
	Adventitia	+	-	-	2+	2+	2+	2+	3+	2+
	Out of Adventitia	-	-	-	+	+	2+	2+	2+	2+
Hemorrhage	Mucosa	NE	NE	NE	NE	NE	NE	NE	NE	NE
	Submucosa	2+	±	+	2+	2+	2+	-	-	2+
	Muscle layer	+	±	+	2+	2+	2+	2+	+	2+
	Adventitia	+	±	±	2+	2+	2+	2+	2+	3+
	Out of Adventitia	-	-	-	2+	+	2+	2+	2+	3+
	Aorta	-	-	-	-	-	+	-	-	-
Necrosis	Mucosa	-	-	-	3+	-	-	-	-	-
	Submucosa	2+	+	+	3+	2+	3+	3+	3+	2+
	Muscle layer	2+	-	+	3+	2+	3+	3+	3+	3+
	Adventitia	NE	NE	NE	NE	NE	NE	NE	NE	NE
	Out of Adventitia	-	-	-	-	+	+	+	2+	2+
	Aorta	-	-	-	-	-	+	-	-	-

Notes). -: No abnormal changes, ±: Very slight, + : Slight, 2+: Moderate, 3+: Marked, NE: Not examined.
Cellular infiltration was observed in the mucosa, submucosa, muscle layer, and adventitia in the 25 J/cm^2 group. Cellular infiltration was observed within and outside of the adventitia in the 50 J/cm^2 and 100 J/cm^2 groups. Fibrosis and hemorrhage were observed in the submucosa, muscle layer, and adventitia in the 25 J/cm^2 group, and fibrosis and hemorrhage were observed within and outside of the adventitia in the 50 J/cm^2 and 100 J/cm^2 groups. Necrosis was observed in the submucosa and muscle layer in the 25 J/cm^2 group and within and outside of the adventitia in the 50 J/cm^2 and 100 J/cm^2 groups. These changes tended to become more severe as the irradiation dose increased.
doi:10.1371/journal.pone.0038308.t002

adventitia in the 50 J/cm^2 and 100 J/cm^2 groups. These changes tended to be more severe as the irradiation dose increased.

No infiltration of inflammatory cells, fibrosis, hemorrhage, or necrosis was observed in the extra-adventitial tissue in the 25 J/cm^2 group. These changes were seen in the 50 J/cm^2 and 100 J/cm^2 groups, and they tended to be more severe as the laser dose increased. In one dog in the 50 J/cm^2 group, hemorrhage from the aorta, which had adhered to the esophagus, was accompanied by necrosis. Inflammatory changes and necrosis of the extra-adventitial tissue were observed histologically in the 100 J/cm^2 group.

Discussion

The effects of Photofrin PDT, a first-generation PDT, depend on the selective retention of the photosensitizer in the neoplastic tissue. Laserphyrin PDT, a second-generation PDT, induces a phototoxic effect when the timing of photoactivation is associated with the peak plasma level of the photosensitizer. Therefore, in normal tissue irradiated by Laserphyrin photoactivation, phototoxicity would be expected even in normal tissue. The relationship between tissue damage and the dose of laser irradiation by Laserphyrin photoactivation remains unknown in the esophagus. To apply Laserphyrin PDT as a treatment option for esophageal cancer, the relationship between the laser irradiation dose and the damage to normal tissue and neoplastic tissue must be known.

In the present study, we evaluated, for the first time, the safety of Laserphyrin photoactivation in the normal esophageal wall. This *in vivo* study using a canine model showed that increasing the dose of Laserphyrin irradiation from 25 J/cm^2 to 100 J/cm^2 caused more severe inflammatory changes, as shown by the laboratory data, macroscopic damage, and histological necrosis. Macroscopically, the ulceration and ischemic changes around the ulcer became more severe as the dose of laser irradiation increased. Pathologically, these changes were confined to the mucosa after irradiation at 25 J/cm^2, whereas they appeared as necrosis in the muscle layer after irradiation at 50 J/cm^2, and the necrosis extended to the extra-adventitial tissue after irradiation at 100 J/cm^2.

Adverse effects, such as appetite loss, nausea, increased inflammatory reaction, and an increase in LDH level, were confirmed, and these were related to the degree of tissue injury in the esophagus. Clinical symptoms were aggravated as the dose of laser irradiation increased. Considering these effects together, we conclude that an irradiation energy of 25 J/cm^2 was within the safe range of Laserphyrin photoactivation in the canine normal esophagus. Tissue fragility differs between humans and dogs, and

this dosage cannot be applied directly to humans. Previous data on the normal canine bronchi and a clinical study of human lung cancer suggest that similar effects should be expected in humans after double-irradiation doses that were used in dogs (unpublished data). Therefore, we recommend that human clinical trials in the esophagus should start with an irradiation dose of 50 J/cm^2, which is similar to that used to treat lung cancer.

Laserphyrin PDT is expected to have a greater effect on the deep-tissue layers compared with Photofrin PDT [13,14]. Laserphyrin PDT provides excellent clinical treatment responses in patients with early lung cancer [15–17]. Laserphyrin PDT might produce similar clinical effects when used to treat esophageal disease, especially for salvage treatment after CRT for esophageal cancer. If so, this would be advantageous for patients because of the lower risk of phototoxicity and shorter interval of light shielding.

This study has some limitations because we could not evaluate the actual tumoricidal effect on esophageal cancer. However, it is difficult to simulate exactly the human pathology and treatment in an animal model. Tissue thickness and fragility differ between humans and animal models. Establishing an esophageal cancer model is difficult in a large animal model. To evaluate the direct effects of Laserphyrin PDT on human esophageal cancer, we carefully run a well-controlled clinical trial. Another limitation was that we did not evaluate the effect by dose escalation of Laserphyrin. However, the recommended timing of the irradiation in humans has been established at a concentration of 20 μg/ml in the plasma. Therefore, we believe that there is no need to estimate another dose in clinical applications.

In conclusion, we have for the first time determined the safety range of 25 J/cm^2 for irradiation with a diode laser in the canine esophagus when the plasma concentration was around 20 μg/ml. This information will be useful for planning clinical studies to examine the effects of Laserphyrin PDT on various esophageal diseases.

Materials and Methods

Animals

Male Beagle dogs weighing 12.0–14.7 kg and 13–14 months old were used in this study. The site of laser irradiation was determined as the area at 9 o'clock 5 cm oral to the esophagogastric junction, where it was possible to stably hold an endoscope and irradiate with a laser. The sites at 5 o'clock and 3 o'clock were marked in advance with a clip and tattooing as the landmarks for irradiation.

This preclinical research was done at the Shinn Nihon Biomedical Laboratories, Ltd. (Kagoshima, Japan) according to the Good Laboratory Practice Guideline. All research and experimental protocols were conducted according to the Regulation for the Care and Use of Laboratory Animals of Shinn Nihon Biomedical Laboratories and were approved by the Animal Care and Use Committee of Shinn Nihon Biomedical Laboratories, Ltd. (Approval ID: No. 704-006).

Photosensitizer

Laserphyrin (Meiji Seika Pharma, Tokyo, Japan) is a water-soluble photosensitizer with a molecular weight of 799.69 and has a chlorine annulus. Its maximum absorption peak is at a wavelength of 407 nm, and there is a second peak at 664 nm. Laserphyrin has high tumor affinity and is excited by visible red light with a longer wavelength of 664 nm, enabling deeper penetration into the tissue [12,13].

Laser Unit and Endoscope

A diode laser (ZH-L5011HJP, Panasonic Health Care, Yokohama, Japan) emitting continuous-wave laser light at a wavelength of 664 nm was used as the laser source for the excitation of Laserphyrin. We used a straight-type fiber-optic tip for the irradiation. An Olympus GIF-XQ240 endoscope and a Panasonic PD laser device (ZH-L5011HJP) were used.

Protocol of Laserphyrin Photoactivation in the Normal Esophagus

We fixed the dose of Laserphyrin because the profile of the systematic side effects is known and has been confirmed for lung cancer in a clinical study [14]. The approved dose of Laserphyrin for humans is 1 mg/kg, and the interval between Laserphyrin injection and laser exposure is 4–6 hours. In this condition, the blood concentration of Laserphyrin is about 20 μg/ml (unpublished data by Meiji Pharma, Tokyo).

In this animal model, Laserphyrin was administered intravenously into the cephalic vein or saphenous vein at a dose of 20 mg/kg. The time interval between photosensitizer injection and laser exposure was 60 min because the concentration of Laserphyrin in the blood during this interval was equal to that in humans (20 μg/dl) (data not shown).

