Table 7 Three-year outcomes after PCI and CABG in patients with heart failure

	Number of patients (event/total)				Comparison of PCI vs CABG			
	PCI (n=812)		CABG (n=421)		Hazard ratio	95%CI		p
Death	172	21%	71	17%	1.68	1.10	2.56	0.02
Death, stroke, or MI	219	27%	91	22%	1.82	1.25	2.66	<0.01
Cardiac death	110	14%	42	10%	2.06	1.20	3.54	0.01
Arrhythmia death	28	3%	2	0%	9.57	1.92	47.72	0.01
Readmission for heart failure	176	22%	53	13%	1.67	1.07	2.59	0.02
Stroke	53	7%	24	6%	1.67	0.80	3.50	0.17
Myocardial infarction	32	4%	12	3%	4.48	1.37	14.69	0.01
Any revascularization	152	19%	16	4%	5.47	2.87	10.43	<0.01

た．総死亡に関する結果は海外の大規模観察研究のサブ解析結果と一致するメッセージであり，本邦においても死亡というハードエンドポイント評価でCABGはPCIにくらべ良好なアウトカムをもたらすことが示されたことは重要である．当研究は観察研究のサブ解析という位置づけではあるが，本邦のオリジナルエビデンスとして重要であることは疑いがないであろう．今後は本邦独自の低左心機能患者を対象としたRCTや観察研究のエビデンスを発信し，本邦独自のガイドラインの作成に結びつけることが期待される．

V. エビデンスの確立に向けて

本総説でも述べたとおり，虚血性心疾患を有する低左心機能患者に対する至適冠血行再建についてのエビデンスは現状では十分に確立されていない．最近の大規模試験からエビデンスからもわかるように，PCI vs CABGのディスカッションとなる対象患者は3枝や左主幹部などの複雑病変を有する患者になると思われる．しかし複雑病変を有する低左心機能患者は，虚血性僧帽弁閉鎖不全を有することも多く，現時点では虚血性僧帽弁閉鎖不全に対する冠血行再建や弁治療の適応についても十分なエビデンスを確立できていない状況であり，それらを十分に踏まえた上でPCI vs CABGのスタディデザインを構築していく必要がある．また低左心機能の定義についても，2013年ACCF/AHA心不全のガイドライン[24]で指摘されているように，LVEF 35～50%の軽～中等度収縮障害の患者と，STICH試験[7]で対象とされているようなLVEF 35%未満の患者では，予後が大きく異なるため，LVEFに準じた治療エビデンスの確立が必要となっている．さらに現在までの報告の多くが左室収縮不全(LVEF低下)についてのエビデンスであるが，実際の臨床では左室収縮力の維持された心不全，いわゆる拡張不全による患者多数存在することが知られており，そのような患者に対するエビデンスも今後確立していく必要があるであろう．

VI. おわりに

虚血性低左心機能患者に対する至適冠血行再建ついてのエビデンスは現状では十分に確立されておらず，ガイドラインにおいても最終的にはハートチームの判断に委ねられているのが現状である．虚血性低左心機能患者の予後は不良であり治療目的を達成するには，冠危険因子の是正・運動療法・薬物療法を基本として，冠動脈疾患の重症度，僧帽弁閉鎖不全の有無などを考慮して総合的に冠血行再建の適応を判断する必要がある．今後はそれらのエビデンスをさらに積み重ね，PCI vs CABGを含めた集学的治療戦略を確立することが重要であると考えられる．

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RESEARCH ARTICLE Open Access

A randomized controlled clinical trial of topical insulin-like growth factor-1 therapy for sudden deafness refractory to systemic corticosteroid treatment

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Abstract

Background: To date, no therapeutic option has been established for sudden deafness refractory to systemic corticosteroids. This study aimed to examine the efficacy and safety of topical insulin-like growth factor-1 (IGF-1) therapy in comparison to intratympanic corticosteroid therapy.

Methods: We randomly assigned patients with sudden deafness refractory to systemic corticosteroids to receive either gelatin hydrogels impregnated with IGF-1 in the middle ear (62 patients) or four intratympanic injections with dexamethasone (Dex; 58 patients). The primary outcome was the proportion of patients showing hearing improvement (10 decibels or greater in pure-tone average hearing thresholds) 8 weeks after treatment. The secondary outcomes included the change in pure-tone average hearing thresholds over time and the incidence of adverse events.

Results: In the IGF-1 group, 66.7% (95% confidence interval [CI], 52.9–78.6%) of the patients showed hearing improvement compared to 53.6% (95% CI, 39.7–67.0%) of the patients in the Dex group ($P = 0.109$). The difference in changes in pure-tone average hearing thresholds over time between the two treatments was statistically significant ($P = 0.003$). No serious adverse events were observed in either treatment group. Tympanic membrane perforation did not persist in any patient in the IGF-1 group, but did persist in 15.5% (95% CI, 7.3–27.4%) of the patients in the Dex group ($P = 0.001$).

Conclusions: The positive effect of topical IGF-1 application on hearing levels and its favorable safety profile suggest utility for topical IGF-1 therapy in patients with sudden deafness.

Trial registration: UMIN Clinical Trials Registry Number UMIN000004366, October 30th, 2010.

Keywords: Dexamethasone, Drug delivery system, IGF-1, Local application, Sudden sensorineural hearing loss

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Background

Sudden sensorineural hearing loss (SSHL), an unexplained unilateral hearing loss with an onset of less than 72 h, is a common disease with acute onset hearing impairment. The incidence of SSHL is reportedly 5 to 20 patients per 100,000 persons per year [1]. Approximately 35,000 patients with SSHL consult a doctor each year in Japan [2]. The standard treatment for SSHL is systemic corticosteroid treatment [3,4]. Hearing improvement after systemic corticosteroids occurs in 50% of the patients, but approximately 20% of the patients show no response [5]. Further, systemic corticosteroid treatment often causes adverse events [6] that can occasionally be life-threatening [7]. As an alternative for systemic corticosteroids, intratympanic corticosteroid treatment by direct injection into the middle ear has recently gained wide popularity because of the low risk for systemic adverse events and because of the potential delivery of high concentrations of a corticosteroid into the inner ear [8]. Intratympanic corticosteroid therapy is commonly used for the treatment of SSHL, after the failure of systemic corticosteroid treatment [9-15]. However, the supporting evidence for its use is weak because of the limitation in the study design and power [16].

A major difficulty in treating sensorineural hearing loss is the poor regeneration capacity of the mammalian cochlea. Therefore, protecting the cochlea from irreversible degeneration is a practical strategy. Several growth factors have been investigated for their protective effects on the sensory hair cells of the cochlea [17,18]. The focus of this study was on insulin-like growth factor-1 (IGF-1), which has been used for the treatment of insulin-resistant diabetes and dwarfism. IGF-1 also plays crucial roles in both the development and maintenance of the cochlea [19,20]. We used a gelatin hydrogel, which enables the sustained release of proteins or peptides, for the delivery of IGF-1 into the cochlear fluid [21]. We previously performed a successful series of animal experiments using this method [21,22]. A prospective, single-armed clinical trial in patients with SSHL refractory to systemic corticosteroids was then performed, the results of which indicated the safety and efficacy of topical IGF-1 therapy in comparison to the historical control of hyperbaric oxygen therapy [23].

The goal of the current study was to investigate the efficacy and safety of topical IGF-1 therapy as a novel therapeutic option for SSHL. We conducted a multicenter, randomized clinical trial to compare topical IGF-1 therapy and intratympanic corticosteroid therapy for treating SSHL refractory to systemic corticosteroids.

Methods

Study design and patients

This was a multicenter, randomized, open, parallel-group trial. The study was conducted from November 2010 through October 2013 at 9 tertiary referral hospitals in Japan. The trial followed the guiding principles of the Declaration of Helsinki. The study protocol, manual of procedures, and informed consent form were approved by the institutional review boards of all participating sites (Ethical Committee of the Graduate School of Medicine, Kyoto University [C470], Ethical Committee of the Graduate School of Medicine, Hirosaki University [2011–145], Ethical Committee of University of Tsukuba Hospital [H23-13], Ethical Committee of Toranomon Hospital [2011-4-15], Ethical Committee of Shinshu University Hospital [1705], Ethical Committee of Nagoya City University Hospital [45-11-0005], Ethical Committee of Kobe City Medical Center General Hospital [1], Ethical Committee of Ehime University Hospital [1105003], and Ethical Committee of the Graduate School of Medicine, Kyushu University [23011]). All patients provided written informed consent. Eligible participants were all adults, 20 years or older, who had SSHL defined as a unilateral sensorineural hearing loss of at least 30 decibels (dB) sound pressure level (SPL) over at least three test frequencies that developed within 3 days. They also met the following eligibility criteria: they had been diagnosed as having SSHL within 25 days of onset; they presented with an abnormality in the distortion product of otoacoustic emissions; and they showed less than 30 dB hearing improvement in the mean hearing level, based on pure-tone audiometry (PTA) at five tested frequencies (0.25 kHz, 0.5 kHz, 1.0 kHz, 2.0 kHz, and 4.0 kHz) after more than 7 days of systemic corticosteroid treatment. Similar to our previous trial [23], we excluded patients with active chronic otitis media, acute otitis media, otitis media with effusion or dysfunction of the auditory tube, malignant tumors, and systemic diseases. All patients underwent imaging examinations to rule out retrocochlear pathology.

Randomization and masking

Patients were randomly assigned (1:1) to receive either topical IGF-1 therapy or intratympanic dexamethasone (Dex) therapy. Randomization was performed centrally with stratification by the study sites and the mean hearing thresholds, based on the PTA at the five frequencies tested at registration (lower than 90 dB SPL vs. 90 dB SPL or higher). The randomization sequence was generated by a third-party contract clinical research organization (independent from the trial investigators). Local investigators used a web-based system during enrolment, which then automatically assigned patients to either treatment group. Besides central randomization, no further masking was used in this open-label study.