To investigate the tissue damage in relation to the energy of the diode laser, dose escalation was set at 3 energy levels. The initial dose was set at an energy level of 25 J/cm^2, and the second and the third were at 50 J/cm^2 and 100 J/cm^2, respectively. These three dosage groups comprised three dogs in each group.

Laser irradiation was applied under general anesthesia using isoflurane. The target area was 1 cm^2 in the normal esophageal mucosa in the 9 o'clock direction and 5 cm from the esophagogastric junction (Figure 5). To avoid missing the location of the irradiated area, the sites at 5 o'clock and 3 o'clock were marked in advance with a clip and tattooing by Indian ink, respectively, as the landmarks for irradiation. The dogs were fasted, and only water was given on the day of irradiation (day 0) and the next day (day 1), and a liquid meal was fed from day 2.

Evaluation of the Effect of Laserphyrin Photoactivation in the Normal Esophagus

To evaluate the response to Laserphyrin photoactivation, changes on the surface of the whole esophagus including the irradiated area were examined endoscopically on days 0, 1, and 7. After the endoscopic examination on day 7, pentobarbital sodium was injected intravenously to provide anesthesia, and the dogs were exsanguinated for euthanasia. The internal organs and tissues were observed macroscopically, fixed in formalin, and subjected to hematoxylin and eosin staining for pathology evaluation. To measure the Laserphyrin concentration in the blood, blood was collected at the time of laser irradiation, which was 60 min after administration.

To evaluate the systemic effects of Laserphyrin, blood was collected from the external jugular vein before irradiation (day 0) and on postirradiation days 1 and 7, and the inflammatory reactions and hematological adverse effects were examined. To determine whether there was reduced oral food intake, the amount of leftover food from the previous day was recorded from day 2 to day 7 and thereafter. Body weight was measured before irradiation on day 0 and on day 7.

Acknowledgments

We thank Emiko Haruyama and Koshiro Kamura for their invaluable support in the conduct of this study.

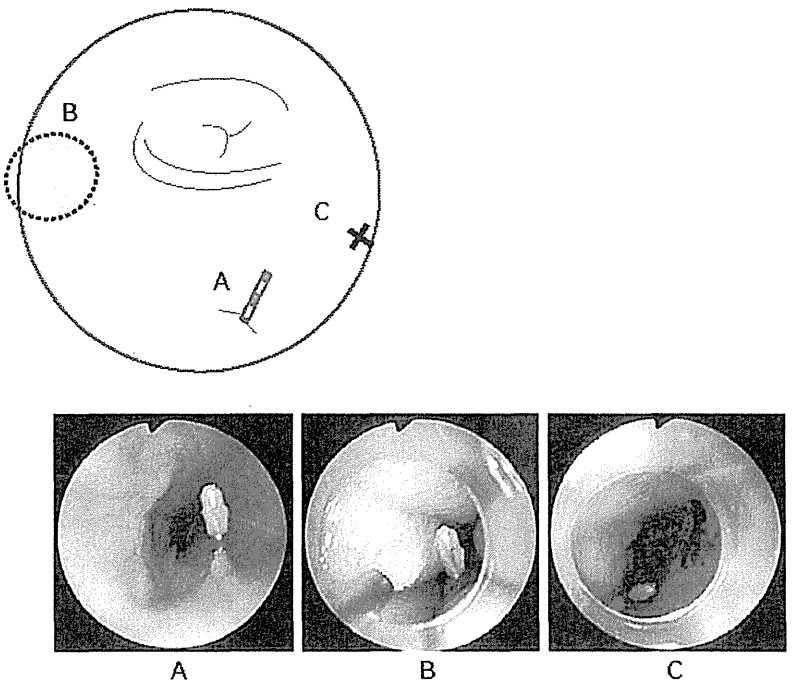

Figure 5. Standard operation procedure for laser irradiation. The site of laser irradiation was fixed at 9 o'clock (A) in all animals because this direction was easiest for holding an endoscope stably and irradiating with a laser. Before laser irradiation, the site at 5 o'clock (B) was marked in advance with a clip. After completion of the laser irradiation (C), the site at 3 o'clock (D) was tattooed to identify the irradiation site for follow-up and autopsy.
doi:10.1371/journal.pone.0038308.g005

Author Contributions

Conceived and designed the experiments: TH MM TT YE. Performed the experiments: TH MM YE YY TY. Analyzed the data: TH MM YY. Contributed reagents/materials/analysis tools: TH MM YY. Wrote the paper: TH MM. Special advice for experiments: SM TC.

References

1. Manyak MJ, Russo A, Smith PD, Glatstein E (1988) Photodynamic therapy. J Clin Oncol 6: 380–391.
2. Pass HI (1993) Photodynamic therapy in oncology: mechanism and clinical use. J Natl Cancer Inst 85: 443–456.
3. Dolmans DE, Fukumura D, Jain RK (2003) Photodynamic therapy for cancer. Nature Reviews Cancer 3: 380–387.
4. Yoshida K, Suzuki S, Mimura S, Ichii M, Sakai H, et al. (1993) Phase III clinical trial on photodynamic therapy (PDT) with porfimer sodium (PHE) and excimer dye laser (PDT EDL-1) for superficial esophageal cancer. Gan to Kagakuryoho 20: 2063–2066 [in Japanese].
5. Savary JF, Grosjean P, Monnier P, Fontolliet C, Wagnieres G, et al. (1998) Photodynamic therapy of early squamous cell carcinomas of the esophagus: a review of 31 cases. Endoscopy 30: 258–265.
6. Overholt BF, Panjehpour M, Haydek M (1999) Photodynamic therapy for Barrett's esophagus: follow-up in 100 patients. Gastrointest Endosc 49: 1–7.
7. Yano T, Muto M, Minashi K, Ohtsu A, Yoshida S (2005) Photodynamic therapy as salvage treatment for local failures after definitive chemoradiotherapy for esophageal cancer. Gastrointest Endosc 62: 31–36.
8. Yano T, Muto M, Minashi K, Onozawa M, Nihei K, et al. (2011) Long-term results of salvage photodynamic therapy for patients with local failure after chemoradiotherapy for esophageal squamous cell carcinoma. Endoscopy 43: 657–663.
9. Swisher SG, Deford L, Merriman KW, Walsh GL, Smythe R, et al. (2000) Effect of operative volume on morbidity, mortality, and hospital use after esophagectomy for cancer. J Thorac Cardiovasc Surg 119: 1126–1134.
10. Swisher SG, Wynn P, Putnam JB, Mosheim MB, Correa AM, et al. (2002) Salvage esophagectomy for recurrent tumors after definitive chemotherapy and radiotherapy. J Thorac Cardiovasc Surg 123: 175–183.
11. Dougherty TJ, Cooper MT, Mang TS (1990) Cutaneous phototoxic occurrence in patients receiving Photofrin. Lasers Surg Med 10: 485–8.
12. Dougherty TJ, Marcus SL (1992) Photodynamic therapy. Eur J Cancer 28A: 1734–1742.
13. Aizawa K, Okunaka T, Ohtani T, Kawabe H, Yasunaka Y, et al. (1987) Localization of mono-L-aspartyl chlorin e6 (NPe6) in mouse tissues. Photochem Photobiol 46: 789–794.
14. Nelson J, Roberts WG, Berns MW (1987) In vivo studies on the utilization of mono-L-aspartyl chlorin (NPe6) for the photodynamic therapy. Cancer Res 47: 4681–4685.
15. Kato H, Furuyama K, Sato M, Okunaka T, Kusunoki Y, et al. (2003) Phase II clinical study of photodynamic therapy using mono-L-aspartyl chlorine e6 and diode laser for early superficial squamous cell carcinoma of the lung. Lung Cancer 42: 103–111.
16. Usuda J, Tsutsui H, Honda H, Ichinose S, Ishizumi T, et al. (2007) Photodynamic therapy for lung cancers based on novel photodynamic diagnosis using talaporfin sodium (NPe6) and autofluorescence bronchoscopy. Lung Cancer 58: 317–323.
17. Usuda J, Ichinose S, Ishizumi T, Hayashi H, Ohtani K, et al. (2010) Outcome of photodynamic therapy using NPe6 for bronchogenic carcinomas in central airways >1.0 cm in diameter. Clin Cancer Res 16: 2198–2204.