Procedures

The treatment was performed within 7 days of enrolment and systemic corticosteroid treatment was completed by

the time of enrolment. Gelatin hydrogels were produced from porcine skin gelatin (Nitta Gelatin Inc., Osaka, Japan), based on a previously described method [23,24]. Mecasermin (Somazon [10 mg for injection]; Astellas Pharma, Inc., Tokyo, Japan), a recombinant human IGF-1, was dissolved in physiological saline at a final concentration of 10 mg/mL. Sixty minutes before application, a 30 μL sample of mecasermin solution was mixed with 3 mg of gelatin hydrogel. After tympanostomy under local anesthesia with 1% lidocaine, the hydrogel (which contained 300 μg of mecasermin) was placed in the round window niche of the middle ear; a single application was used. The control group received four 0.5 mL doses containing 3.8 mg/mL dexamethasone sodium phosphate (Orgadrone injection [1.9 mg]; MSD, Inc., Tokyo, Japan) that was injected into the middle ear through the tympanic membrane. In the literature [9-15], a variety of regimens for intratympanic injections of corticosteroids have been used. Considering practical use in common clinical settings, we chose four doses. Injections were performed over 4 consecutive days in principle. Within at least 7 days, four injections were administered. Patients were placed in the supine position with the affected ear slightly raised and remained in this position for 30 min after the injection. For 16 weeks after treatment, the patients were examined at the outpatient clinics of the participating sites. The PTA was measured on the day of enrolment, and then at 1, 2, 4, 8, 12, and 16 weeks after treatment. During the observation period, which totaled 16 weeks, all adverse events were recorded.

Outcomes

The primary outcome measure was the proportion of patients showing hearing improvement of 10 dB or greater in the mean hearing level. Hearing improvement was based on PTA at five tested frequencies and was defined as better than slight recovery, based on the criteria for hearing improvement determined by the Sudden Deafness Research Committee of the Japanese Ministry of Health, Labor and Welfare in 1984 (Table 1) at 8 weeks after treatment. Briefly, the complete recovery includes patients showing recovery of a hearing level within 20 dB SPL or to the similar level to the opposite side, the marked recovery is more than 30 dB recovery, and the no recovery is less recovery than 10 dB. Secondary outcome measures included the change in the pure-tone average hearing thresholds over time (i.e., from the first audiogram to the 16-week follow-up audiogram), the proportion of patients showing hearing improvement at 12 and 16 weeks after treatment, and the incidence of adverse events during the observation period. In addition to checking vital signs in the physical examination at each visit, laboratory studies were performed at registration, and then at 1 week, 8 weeks, and 16 weeks after treatment.

Table 1 Criteria for hearing improvement determined by Sudden Deafness Research Committee of the Japanese Ministry of Health, Labour and Welfare in 1984

Complete recovery	Recovery of a hearing level within 20 decibels (dB) at all five frequencies tested (0.25, 0.5, 1.0, 2.0 and 4.0 kHz) or recovery to the same level as the opposite side in pure-tone auditometry
Marked recovery	30 dB and over recovery in the mean hearing level at the five frequencies tested
Slight recovery	Recovery of better than 10 dB and less than 30 dB in the mean hearing level at the five frequencies tested
No response	Less recovery than 10 dB in the mean hearing level at the five frequencies tested

Statistical analysis

The null hypothesis was that the effect of topical IGF-1 treatment on the proportion of patients showing hearing improvement would not be superior to intratympanic injection of Dex. The sample size was determined based on our previous findings and on published papers. In our previous clinical trial of topical IGF-1 therapy, no patient that had been enrolled later than 26 days after sudden hearing loss recovered their hearing [23]. The proportion of patients with hearing improvement after topical IGF-1 treatment was 62.5% (10 of 16 patients) at 12 weeks and 68.8% (11 of 16 patients) at 24 weeks, using the eligibility criteria of patients with SSHL within 25 days after onset of sudden hearing loss. Based on these findings, we hypothesized that the expected proportion of patients showing hearing improvement with topical IGF-1 therapy would be 65%. To determine the expected proportion of patients showing hearing improvement for intratympanic Dex therapy, we referred to the information in several publications [9-15] that were available in October 2010 and used the following criteria: i) the sample size had more than 10 participants and ii) intratympanic Dex therapy was a salvage treatment (Table 2). In these previous reports, the mean proportion of patients showing hearing improvement was 39% (range, 21–58%). Therefore, the proportion of patients who would show hearing improvement with intratympanic Dex therapy was expected to be approximately 40%. The sample size was based on the continuity-adjusted arcsine test with a one-sided significance level of 0.05 and a power of 0.80. The required sample size was 120 participants, assuming that 5% of the patients would be excluded from the analysis.