Yano et al. Radiation Oncology 2012, 7:113
http://www.ro-journal.com/content/7/1/113

RADIATION ONCOLOGY

RESEARCH Open Access

Phase I study of photodynamic therapy using talaporfin sodium and diode laser for local failure after chemoradiotherapy for esophageal cancer

Tomonori Yano[1†], Manabu Muto[2,7*†], Kenichi Yoshimura[3†], Miyuki Niimi[3†], Yasumasa Ezoe[4†], Yusuke Yoda[1†], Yoshinobu Yamamoto[5†], Hogara Nishisaki[5†], Koji Higashino[6†] and Hiroyasu Iishi[6†]

Abstract

Background: Photodynamic therapy (PDT) is a less invasive and effective salvage treatment for local failure after chemoradiotherapy (CRT) for esophageal cancer, however it causes a high rate of skin phototoxicity and requires a long sun shade period. Talaporfin sodium is a rapidly cleared photosensitizer that is expected to have less phototoxicity. This study was undertaken to clarify the optimum laser fluence rate of PDT using talaporfin sodium and a diode laser for patients with local failure after CRT or radiotherapy (RT) for esophageal cancer.

Methods: This phase I, laser dose escalation study used a fixed dose (40 mg/m^2) of intravenous talaporfin sodium administered 4 to 6 hours before irradiation in patients with local failure limited to T2 after CRT or RT (≥ 50 Gy). The primary endpoint was to assess the dose limiting toxicity (DLT) of PDT, and the secondary endpoints were to evaluate the adverse events and toxicity related to PDT. The starting fluence of the 664 nm diode laser was 50 J/cm^2, with an escalation plan to 75 J/cm^2 and 100 J/cm^2.

Results: 9 patients with local failure after CRT or RT for ESCC were enrolled and treated in groups of 3 individuals to the third fluence level. No DLT was observed at any fluence level. Phototoxicity was not observed, but one subject had grade 1 fever, three had grade 1 esophageal pain, and 1 had grade 1 dysphagia. Five of 9 patients (55.6%) achieved a complete response after PDT.

Conclusions: PDT using talaporfin sodium and a diode laser was safe for local failure after RT in patients with esophageal cancer. The recommended fluence for the following phase II study is 100 J/cm^2.

Keywords: Photodynamic therapy, Esophageal cancer, Talaporfin sodium, Salvage treatment, Phase I study

Background

Chemoradiotherapy (CRT) is one of the curative treatment options for esophageal cancer. However, local failure without distant metastasis after completion of CRT occurs in more than 40% of patients and this remains a major problem to achieve a cure [1]. Although salvage esophagectomy is generally indicated in such cases, it carries with it a high morbidity and mortality [2-5]. If the patients with local failure after completion of CRT have T2 or earlier T-stage residual tumors or for those without lymph node metastasis, salvage esophagectomy has curative potential [2-5]. Onozawa et al. reported that regional nodal failure within the field of elective lymph node irradiation was rare in patients achieving a complete response (CR) after CRT (1%; 95% confidence interval [CI], 0%–5%) for esophageal squamous cell carcinoma (ESCC) [6]. These data might indicate that if both lymph node and distant metastasis were controlled by CRT, local salvage treatment that targeted only the primary site could be a minimally invasive curative treatment option in carefully selected patients.

To develop such a treatment option for local failure, we have introduced photodynamic therapy (PDT) as a

* Correspondence: mmuto@kuhp.kyoto-u.ac.jp
†Equal contributors
[2]Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, Kyoto, Japan
[7]Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan
Full list of author information is available at the end of the article

salvage treatment [7,8], and reported the results of the phase II study of salvage PDT for local residual T1 tumors after CRT [9]. In this study, 76% (19/25) of the patients could achieve CR and suffered only modest rates of adverse events and complications. These data suggest salvage PDT could be a curative treatment option for patients with local failure after CRT.

However, first generation PDT using porfimer sodium (Photofrin Injection, Pfizer Japan Inc., Tokyo, Japan) has some problems such as a high risk of skin phototoxicity requiring a long sun shade period (4–6 weeks), and the need for a large and expensive excimer dye laser system.

In contrast, second generation PDT using talaporfin sodium (Laserphyrin for Injection, Meiji Seika Pharma Co., Ltd., Tokyo, Japan) is featured as possessing a more rapid clearance from the skin and a longer absorption band (664 nm) compared with porfimer sodium. It is also theoretically expected to have a lower rate of phototoxicity with a shorter sun shade period and be more effective in deeper layers of tissue. Furthermore, the diode laser system (PD laser, Panasonic Healthcare Co., Ltd., Ehime, Japan) that emits 664 nm laser light and excites the talaporfin sodium is a much smaller system compared with the excimer dye laser system. In a clinical trial for early lung cancer treatment, PDT using talaporfin sodium demonstrated a high response rate (95%), similar to PDT using porfimer sodium, and modest skin photosensitivity with a 2 week sun shade period [10]. However, PDT using talaporfin sodium and diode laser is approved only for early lung cancer in Japan, and its safety and efficacy have not been clinically evaluated for other cancers.

The aim of this phase I study was to clarify the optimum laser irradiation fluence rate, and to evaluate the safety of PDT using talaporfin sodium and diode laser for patients with local failure after CRT or radiotherapy (RT) for esophageal cancer.

Methods

This study was a multi-institutional open label phase I laser dose escalation study with a fixed dose of talaporfin sodium for patients with local failure after CRT or RT for ESCC. The study protocol was approved by the institutional review board of all participating institutions. This study was conducted in accordance with the Ethical Guideline for Clinical Research by the Ministry of Health, Labour and Welfare and the Declaration of Helsinki. The study was also registered with the University Hospital Medical Information Network (UMIN) Clinical Trials Registry, and the identification number is UMIN000003970.

Patients

The eligibility criteria of this study were as follows: 1) local failure after CRT or RT(≥ 50 Gy) for esophageal cancer; 2) refusal of salvage esophagectomy or lack of tolerability for salvage esophagectomy; 3) local failed lesions limited within the muscularis propria (T2); 4) local failed lesions that were not involved in the cervical esophagus, 5) longitudinal lesion length of shorter than 3 cm and less than one half the circumference of the lumen; 6) no more than 2 failure lesions; 7) enrollments of patients with lymph node or distant metastasis were accepted, except for those with indication for systemic chemotherapy 8) local failure lesions which meet at least one of the following criteria; a) histologically proven carcinoma by biopsy specimen, b) emerged ulceration in the lesions, c) enlarged submucosal tumor like protrusion in the lesion, d) presence of heteroechoic solid component with endoscopic ultrasound (EUS) observation, 9) age ≥ 20, 10) Eastern Cooperative Oncology Group performance status ≤ 2; 11) adequate bone marrow function (white blood cell count ≥2000/mm^3 and ≤ 12,000/mm^3, hemoglobin >8.0 g/dL, platelet count ≥75,000/mm^3), renal function (serum creatinine level ≤ 2.0 mg/dL), and liver function (serum total bilirubin level ≤ 2.0 mg/dL, both alanine aminotransferase and aspartate aminotransferase ≤ 100 IU/L); and 12) provision of written informed consent.

The exclusion criteria were 1) significant cardiovascular diseases (uncontrolled hypertension, myocardial infarction, unstable angina, congestive heart failure), uncontrolled diabetes mellitus, or severe liver cirrhosis; 2) systemic infection requiring antibiotics; 3) inability to obey the sun shade restrictions; 4) additional PDT just after salvage endoscopic mucosal resection or endoscopic submucosal dissection for local failures; 5) baseline lesions before CRT or RT judged to involve the aorta; 6) porphyria; 7) preexisting of sun photosensitivity; 8) previous treatment with PDT using talaporfin sodium, or treatment with PDT using porfimer sodium at least 3 months before enrollment; 9) pregnant or nursed women, or unwillingness to use of contraception; and 10) judged by investigator that enrollment was inappropriate for the patient.

Study design

In the present study, the dose of talaporfin sodium was the same as that used for lung cancer, because the safety profile of the 40 mg/m^2 dose was already clarified in the PDT regimen for lung cancer. Therefore, we planned this laser fluence escalation study to find the optimum fluence level of diode laser treatment for local failure after CRT or RT for esophageal cancer. The primary endpoint of this study was to assess the dose limiting toxicity (DLT) related with PDT at each level. The secondary endpoints were to evaluate the adverse events and toxicity related to PDT. The starting fluence of the diode laser was 50 J/cm^2 (level 1), with the escalation

plan increasing the fluence to 75 J/cm^2 (level 2) and 100 J/cm^2 (level 3). The starting fluence of 50 J/cm^2 was chosen based on results of a pre-clinical study using canine esophagus model [11]. A minimum of 3 patients were assessed for toxicity at each level. If a DLT was not observed within 28 days after laser irradiation, then the level was raised. If a DLT was observed in one of the 3 patients, an additional 3 patients were treated at the same fluence level. The maximam tolerated dose (MTD) was defined at each level when DLT was observed in 2 or more of the 3 patients, or 3 or more of the two 3 patient groups that were treated at the same fluence level. The recommended dose (RD) for further study in phase II was defined as just below the level of the MTD. If the DLT was not observed in the level, then level 3 (100 J/cm^2) was defined as the RD for the phase II study.