All statistical analyses were performed on an intention-to-treat basis. The safety analyses were conducted on all patients who underwent randomization and received at least one dose of the study drugs. Efficacy was analyzed in all patients except those who had been excluded from the

Table 2 Previous studies of intratympanic dexamethasone therapy as a salvage treatment

	No. of patients	PTA recovery	Dexamethasone concentration	Doses	Proportion of recovery (%)
Ho et al. [9]	15	28 dB	4 mg/mL	3 doses, once a week	53%
Choung et al. [10]	34	9 dB	5 mg/mL	4 doses, twice a week	39%
Roebuck et al. [11]	31	12 dB	24 mg/mL	Single	29%
Haynes et al. [12]	40	5 dB	24 mg/mL	Single	27.5%
Plontke et al. [13]	11	14 dB	4 mg/mL	Pump for 14 days	45%
Kakehata et al. [14]	24	17 dB	4 mg/mL	15 doses	58%
Lee et al. [15]	34	NA	4 mg/mL	6 doses for 14 days	21%
Mean (95% CI)	27 (21–33)				39% (30%–48%)

PTA, Pure-tone audiometry; NA, Not available; CI, Confidential interval.

safety analyses due to eligibility violations. The differences between treatments in the proportion of patients showing hearing improvement at 8, 12, and 16 weeks after treatment were evaluated with Fisher's exact test at a one-sided significance of 0.05. The difference between treatments in changes in pure-tone average hearing thresholds over time was investigated with repeated measures linear mixed model containing terms for treatment, time, and treatment-by-time interaction with an unstructured covariance structure [25]. The effect of treatment-by-treatment interaction was analyzed with the *t*-test at a one-sided significance of 0.05. The differences between treatments in the incidence of adverse events and in the baseline characteristics were analyzed with either Fisher's exact test or the *t*-test at a two-sided significance of 0.05. Statistical analyses were performed by using SAS version 9.3 software (SAS Institute, Inc., Cary, NC, USA).

Results

Study overview

Patients from nine participating sites were enrolled between March 2011 and October 2013. The recruitment and enrollment period was originally planned to close in February 2013, but it was extended to meet the recruitment targets. There were 120 patients who consented to participate (Figure 1). All patients were randomized to either the IGF-1 group or the Dex group; 118 patients were included in the safety analysis (60 IGF-1, 58 Dex) because 2 patients from the IGF-1 group withdrew consent. Of the 118 patients who were included, 4 patients completed the treatments, but missed the 8-week follow-up (2 IGF-1, 2 Dex) and 1 patient was excluded owing to examiner error (1 IGF-1).

Baseline characteristics were comparable between the two treatment groups (Table 3). Between the IGF-1 and Dex groups, no significant baseline differences in demographics, physical characteristics, ear examination, or PTA thresholds were found except the proportion of patients presenting with aural fullness (Table 3). The mean age of all participants was 49.3 years, and 45.8% of the participants were male. The mean PTA thresholds in the affected and unaffected ears were 85.2 dB SPL (95% confidence interval [CI], 81.3–89.1) and 18.1 dB SPL (95% CI, 14.9–21.3), respectively. The mean number of days

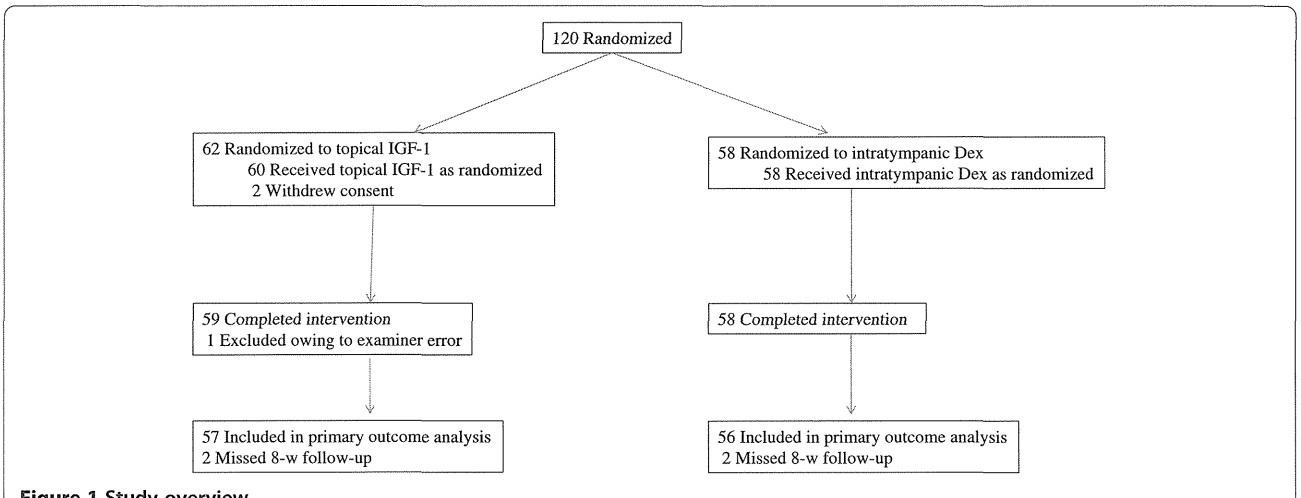

Figure 1 Study overview.