Staging

In this study, clinical stage was determined according to the TNM classification of the International Union Against Cancer 6th edition [12] and the *Japanese Classification of Esophageal Cancer*, 10th edition, revised version [13]. Clinical T stage was evaluated by endoscopy, EUS, and computed tomography (CT) of the chest. Clinical N and M stages were evaluated by EUS and CT of the neck, chest, and abdomen.

Treatment and surveillance

All PDT procedures were performed in an inpatient setting. One hundred milligrams of talaporfin sodium were dissolved in 4 mL of saline, and a 40 mg/m^2 dose was slowly injected intravenously. Four to 6 hours after administration of talaporfin sodium, the local failure lesion was irradiated with diode laser at a 664 nm wavelength. The diode laser light was delivered via a straight type fiber without any balloon or light diffuser through the operative channel of the scope. A plastic attachment was fitted to the tip of the scope to keep it facing the lesion and to maintain the distance between the tip of the straight type fiber and the surface of the lesion during the procedure. If the lesions were larger than 1 cm^2, multiple treatment fields were overlapped to cover the entire lesion. In this phase I study, the starting fluence was 50 J/cm^2 (level 1), with a fixed fluence rate of 150 mW/cm^2. If the post laser treatment change (e.g. ischemic change of mucosa) by endoscopic observation was suspected to be insufficient, additional laser irradiation was performed on the next day as a second session.

Before enrollment, patients were evaluated with a physical examination, performance status, dysphagia score [14] assessment, complete blood count, blood chemistry, electrocardiogram, and a chest-X ray study. After patient enrollment, adverse events were observed and graded until 28 days after laser irradiation. Patients were assessed through physical examinations, measurements of hematological and biochemical variables in the blood, chest X-ray studies, and endoscopic examinations, at least once a week until 28 days after PDT. All patients were instructed to avoid direct exposure to sunlight for 2 weeks after the injection of talaporfin sodium to protect them from skin photosensitization. Patients were allowed to discharge 2 weeks after laser irradiation if there were no complications related to PDT. Adverse events and toxicity were graded according to the Common Terminology Criteria for Adverse Events (CTCAE), version 4.0 [15].

DLT was defined as follows; 1) pain that requires administration of opioid analgesics for relief and persists for 4 days or more; 2) grade 2 or higher fever that persists 4 days or more, in spite of antipyretic administration; 3) grade 3 or higher esophageal fistula without any evidence of disease progression; 4) grade 3 or higher esophageal stenosis without any evidence of disease progression; 5) grade 3 or higher esophageal hemorrhage without any evidence of disease progression, 6) grade 4 or higher non-hematological toxicity.

Efficacy

Treatment efficacy and toxicity at the primary site were evaluated by endoscopy every week for the first 4 weeks after PDT, and every 2 weeks thereafter until the efficacy was confirmed. The clinical criteria of CR at the primary site after PDT were as follows: 1) residual lesion not observed; 2) disappearance of post PDT ulceration and confirmation of the scar formation; and 3) histological confirmation of the absence of cancer cells by biopsy. In addition, when the ulceration or erosion was not cured within 6 months after PDT, but the biopsy specimen did not continuously reveal residual cancer cells, treatment efficacy was assessed as uncertain CR and they were evaluated as a CR case.

CT was used to evaluate distant organ or lymph node metastasis every 3 months after PDT. In this study, lymph node metastasis was diagnosed clinically if the lymph node was larger than 10 mm in diameter on CT, and distant metastasis was diagnosed if the emergent metastatic lesion was confirmed with CT.

Results

Patients characteristics

Between October 2010 to May 2011, a total of 9 patients with local failure after CRT or RT for ESCC were enrolled and treated in groups of 3 individuals using up to the third fluence level. Baseline patient and lesion characteristics before CRT or RT are shown in Table 1. All of the patients were male, and their median age was 72 years old (range: 58–83). The tumor location was the upper esophagus in one patient, middle esophagus in

Table 1 Baseline patient and lesion characteristics before RT

	Age (y)	Gender	clinical stage	histology	Location
Level 1 (50 J/cm^2)					
No.1	74	Male	T3N1M1b	SCC	Middle
No.2	83	Male	T3N0M0	SCC	Middle
No.3	72	Male	T2N0M0	SCC	Middle
Level 2 (75 J/cm^2)					
No.4	58	Male	T3N1M0	SCC	Middle
No.5	81	Male	T1N0M0	SCC	Lower
No.6	67	Male	T1N0M0	SCC	Middle
Level 3 (100 J/cm^2)					
No.7	77	Male	T1N0M0	SCC	Upper
No.8	66	Male	T2N1M0	SCC	Lower
No.9	65	Male	T1N0M0	SCC	Lower

5 patients, and lower esophagus in 3 patients. The baseline clinical stages before CRT or RT were: stage I in four, stage II in three, stage III in one, and stage IVb in one patient. The initial T stage was T1 in 4 patients, T2 in 2 patients, and T3 in 3 patients. Patient and lesion characteristics before PDT are shown in Table 2. Seven of the 9 patients were treated with CRT, and the other two were treated with RT alone. The irradiation dose for the patients was 60 Gy in 7 patients, 70 Gy in one patient, and 50.4 Gy in one patient, respectively. The majority of their chemotherapeutic regimen was cisplatin plus continuous infusion of fluorouracil. Their failure patterns were recurrence after achieving a CR with CRT in 7 patients, and residual tumors just after CRT in 2 patients. All local failure lesions enrolled in this study were histologically proven solitary lesion within the radiation field. The median tumor length was 1.0 cm (range: 0.8-3.0 cm), and the circumference of the lumen was 7 patients in < 1/4, and 2 patients in 1/4-1/2. The T stage of the recurrent lesion was T1 in 6 patients, and T2 in 3 patients. None of them had clinical lymph node or distant metastasis on CT evaluation before PDT.

Toxicity

All 9 patients received administration of talaporfin sodium and diode laser irradiation, therefore they were assessable for DLT. No dermatological adverse events related to talaporfin sodium, such as allergic reaction or photosensitivity, were not observed in any patients. DLT was not observed in any patients at any level of treatments. The hematological and non-hematological toxicities in this study are summarized in Table 3. The hematological toxicity related to PDT was not observed in all patients, and when present, the non-hematological toxicity was generally mild and graded 1 as follows; 1 patient (11%) with grade 1 fever, 3 patients (33%) with grade 1 esophageal pain, and 1 patient (11%) with grade 1 dysphagia.

Efficacy

The details of the procedure and treatment efficacy of PDT are summarized in Table 4. The esophageal surface areas that were treated ranged 2–3 cm^2 in level 1, 2–4 cm^2 in level 2, 1–2 cm^2 in level 3, respectively. The total irradiation dose ranged 100–150 J in level 1, 150–300 J in level 2, and 100–200 J in level 3, respectively. A CR was achieved in 5 of 9 patients (55.6%, 95% Confidence interval [CI]: 21.2–86.3). The CR rate of each fluence level was 33.3% (1/3) in level 1, 66.7% (2/3) in level 2, and 66.7% (2/3) in level 3, respectively. Total 2 of 9 patients irradiated with another session on the next day. One was at the level 1 (patient No.3), and the other was at level 3 (patient No.8). Patient No.3 could be achieved CR, whereas No.8 could not be. There was no severe toxicity with additional irradiation on the next day.

The median period between PDT and the confirmation of CR at primary site was 39 days (range: 29–99). A representative case of a patient who achieved CR is shown in Figure 1. Furthermore, the CR rate of patients with T1 local failure was 66.7% (4/6), and that of patients with T2 local failure was33.3% (1/3). At the median follow up period of 275 days (range: 91–371), of the 5 patients who achieved CR with PDT, local recurrence was detected in 2, and the lymph node metastasis was detected in one patient.

Discussion

In this study, we evaluated for the first time the efficacy and safety of PDT using talaporfin sodium and diode laser for patients with ESCC as well as for salvage treatment for the local failure after CRT or RT. We did not experience any DLT at any levels of laser irradiation and also had a promising CR rate, therefore PDT using talaporfin sodium and diode laser for esophageal cancer could be a tolerable therapeutic option.

In the present study, skin photosensitivity did not develop in any patients with 2 weeks of sun shade period. As for other adverse events, we experienced one patient (11%) in fever, 3 (33%) in esophageal pain, and 1 (11%) in dysphagia. However, these toxicities were grade 1. In contrast, in a prior phase II study of PDT using porfimer sodium and excimer dye laser, approximately 32% of the patients experienced photosensitivity in spite of 4 to 6 week sun shade period. Furthermore, we observed esophageal pain (61%), pharyngolaryngeal pain (17%), dysphagia (39%), and fever (48%) [9]. These toxicities were grade 1 or 2. These results indicated that talaporfin -based PDT had merits of less skin photosensitivity and a shorter sun shade period. However, because the esophageal surface area irradiated (1–4 cm^2 vs 3–9 cm^2)

Table 2 Patient and lesion characteristics before PDT

	radiation dose (Gy)	concomitant chemotherapeutic regimen	pattern of failure	number of lesion	histological confirmation	tumor length (cm)	lesion circumference of the lumen	clinical stage
Level 1 (50 J/cm^2)								
No.1	60	cisplatin + 5FU	residue	1	positive	2.0	<1/4	T2N0M0
No.2	60	nedaplatin + 5FU	recurrence	1	positive	1.0	<1/4	T1N0M0
No.3	60	5FU	residue	1	negative	1.5	<1/4	T2N0M0
Level 2 (75 J/cm^2)								
No.4	60	cisplatin + 5FU	recurrence	1	positive	1.0	<1/4	T1N0M0
No.5	60	-	recurrence	1	positive	0.8	<1/4	T1N0M0
No.6	60	-	recurrence	1	positive	2.0	1/4-1/2	T1N0M0
Level 3 (100 J/cm^2)								
No.7	60	cisplatin + 5FU	recurrence	1	positive	1.0	1/4-1/2	T1N0M0
No.8	50.4	cisplatin + 5FU	recurrence	1	positive	3.0	<1/4	T2N0M0
No.9	70	cisplatin + 5FU	recurrence	1	positive	1.0	<1/4	T1N0M0

and the total laser irradiation dose (100–300 J vs 225–675 J) were quite different between each study, the toxicity related with laser irradiation should be evaluated in the next phase II study.

While this study was a phase I study, 5 of 9 patients (55.6%) achieved a CR. The CR rate for the T1 residual tumors was 66.7%, and that of T2 was 33.3%. These data were similar to the efficacy with PDT using porfimer sodium with excimer dye laser. This indicated that PDT had a high potential for local control for ESCC. Although further evaluation for the efficacy of talaporfin-based PDT is necessary in the next step with larger subjects, talaporfin-based PDT demonstrated promising anti-tumor effect for the ESCC.

Second-line systemic chemotherapy is also a treatment option for patients with treatment failure after CRT for ESCC. However, systemic chemotherapy has not demonstrated a sufficient effect in studies; the overall response rate ranged 0%–16%, and the complete response rate ranged 0%–6% [16-19]. Even for the primary site, it is quite difficult to achieve CR. This suggests that second-line systemic chemotherapy is most likely a palliative treatment option. Taken together, salvage PDT could be a curative treatment option for local failure after CRT.

For treatment failure after CRT for ESCC, salvage surgery is generally indicated. However, it demonstrated a high rate of complications and treatment-related mortality ranging from 8% to 22% [2-5]. Therefore, the indications for salvage surgery should be carefully considered. In this study, there were no severe adverse events related to talaporfin sodium-based PDT. Although there was one case of treatment related death in the prior phase II study of PDT using porfimer sodium, the rate of

Table 3 Hematological and non-hematological toxicity

	Level 1(50 J/cm^2), n = 3				Level 2(75 J/cm^2), n = 3				Level 3(100 J/cm^2), n = 3				Total (any grade, %)
	Grade				Grade				Grade				
	1	2	3	4	1	2	3	4	1	2	3	4	
Anemia	0	0	0	0	0	0	0	0	0	0	0	0	0
White bloode cell decreased	0	0	0	0	0	0	0	0	0	0	0	0	0
Neutrophil count decreased	0	0	0	0	0	0	0	0	0	0	0	0	0
Platelet count decreased	0	0	0	0	0	0	0	0	0	0	0	0	0
Fever	0	0	0	0	1	0	0	0	0	0	0	0	1(11%)
Esophageal pain	1	0	0	0	2	0	0	0	0	0	0	0	3(33%)
Pharyngolaryngeal pain	0	0	0	0	0	0	0	0	0	0	0	0	0
Dysphagia	1	0	0	0	0	0	0	0	0	0	0	0	1(11%)
Anorexia	0	0	0	0	0	0	0	0	0	0	0	0	0
Nausea	0	0	0	0	0	0	0	0	0	0	0	0	0
Photosensitivity	0	0	0	0	0	0-	0	0	0	0	0	0	0

Table 4 The details of procedure and the efficacy of PDT

	treated surface area (cm2)	total irradiation dose (J)	response of PDT	the period between PDT and CR (days)	local recurrence after CR	metastasis after CR
Level 1 (50 J/cm^2)						
No.1	3	150	nonCR	-	-	-
No.2	2	100	nonCR	-	-	-
No.3	3	150	CR	99	No	No
Level 2 (75 J/cm^2)						
No.4	2	150	nonCR	-	-	-
No.5	2	150	CR	63	No	Yes (LN)
No.6	4	300	CR	29	No	No
Level 3 (100 J/cm^2)						
No.7	1	100	CR	39	Yes	No
No.8	2	200	nonCR	-	-	-
No.9	1	100	CR	33	Yes	No

CR complete response, LN lymph node.

treatment related death was only 4% (1/25). We believe, based on these results, that salvage PDT is a less life-threating treatment option than salvage surgery.

Before starting this study, we performed a pre-clinical study of PDT using talaporfin sodium and diode laser for canine esophagus [11]. In that pre-clinical study, laser irradiation was escalated with three levels of fluence 25, 50, 100 J/cm^2 after administration of talaporfin sodium for three dogs at each levels, and pathologically evaluated one week after irradiation. The ulceration and ischemic changes around the ulcer became more severe as the dose of laser irradiation increased. Pathologically, these changes were confined to the mucosa after irradiation at 25 J/cm^2, whereas they appeared as necrosis in the muscle layer after irradiation at 50 J/cm^2, and the necrosis extended to the extra-adventitial tissue after irradiation at 100 J/cm^2. Therefore, we concluded that 25 J/cm^2 was within the safe range in normal canine esophagus. However, this dosage cannot directly apply for human, because the thickness is different between human and dog esophagus. Previous data on the normal canine bronchi and a clinical study of human lung cancer suggest that similar effects should be expected in humans after double-irradiation doses that were used in

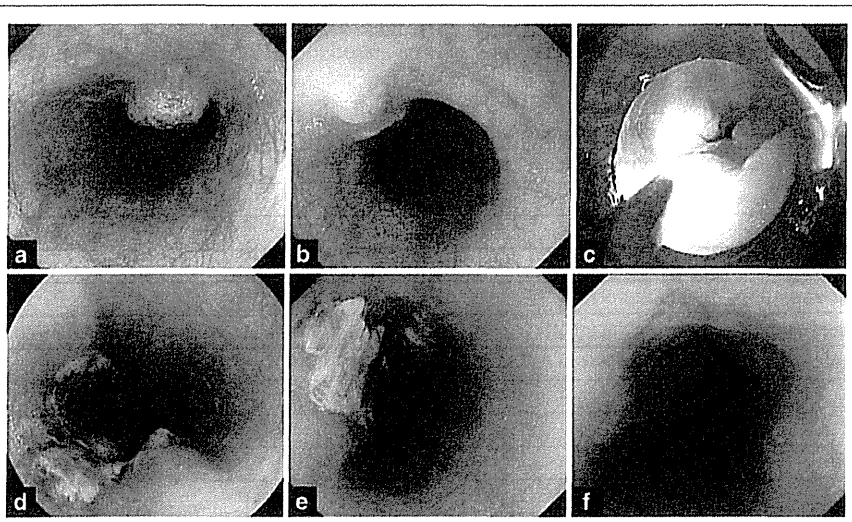

Figure 1 A patient who achieved complete response with photodynamic therapy. a: before chemoradiotherapy. **b**: local residue was detected after chemoradiotherapy. **c**: during PD laser irradiation. **d**: two days after PDT, ischemic change was observed at the laser irradiated site. **e**: two weeks after PDT, deep ulceration was observed at the laser irradiated site. **f**: CR was achieved 10 weeks after PD.

dogs. Therefore, we recommend that human clinical trials in the esophagus should start with an irradiation dose of 50 J/cm^2, and it might be appropriated from the safety profile of present study. In conclusion, PDT using talaporfin sodium and diode laser irradiation demonstrated an acceptable safety profile with manageable adverse events and promising efficacy for local failure after RT for patients with esophageal cancer. From the results of this study, 100 J/cm^2 was selected as a recommended fluence for the next phase II study.