Table 3 Baseline characteristics of patients

Characteristics	Intratympanic Dex (n = 58)	Topical IGF-1 (n = 60)	*P* value
Age-yr, mean ± SD	50.1 ± 13.0	48.6 ± 14.0	0.557
Male sex-no. (%)	26 (44.8)	28 (46.7)	0.855
No. of days for study entry from onset, mean (95% CI)	16.3 (15.1–17.5)	15.8 (14.6–17.0)	0.574
Hearing improvement by pre-treatment-no. (%)			
>10 dB, <30 dB	13 (22.4)	17 (28.3)	0.528
<10 dB	45 (77.6)	43 (71.7)	
Hearing-dB pure-tone average, mean (95% CI)			
Affected ear	84.8 (79.1–90.4)	85.6 (80.0–91.2)	0.835
Unaffected ear	15.8 (12.4–19.2)	20.4 (15.0–25.7)	0.160
Other symptoms-no. (%)			
Dizziness/Vertigo	23 (39.7)	31 (51.7)	0.202
Tinnitus	49 (84.5)	51 (85)	>0.999
Aural fullness	44 (75.9)	32 (53.3)	0.013*

An asterisk indicates statistical significance with Fisher's exact test. Dex: dexamethasone.

from symptom onset to study entry was 16.0 days (95% CI, 15.2–16.9). At enrolment, dizziness or vertigo was present in 45.8% of the patients, tinnitus was present in 84.8% of the patients, and aural fullness was present 64.4% of the patients.

Primary outcome

The primary outcome was the proportion of patients showing hearing improvement (10 dB or greater in pure-tone average hearing thresholds) at 8 weeks after treatment. In the Dex group, 53.6% (95% CI, 39.7–67.0%) of patients showed hearing improvement at 8 weeks after treatment, whereas in the IGF-1 group, 66.7% (95% CI, 52.9–78.6%) of patients showed hearing improvement (Table 4). The null hypothesis for the primary outcome was not rejected ($P = 0.109$). However, a trend was observed in the higher proportion of patients in the IGF-1 group showing complete or marked recovery (30 dB or greater in pure-tone average hearing thresholds) over that in the Dex group (Table 2).

Secondary outcomes

The changes in the pure-tone average hearing thresholds occurring over time in both treatments are shown in Figure 2. The difference in the changes in the pure-tone average hearing thresholds over time between the treatments was statistically significant with repeated measures linear mixed model containing terms for treatment, time, and treatment-by-time interaction with an unstructured covariance structure (the effect for the interaction term [standard error]: −0.28 [0.10], $P = 0.003$). This demonstrated that pure-tone average hearing thresholds of the IGF-1 group significantly reduced over time, if changes in pure-tone average hearing thresholds over time in the Dex group were set to zero.

The proportions of patients showing hearing improvement (i.e., 10 dB or greater) at 12 weeks and 16 weeks after treatment were estimated as the secondary outcomes (Table 4). The null hypothesis for the proportions of patients showing hearing improvement at 12 weeks or 16 weeks after treatment was not rejected ($P = 0.066$ for 12 weeks; $P = 0.116$ for 16 weeks). At 12 and 16 weeks after treatment, there was a trend in the IGF-1 group showing a larger number of patients with complete and marked recovery when compared to the Dex group (Table 4).

Adverse events

No serious adverse events were observed in either treatment group. During the observation period, moderate adverse events occurred in 43.1% (95% CI, 30.2–56.8) of the patients in the Dex group and in 35.0% (95% CI, 23.1–48.4) of the patients in the IGF-1 group (Table 4). No significant difference in the incidence of adverse events was found between treatments ($P = 0.452$). Most adverse events, such as otitis media, otitis externa, tinnitus, and nausea or vomiting disappeared during the observation period. However, tympanic membrane perforation persisted in 15.5% (95% CI, 7.3–27.4%) of the patients in the Dex group at the end of the observation period. On the other hand, no patient in the IGF-1 group showed residual perforation in the tympanic membrane. The difference in the incidence of tympanic membrane perforation was statistically significant ($P = 0.001$).

Discussion

This is the first randomized controlled clinical trial to test the efficacy of a growth factor for the treatment of sensorineural hearing loss. In the current study, we locally applied IGF-1 to patients with SSHL refractory to

Table 4 Primary and secondary outcomes

	Intratympanic Dex	Topical IGF-1	*P* value
Primary outcome			
Proportion of patients showing hearing recovery at 8 weeks	53.6% (30/56)	66.7% (38/57)	0.109
	[95% CI: 39.7–67.0]	[95% CI: 52.9–8.6]	
Complete recovery	0.0% (0/56)	3.5% (2/57)	
Marked recovery	16.1% (9/56)	24.6% (14/57)	
Slight recovery	37.5% (21/56)	38.6% (22/57)	
No recovery	46.4% (26/56)	33.3% (19/57)	
Secondary outcomes			
Proportion of patients showing hearing recovery at 12 weeks	55.4% (31/56)	70.7% (41/58)	0.066
	[95% CI: 41.5–68.7]	[95% CI: 57.3–81.9]	
Complete recovery	0.0% (0/56)	5.2% (3/58)	
Marked recovery	21.4% (12/56)	31.0% (18/58)	
Slight recovery	33.9% (19/56)	34.5% (20/58)	
No recovery	44.6% (25/56)	29.3% (17/58)	
Proportion of patients showing hearing recovery at 16 weeks	54.7% (29/53)	67.9% (36/53)	0.116
	[95% CI: 40.4–68.4]	[95% CI: 53.7–80.1]	
Complete recovery	0.0% (0/53)	5.7% (3/53)	
Marked recovery	22.6% (12/53)	24.5% (13/53)	
Slight recovery	32.1% (17/53)	37.7% (20/53)	
No recovery	45.3% (24/53)	32.1% (17/53)	
Adverse events			
Serious	0.0% (0/58)	0.0% (0/59)	>0.999
Non-serious	43.1% (25/58)	35.0% (21/59)	0.452
Tympanic membrane perforation	15.5% (9/58)	0.0% (0/59)	0.001*
Otitis media	1.7% (1/58)	6.8% (4/59)	0.364
Otitis externa	0.0% (0/58)	1.7% (1/59)	>0.999
Tinnitus	8.6% (5/58)	0.0% (0/59)	0.027*
Nausea/Vomit	3.4% (2/58)	3.4% (2/59)	>0.999
Others	24.1% (14/58)	30.5% (18/59)	0.535