Abbreviations

CRT: Chemoradiotherapy; CR: Complete response; ESCC: Esophageal squamous cell carcinoma; PDT: Photodynamic therapy; RT: Radiotherapy; UMIN: University hospital Medical Information Network; EUS: Endoscopic ultrasound; DLT: Dose limiting toxicity; MTD: Maximam tolerated dose; CTCAE: Common terminology criteria for adverse events.

Competing interests

There are no financial disclosure or conflict of interest relevant to this study.

Author details

[1]Department of Gastroenterology and Gastrointestinal Oncology, National Cancer Center Hospital East, Kashiwa, Japan. [2]Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, Kyoto, Japan. [3]Translational Research Center, Kyoto University Hospital, Kyoto, Japan. [4]Department of multidisciplinary cancer treatment, Kyoto University Graduate School of Medicine, Kyoto, Japan. [5]Department of Gastroenterological oncology, Hyogo Cancer Center, Hyogo, Japan. [6]Department of Gastrointestinal Oncology, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan. [7]Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan.

Authors' contributions

TY, MM, YE,YYO, YYA, HN, KH, HI are responsible for patient accrural, treatment and follow up. TY, MM, KY, MN are responsible for study design, protocol and draft of the manuscript. KY is a statisticain and MN is a cordinator of this study. MM organised this group and study. All authors read and appoved the final manuscript.

Financial support

This study was supported by Research grant of Ministry of Health, Labour and Welfare of Japan.

Received: 4 April 2012 Accepted: 23 July 2012
Published: 23 July 2012

References

1. Herskovic A, Martz K, Al-Sarraf M, Leichman L, Brindle J, Vaitkevicius V, Cooper J, Byhardt R, Davis L, Emami B: **Combined chemotherapy and radiotherapy compared with radiotherapy alone in patients with cancer of the esophagus.** *N Engl J Med* 1992, **326**:1593–8.
2. Swisher SG, Wynn P, Putnum JB, Mosheim MB, Correa AM, Komaki RR, Ajani JA, Smythe WR, Vaporciyan AA, Roth JA, Walsh GL: **Salvage esophagectomy for recurrent tumors after definitive chemotherapy and radiotherapy.** *J Thorac Cardiovasc Surg* 2002, **123**:173–83.
3. Miyata H, Yamasaki M, Takiguchi S, Nakajima K, Fujiwara Y, Nishida T, Mori M, Doki Y: **Salvage esophagectomy after definitive chemoradiotherapy for thoracic esophageal cancer.** *J Surg Oncol* 2009, **100**:442–6.
4. Tachimori Y, Kanamori N, Uemura N, Hosokawa N, Igaki H, Kato H: **Salvage esophagectomy after high-dose chemoradiotherapy for esophageal squamous cell carcinoma.** *J Thorac Cardiovasc Surg* 2009, **137**:49–54.
5. Chao YK, Chan SC, Chang HK, Liu YH, Wu YC, Hsieh MJ, Tseng CK, Liu HP: **Salvage surgery after failed chemoradiotherapy in squamous cell carcinoma of the esophagus.** *Eur J Surg Oncol* 2009, **35**(3):289–94.
6. Onozawa M, Nihei K, Ishikura S, Minashi K, Yano T, Muto M, Ohtsu A, Ogino T: **Elective nodal irradiation (ENI) in definitive chemoradiotherapy (CRT) for squamous cell carcinoma of thoracic esophagus.** *Radiother Oncol* 2009, **92**:266–9.
7. Yano T, Muto M, Minashi K, Ohtsu A, Yoshida S: **Photodynamic therapy as salvage treatment for local failures after definitive chemoradiotherapy for esophageal cancer.** *Gastrointest Endosc* 2005, **62**:31–6.
8. Yano T, Muto M, Minashi K, Onozawa M, Nihei K, Ishikura S, Kaneko K, Ohtsu A: **Long-term results of salvage photodynamic therapy for patients with local failure after chemoradiotherapy for esophageal squamous cell carcinoma.** *Endoscopy* 2011, **43**(8):657–63.
9. Yano T, Muto M, Minashi K, Iwasaki J, Kojima T, Fuse N, Doi T, Kaneko K, Ohtsu A: **Photodynamic therapy as salvage treatment for local failure after chemoradiotherapy in patients with esophageal squamous cell carcinoma: a phase II study.** *Int J Cancer* 2011. doi:10.1002/ijc.27320 [Epub ahead of print].
10. Kato H, Furukawa K, Sato M, Okunak T, Kusunoki Y, Kawahara M, Fukuoka M, Miyazawa T, Yana T, Matsui K, Shiraishi T: **Phase II clinical study of photodynamic therapy using mono-L-asparthyl chlorin e6 and diode laser for early superficial squamous cell carcinoma of the lung.** *Lung Cancer* 2003, **42**:103–111.
11. Horimatsu T, Muto M, Yoda Y, Yano T, Ezoe Y, Miyamoto S, Chiba T: **Tissue damage in the canine normal esophagus by photoactivation with talaporfin sodium (Laserphyrin): a preclinical study.** *PLoS ONE* 2012, 7(6):e38308. doi:10.1371/journal.pone.0038308.
12. Sobin LH, Wittekind C (Eds): *TNM Classification of Malignant Tumours*. 6th edition. New York: Wiley-Liss; 2002.
13. *Guidelines for the Clinical and Pathologic Studies on Carcinoma of the Esophagus*. 10th edition.: The Japan Esophageal Society; 2007.
14. Atkinson M, Ferguson R, Ogylvie AC: **Management of malignant dysphagia by intubation at endoscopy.** *J Roy Soc Med.* 1979, **27**:894–7.
15. *Common Terminology Criteria for Adverse Events (CTCAE) v 4.0 published May 28,2009*. URL http://ctep.cancer.gov/protocolDevelopment/electronic_applications/ctc.htm#ctc_40.
16. Conroy T, Etienne PL, Adenis A, Wagener DJ, Paillot B, François E, *et al*: **Phase II trial of vinorelbine in metastatic squamous cell esophageal carcinoma.** *J Clin Oncol* 1996, **14**:164–70.
17. Lordick F, von Schilling C, Bernhard H, Hennig M, Bredenkamp R, Peschel C: **Phase II study of irinotecan plus docetaxel in cisplatin-pretreated relapsed or refractory oesophageal cancer.** *Br J Cancer* 2003, **89**:630–3.
18. Muro K, Hamaguchi T, Ohtsu A, Boku N, Chin K, Hyodo I, Fujita H, Takiyama W, Ohtsu T: **A phase II study of single-agent docetaxel in patients with metastatic esophageal cancer.** *Ann Oncol* 2004, **15**:955–9.
19. Park BB, Im YH, Hwang IG, Lee SC, Ahn JS, Ahn MJ, Lim HY, Kang WK, Park K, *et al*: **Salvage chemotherapy with mitomycin C, ifosfamide, and cisplatin (MIC) for previously treated metastatic or recurrent esophageal squamous cell carcinoma.** *Invest News Drugs* 2008, **26**:387–92.

doi:10.1186/1748-717X-7-113
Cite this article as: Yano *et al.*: Phase I study of photodynamic therapy using talaporfin sodium and diode laser for local failure after chemoradiotherapy for esophageal cancer. *Radiation Oncology* 2012 7:113.

Biochemical and Biophysical Research Communications 423 (2012) 642–646

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Stability of acetaldehyde-derived DNA adduct in vitro

Kimiko Hori [a], Shin'ichi Miyamoto [a], Yoshiyuki Yukawa [a], Manabu Muto [a,*], Tsutomu Chiba [a], Tomonari Matsuda [b]

[a] Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, 54 Shogoin-kawaharacho, Sakyo-ku, Kyoto 606-8507, Japan
[b] Research Center for Environmental Quality Management, Kyoto University, 1-2 Yumihama, Ohtsu, Shiga 520-0811, Japan

ARTICLE INFO

Article history:
Received 26 May 2012
Available online 5 June 2012

Keywords:
Alcohol
Acetaldehyde
DNA adduct
N^2-ethylidene-2′-deoxyguanosine
Esophageal cancer
Carcinogenesis

ABSTRACT

Acetaldehyde (AA) derived from alcoholic beverages is a confirmed carcinogen for esophageal and head and neck cancers. AA forms various DNA adducts and is thought to play a crucial role in carcinogenesis. Transient DNA adducts are usually repaired, but the stability of AA-derived DNA adducts has not been elucidated. We investigated the stability of N^2-ethylidene-2′-deoxyguanosine (N^2-ethylidene-dG), a major AA-derived DNA adduct, in cultured cells. First, to determine the optimal concentration of AA for detecting N^2-ethylidene-dG in cell culture, a dose–response study was performed using HL60 cells of the human promyelocytic leukemia cell line. An AA concentration $\geqslant$0.01% (1.8 mM) was required to detect N^2-ethylidene-dG in vitro. We next examined the stability of N^2-ethylidene-dG. After a 1 or 2 h exposure to 0.01% of AA in a tightly sealed bottle, N^2-ethylidene-dG content was measured by sensitive liquid chromatography tandem mass spectrometry immediately, 24 h, and 48 h after exposure. After the 1 h exposure, the mean (± SD) N^2-ethylidene-dG contents were 12.1 ± 1.28, 8.20 ± 0.64, and 6.70 ± 0.52 adducts per 10^7 bases at each postexposure time. After the 2 h exposure, N^2-ethylidene-dG content increased to 21.4 ± 7.50, 10.5 ± 3.61, and 9.83 ± 3.90 adducts per 10^7 bases at each postexposure time. The half-life of this adduct was calculated as ~35 h in independent experiments. These results indicate that AA exposure from daily alcohol consumption may cause DNA damage and may increase the risk of alcohol-related carcinogenesis.