Asterisks indicate statistical significance with Fisher's exact test.

systemic steroids. The null hypothesis of the primary outcome was that the proportion of patients showing hearing improvement after topical IGF-1 therapy would not be better than that after intratympanic Dex therapy. The null hypothesis was not rejected in the present study. The major reason for this is an unexpectedly high proportion of patients showing hearing improvement after intratympanic Dex therapy. The proportion of patients showing hearing improvement after topical IGF-1 therapy was 66.7 to 70.7%, which was nearly identical to our hypothesized value of 65%, whereas the proportion of patients showing hearing improvement after intratympanic Dex therapy (range, 53.6– 55.4%) was higher than our hypothesized value of 40%.

Although the null hypothesis for the primary outcome was not rejected, this randomized controlled trial showed significantly better recovery of pure-tone average thresholds over time in the IGF-1 group, compared to the Dex group. In addition, a trend that the proportion of patients in the IGF-1 group who showed complete or marked recovery was higher than that in the Dex group was observed at 8, 12, or 16 weeks after treatment. Complete recovery of hearing was observed only in the IGF-1 group. These findings strongly suggest the superior efficacy of topical IGF-1 therapy over intratympanic Dex therapy.

In the current study, we used intratympanic corticosteroid therapy as a control treatment because it has widely been accepted as a salvage treatment for SSHL refractory to systemic corticosteroids [9-15]. The current randomized study also provided evidence for the safety and efficacy of intratympanic Dex therapy for SSHL refractory to systemic corticosteroids. Similar to the results

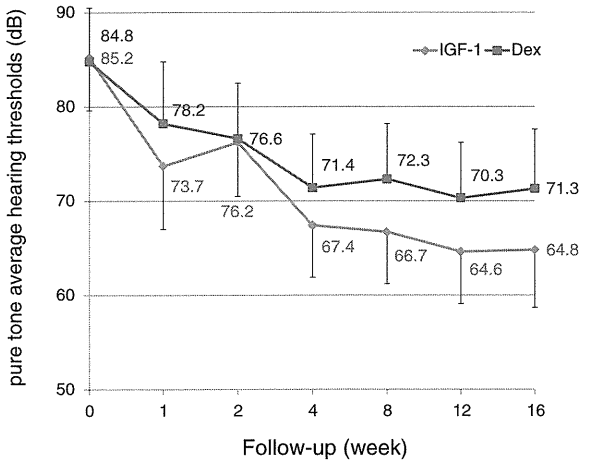

Figure 2 Changes in pure-tone average hearing thresholds over time. The difference between IGF-1 and dexamethasone (Dex) treatments in changes in pure-tone average hearing thresholds over time was statistically significant. Captions in the graph show mean values. Bars represent 95% confidence intervals.

of previous studies [9-15], no serious adverse events occurred in the Dex patient group. However, tympanic membrane perforation persisted in 15.5% of these patients, while tympanic membrane perforation was absent in the IGF-1 patient group. The incidence of tympanic membrane perforation in the Dex group in the present study was higher than the 3.9% incidence in a previous randomized trial of intratympanic corticosteroid therapy as the initial treatment [8]. This may be because of a difference in either the application regimen or the influence of the preceding systemic corticosteroid treatment. It is important to note that intratympanic injection of corticosteroids causes tympanic membrane perforation in a certain proportion of treated patients, while both our previous [23] and present results demonstrated no occurrence of residual perforation in tympanic membranes of patients treated with topical IGF-1 therapy. This indicated the superior safety of topical IGF-1 therapy over intratympanic Dex therapy. On the other hand, the high incidence of tympanic membrane perforation in the Dex group might affect hearing recovery outcomes in the Dex group.

The present findings indicate the efficacy and safety of topical IGF-1 therapy for SSHL. However, topical IGF-1 therapy requires surgical procedures and causes uncomfortable symptoms associated with the local application. In addition, spontaneous recovery of hearing occurs in 30 to 60% of patients with SSHL [5,26-28]. Therefore, with the need for balancing the harmful side effects and the benefits, SSHL patients showing insufficient hearing improvement after the administration of oral corticosteroids or after strict observation for 7 days may be good candidates for topical IGF-1 therapy. On the other hand, IGF-1 is a promoter of cell proliferation in some cellular contents. Therefore, a long-term follow-up of patients may be required. Of note, in our previous clinical trial, we locally applied IGF-1 in the middle ear of 25 patients with refractory SSHL [23]; in the 5-year follow-up, no tumor formation was identified in those patients.