1. Introduction

In 2010, the International Agency for Research on Cancer reported that acetaldehyde (AA) derived from alcoholic beverages is carcinogenic and contributes to esophageal and head and neck cancers. The consumption of alcoholic beverages is related to an increased risk of cancer of the liver, colorectum, and female breast [1,2]. AA, an intermediate of ethanol, is genotoxic and causes mutations, sister chromatid exchanges, micronuclei, and aneuploidy in cultured mammalian cells. AA reacts with DNA to form a variety of adducts, of which deoxyguanosine (dG) is the most reactive. DNA adducts may cause polymerase errors and induce mutations in critical genes that activate proto-oncogenes and inactivate tumor suppressor genes [3,4].

Quantification of AA-derived DNA adducts in human tissues is important for assessing their carcinogenic potential. The major DNA adduct of AA formed upon reaction with dG in vitro is N^2-ethylidene-2′-deoxyguanosine (N^2-ethylidene-dG), an unstable Schiff base at the N^2 position of dG. N^2-ethylidene-dG is quite stable in DNA but decomposes quickly at the nucleoside level. Wang et al. showed that the content of N^2-ethylidene-dG could be measured using liquid chromatography–tandem mass spectrometry (LC/MS/MS) by detection of N^2-ethyl-2′-deoxyguanosine (N^2-Et-dG) after reduction of DNA during isolation and enzymatic hydrolysis [5,6]. We purified DNA using several reagents containing a strong reducing agent, $NaBH_3CN$. During the purification procedure, it was expected that N^2-ethylidene-dG would be converted to stable N^2-Et-dG (Fig. 1). N^2-Et-dG in human liver could be detected a few hundredfold by undergoing this process [7]. This method was used to show that the content of hepatic and gastric N^2-ethylidene-dG is much higher in ethanol-treated *aldehyde dehydrogenase-2*-knockout mice than in control mice [8,9].

However, the biochemical properties of N^2-ethylidene-dG are not understood fully. DNA adducts are usually repaired by various pathways, but their stability has not been investigated. In this study, we analyzed the stability of N^2-ethylidene-dG in vitro.

Abbreviations: AA, acetaldehyde; ALDH2, aldehyde dehydrogenase-2; LC/MS/MS, liquid chromatography–tandem mass spectrometry; N^2-ethylidene-dG, N^2-ethylidene-2′-deoxyguanosine; N^2-Et-dG, N^2-ethyl-2′-deoxyguanosine; α-Me-γ-OH-PdG, α-methyl-γ-hydroxy-1, N^2-propano-2′-dG; N^2-Dio-dG, ^{2}-(2,6-dimethyl-1,3-dioxan-4-yl)-2′-dG; 8-oxo-dG, 8-oxo-7,8-dihydro-2′-dG.

* Corresponding author. Fax: +81 75 751 4303.
E-mail address: mmuto@kuhp.kyoto-u.ac.jp (M. Muto).

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.05.158

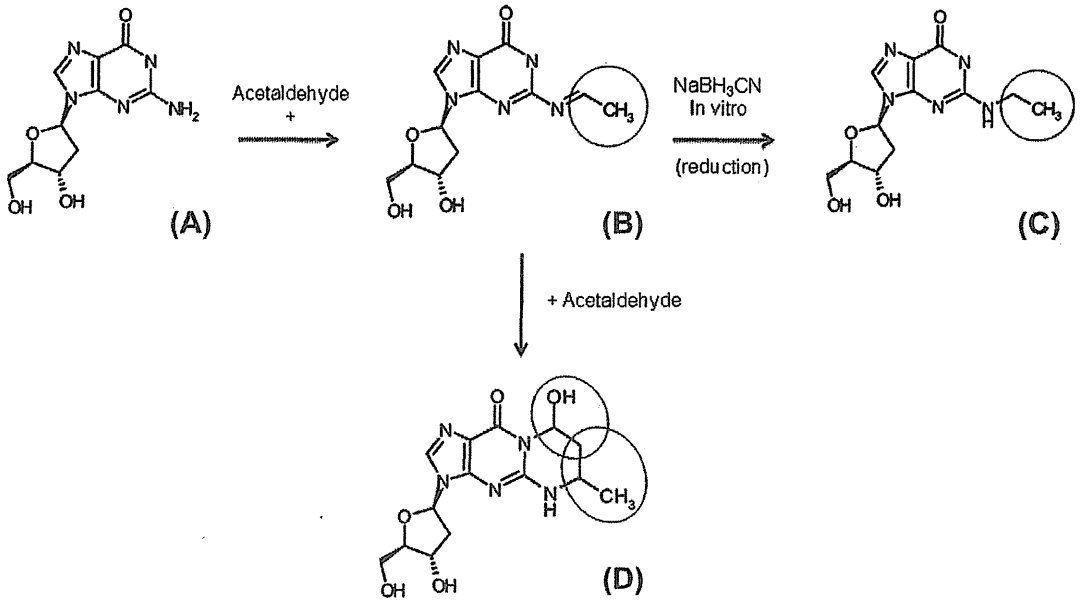

Fig. 1. Formation of acetaldehyde (AA) deoxyguanosine adducts. (A) Deoxyguanosine (dG). (B) N^2-ethylidene-2-deoxyguanosine (N^2-ethylidene-dG). (C) N^2-ethyl-2-deoxyguanosine (N^2-Et-dG). (D) α-Methyl-γ-hydroxy-1, N^2-propano-2-deoxyguanosine (α-Me-γ-OH-PdG). N^2-ethylidene-dG (B) is the major DNA adduct formed by AA and is too unstable in the nucleoside form to be detected directly. However, N^2-ethylidene-dG (B) could be measured by detection of N^2-Et-dG (C) after reduction of DNA with $NaBH_3CN$ during isolation and hydrolysis. The adduct parts are shown inside the circles.

2. Materials and methods

2.1. Cell line

HL60 cells, of the human promyelocytic leukemia cell line (American Type Culture Collection, Manassas, VA), were cultured in suspension in RPMI 1640 medium (Gibco Ltd., Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) in a humidified incubator at 37 °C in 5% CO_2. Tissue culture plastic ware was obtained from Corning Glass Works (Corning, NY).

2.2. DNA isolation

To quantify N^2-ethylidene-dG, DNA was isolated from HL60 cells as described previously [9]. The Puregene DNA Purification System Cell and Tissue kit (Gentra Systems, Inc., Minneapolis, MN) was used. The protocol was basically as described according to the manufacturer's directions, except for the addition of $NaBH_3CN$ to all solutions (final concentration: 100 mM).

After the purification step, DNA was dissolved in 10 mM Tris–HCl/5 mM EDTA buffer (pH 7.0), extracted with chloroform, and precipitated with ethanol.

2.3. DNA digestion

DNA samples (20 μg) were digested to their corresponding 2-deoxyribonucleoside-3-monophosphates by the addition of 15 μl of 17 mM citrate plus 8 mM $CaCl_2$ buffer containing micrococcal nuclease (22.5 U) and spleen phosphodiesterase (0.075 U) plus internal standards. The solutions were mixed and incubated for 3 h at 37 °C, after which alkaline phosphatase (1 U), 10 μl of 0.5 M Tris–HCl (pH 8.5), 5 μl of 20 mM $ZnSO_4$, and 67 μl of distilled water were added, and the solution was incubated for 3 h at 37 °C. The digested sample was extracted twice with methanol. The methanolic fractions were evaporated to dryness, resuspended in 50 μl of distilled water, and subjected to sensitive LC/MS/MS.