Conclusions

We performed a randomized, controlled clinical trial of topical IGF-1 therapy in patients with SSHL refractory to systemic corticosteroids and compared this treatment to intratympanic corticosteroid therapy. Present results suggest the possibility that IGF-1 is superior to intratympanic Dex therapy, but the current study design failed to confirm this possibility. The positive effect of topical IGF-1 application on hearing levels and its favorable safety profile suggest utility for topical IGF-1 therapy as a salvage treatment for SSHL.

Abbreviations

CI: Confidence interval; dB: Decibels; Dex: Dexamethasone; IGF-1: Insulin-like growth factor-1; PTA: Pure-tone audiometry; SSHL: Sudden sensorineural hearing loss.

Competing interests

All authors declare that they have no competing of interests.

Authors' contributions

TN obtained funding. TN, ST, HT, TI-I, TI, ASh, AY, YT, and JI contributed to the research design. AY and YT designed and prepared gelatin hydrogels. HT and ASh undertook and monitored study conduct with supervision from TN. JI, TN, KK, SU, NH, KT, MT, KF, ASa, SK, TS, HH, and NY recruited patients and undertook patient treatments. HT performed data cleaning and preparations for analysis. MY performed statistical analyses and wrote the statistical sections. TN and MY interpreted data. TN wrote the first draft of the manuscript. All authors contributed to the revised draft versions. All authors approved the final version.

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Role of the funding source

This work was initiated by the investigators and was conducted independently of any commercial entities. The drugs were purchased commercially.

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Preclinical Validation of Talaporfin Sodium-Mediated Photodynamic Therapy for Esophageal Squamous Cell Carcinoma

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Abstract

Photodynamic therapy (PDT) kills cancer cells via a photochemical reaction mediated by an oncotropic photosensitizer. Herein, we performed an experimental preclinical study to validate the anti-tumour effect of talaporfin sodium-mediated PDT (t-PDT) for esophageal squamous cell carcinoma (ESCC) cells. We used human ESCC cells derived from various differentiation grades or resistant to 5-fluorouracil (5-FU). The cytotoxic effect of t-PDT was determined by evaluating cell viability, apoptosis and generation of reactive oxygen species (ROS) and DNA double-strand breaks. Furthermore, the anti-tumour effect of t-PDT was assessed using an anchorage-independent cell-growth assay and xenograft transplantation models. t-PDT induced potent cytotoxicity in ESCC cells independent of their differentiation grade or 5-FU resistance. Moreover, t-PDT induced robust apoptosis, as indicated by cell shrinkage, perinuclear vacuolization, nuclear fragmentation and induction of annexin V-positive cells. This apoptotic response was accompanied by concurrent activation of ROS, and induction of DNA double-strand breakage. Importantly, t-PDT suppressed efficiently anchorage-independent cell growth as well as ESCC-xenografted tumor formation. In aggregate, t-PDT showed anti-tumor potential for ESCC cells with various histological grades or chemoresistance, providing a novel translational rationale of t-PDT for the treatment of ESCC.

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information file.

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Introduction

Photodynamic therapy (PDT) is a light-based oncological intervention that uses a tumor-specific photosensitizer and laser irradiation [1]. Briefly, the administration of a tumor-targeting photosensitizing agent followed by irradiation with a specific wavelength generates reactive oxygen species (ROS) that cause DNA damage, resulting in a selective anti-tumor effect [2]. The first clinical trial of PDT was reported by Dougherty *et al* [3], the US Food and Drug Administration approved it as the first drug–device combination [4]. Thereafter, PDT has been used in the treatment of a wide range of cancers, including breast [3], skin [5], lung [6], head and neck [7,8] and gastric [9] cancer.

Regarding esophageal diseases, the efficiency of PDT using some photosensitizers such as a mTHPC (metatetrahydroxyphenylchlorin) or a porfimer sodium has been demonstrated in the treatment of superficial esophageal cancer [10,11] or the prevention of the development of adenocarcinoma from high-grade dysplasia in Barrett's esophagus [12]. In addition, we reported that PDT with porfimer sodium is quite useful as a salvage treatment for ESCC patients with local failure after definitive chemoradiotherapy [13]. However, PDT with the first-generation photosensitizer including porfimer sodium has some major problems, such as a high risk of skin phototoxicity and a need for a large and expensive excimer dye laser system [13].

Recently, a new photosensitizer, talaporfin sodium, and a diode laser system have been developed as a second-generation PDT to circumvent the above-mentioned problems [14]. Talaporfin sodium features rapid clearance from the skin. In addition, the absorption wavelength of talaporfin sodium (664 nm) is longer than that of porfimer sodium (640 nm) [14]. Therefore, theoretically, it is expected to have a low rate of phototoxicity and to be effective on deep tissue layers. Indeed, talaporfin-mediated PDT (t-PDT) exhibited lower skin phototoxicity in a clinical trial for early lung cancer [15]. Furthermore, the diode laser systems are much more convenient devices in terms of size and cost of equipment compared with the excimer dye laser systems.