2.4. Instrumentation

LC/MS/MS analyzes were performed using a Shimadzu LC system (Shimadzu, Kyoto, Japan) interfaced with a Quattro Ultima triple-stage quadrupole MS (Waters Micromass, Manchester, UK). The LC column was eluted over a gradient that began at a ratio of 5% methanol to 95% water; the ratio was changed to 40% methanol over a period of 30 min, to 80% methanol from 30 to 35 min, and finally returned to the original starting condition of 5-95% for the remaining 11 min. The total run time was 46 min. Sample injection volumes of 20 μl each were separated on a Shim-pack XR-DOS column (3.0 mm × 75 mm; Shimadzu, Kyoto, Japan) and eluted at a flow rate of 0.2 ml/min. Mass spectral analyzes were performed in positive ion mode with nitrogen as the nebulizing gas. The ion source temperature was 130 °C, the desolvation gas temperature was 380 °C, and the cone voltage was operated at a constant 35 V. Nitrogen gas was also used as the desolvation gas (700 L/h), and cone gas (35 L/h) and argon were used as the collision gas at a collision cell pressure of 1.5×10^{-3} mbar. Positive ions were acquired in multiple-reaction monitoring (MRM) mode. The MRM transitions monitored were as follows: [$^{15}N_5$]-N^2-Et-dG, m/z 301 → 185 and N^2-Et-dG, m/z 296 → 180. The amount of DNA adduct was quantified by the ratio of the peak area of the target adduct to its stable isotope. QuanLynx (version 4.0) software (Waters Micromass, Milford, MA) was used to create a standard curve and to calculate the adduct concentration. The amount of dG was monitored by a SPD-10A UV–Vis detector (Shimadzu, Kyoto, Japan) placed before the tandem MS.

2.5. DNA adduct standard and its stable isotope

N^2-Et-dG and its [U-$^{15}N_5$]-labeled standard were synthesized as described previously [10].

2.6. Exposure of HL60 cells to AA

Because AA is highly volatile (boiling point, 20.2 °C), a complete closed system was required to expose the cells to AA. Cells were transferred to PBS to remove any factors that might affect cell growth provided by the medium and FBS. A glass vial with a rubber lid was filled with 10 ml of fresh PBS containing 40×10^6 cells without headspace. To ensure airtightness, the rubber lid was sealed tightly with Parafilm (Bemis Company Inc., Neenah, WI). The AA solution was prepared on ice and 0.1 ml of AA solution was added quickly to the vial with an injection needle through the rubber lid.

2.7. Determination of the optimal cultural concentration of AA to detect N^2-ethylidene-dG

To determine the optimal concentration of AA to detect N^2-ethylidene-dG in vitro, the levels of adducts were measured in HL60 cells after a 1 h exposure to various concentrations of AA (1.8 μM (0.00001%), 18 μM (0.0001%), 180 μM (0.001%), and 1.8 mM (0.01%)).

2.8. Calculation of the half-life ($t_{1/2}$) of N^2-ethylidene-dG in vitro

After the 1 or 2 h exposure to AA, cells were washed with fresh PBS, placed in serum-free medium, and cultured under conventional conditions. Cells were collected and DNA was isolated immediately, 24 h, and 48 h after exposure, as shown in Fig. 2. In the control group, the AA solution was replaced by 0.1 ml of PBS. The level of N^2-ethylidene-dG was measured at each postexposure time using LC/MS/MS and its $t_{1/2}$ was calculated.

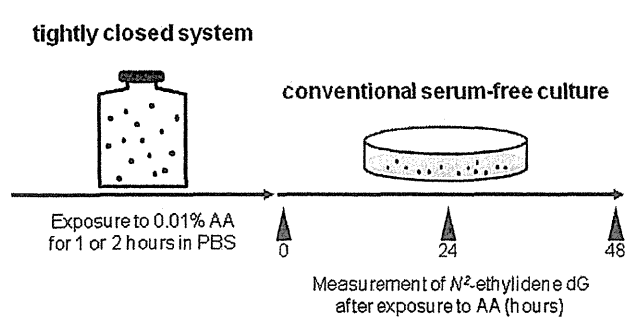

Fig. 2. Experimental procedure. HL60 cells were exposed to acetaldehyde in a tightly closed system because of its high volatility. After exposure, cells were switched to a conventional serum-free culture system (37 °C, 5% CO_2), DNA was collected immediately, 24 h, and 48 h after exposure, and the levels of N^2-ethylidene-dG were measured at each time ($n = 4$). AA, acetaldehyde; PBS, phosphate-buffered saline.

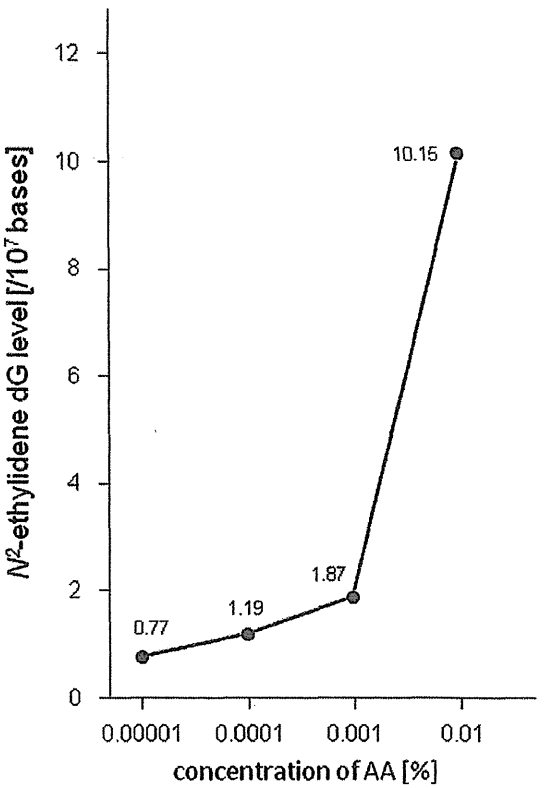

Fig. 3. Determination of the optimal concentration of acetaldehyde (AA) to detect N^2-ethylidene-dG in vitro. HL60 cells were exposed for 1 h to various concentrations of AA. The levels of N^2-ethylidene-dG were measured, and the mean levels ($n = 2$) are shown in the figure at each concentration. AA, acetaldehyde.

3. Results

3.1. Optimal cultural concentration of AA to detect N^2-ethylidene-dG

We confirmed that an AA concentration up to 0.01% (1.8 mM) for 2 h did not lead to cell death but did cause arrest of cell growth (data, not shown). After the 1 h exposure to various concentrations of AA (0.00001% (1.8 μM), 0.0001% (18 μM), 0.001% (180 μM), or 0.01% (1.8 mM)), the mean levels ($n = 2$) of N^2-ethylidene-dG at each time were 0.77, 1.19, 1.87, and 10.15 per 10^7 bases, respectively. Based on these results, we decided that the optimal concentration of AA was 0.01% (1.8 mM) (Fig. 3).

3.2. Half-life of N^2-ethylidene-dG in vitro

In the cells exposed to AA for 1 h (0.01% (1.8 mM) AA, $n = 4$), the mean (± SD) levels immediately, 24 h, and 48 h after exposure were 12.1 ± 1.28, 8.20 ± 0.64, and 6.70 ± 0.52 adducts per 10^7 bases, respectively. In the control group ($n = 2$), the mean levels were 3.26, 3.31, and 3.26 adducts per 10^7 bases, respectively (Fig. 4A). In the cells exposed to AA for 2 h (0.01% (1.8 mM) AA, $n = 4$), the levels were 21.4 ± 7.50, 10.5 ± 3.61, and 9.83 ± 3.90 adducts per 10^7 bases, respectively. In the control group ($n = 2$), the mean levels were 3.19, 2.44, and 2.70 adducts per 10^7 bases, respectively (Fig. 4B).

To estimate the levels of N^2-ethylidene-dG formed from AA alone, the data were corrected by subtracting the control levels from the mean levels in the exposure group at each time. The corrected data were 8.85, 4.89, and 3.45 adducts per 10^7 bases in the 1 h exposure group and 18.2, 8.09, and 7.13 adducts per 10^7 bases in the 2 h exposure group for levels measured immediately, 24 h, and 48 h after exposure, respectively. Fitted curves were obtained from the corrected data. The two fitted curves could be presented as an exponential approximation and its mathematical expression was as follows: $y = 8.50e^{-0.02x}$ ($R^2 = 0.98$) in the 1 h exposure group (Fig. 4A) and $y = 16.2e^{-0.02x}$ ($R^2 = 0.85$) in the 2 h exposure group (Fig. 4B). The same $t_{1/2}$ of approximately 35 h ($t_{1/2} = \log_e 2/0.02 = 0.693/0.02 = 34.65$) was obtained in independent experiments.

4. Discussion

We show for the first time that the $t_{1/2}$ of DNA adducts formed by AA is about 35 h in vitro. There are some limitations in our