Thus, t-PDT with a diode laser may function as an ideal combination among the PDT drugs and devices that are available for the treatment of ESCC. However, an experimental preclinical study remains to be performed to validate the efficacy of t-PDT for ESCC. Here, we determined the cytotoxic effects of t-PDT in

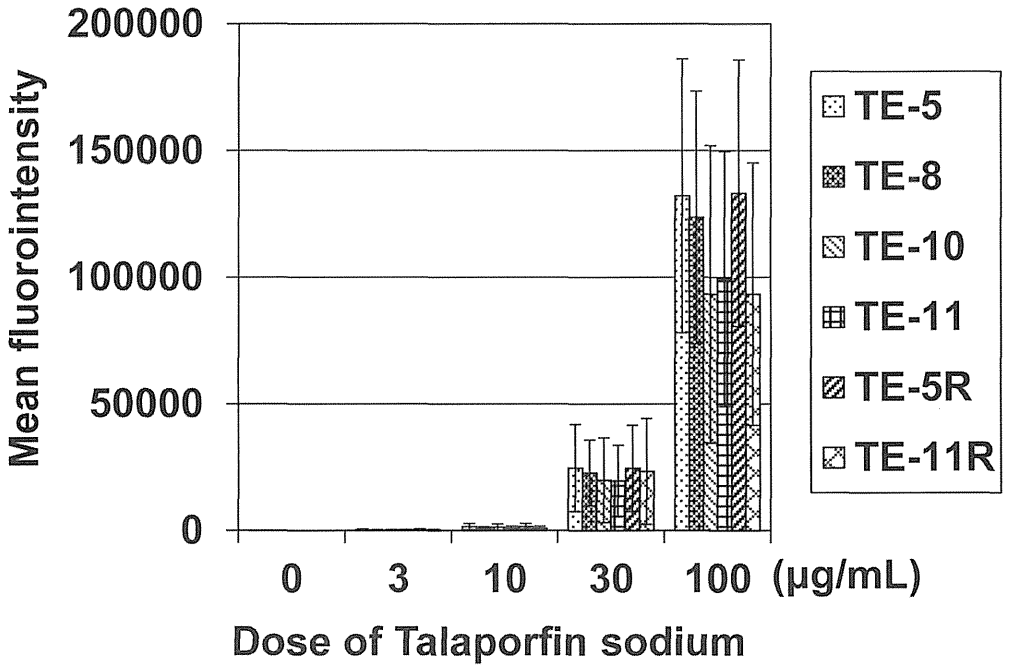

Figure 1. The fluorescence intensity of talaporfin sodium in cultured ESCC cells. The fluorescence intensity of talaporfin sodium in cultured ESCC cells (10000 cells) incubated with the indicated concentrations of talaporfin sodium for 24 h was measured by flow cytometry. As shown, talaporfin sodium was almost equally incorporated to the various ESCC cells *in vitro*.
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culture and xenograft transplantation models using ESCC cells representing various differentiation grades and resistance to 5-fluorouracil (5-FU), which is a key chemotherapeutic drug for ESCC [16]. Moreover, we explored the mechanisms underlying the anti-tumor effect of t-PDT.

Materials and Methods

Cell lines and cell culture

The human ESCC cell lines TE-5 (derived from poorly differentiated ESCC), TE-8 (derived from moderately differentiated ESCC), TE-10 (derived from highly differentiated ESCC) and TE-11 (derived from moderately differentiated ESCC) were obtained from the Riken BioResource Center (Ibaragi, Japan) [17]. TE-5R and TE-11R cells, which are derived from TE-5 and TE-11 cells, respectively, are 5-FU-resistant ESCC cells that were established originally by us via exposure of parental cells to gradually increasing concentrations of 5-FU. TE-5R and TE-11R cells were 15.6-fold or 7.9-fold resistant to 5-FU compared with parental cells, respectively (manuscript in preparation). Of note, TE-11R cells are highly transformed cells with advanced anchorage-independent cell-growth activities, as well as tumorigenicity. Accordingly, we used mainly TE-11R cells to verify the cytotoxic or anti-tumor effect of t-PDT. All ESCC cells were cultured in RPMI1640 medium (Life Technologies Corp., Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS) (Life Technologies Corp.), 100 μg/mL streptomycin and 100 U/mL penicillin (Life Technologies Corp.).

Photosensitizer and laser light delivery system

Talaporfin sodium was obtained from Meiji Seika Pharma Co., Ltd (Tokyo, Japan) [18]. The laser we used in this study was a diode laser system using a semiconductor laser irradiator (Panasonic Healthcare Co., Ltd., Yokohama, Japan) [18]. The details of the settings in the laser system as well as the optimal doses of talaporfin sodium and laser irradiation *in vitro* and *in vivo* were referred to the previously reports [18,19].

Measurement of fluorescence intensity in ESCC cells treated with talaporfin sodium

To show the uptake of talaporfin sodium in cultured ESCC cells, we measured the fluorescence intensity of talaporfin sodium. Cells were treated with the indicated concentrations of talaporfin sodium for 24 h. Cells were washed twice with phosphate-buffered saline (PBS), immersed in 2% FBS/PBS without talaporfin sodium, and then they were followed by the measurement of the mean fluorescence intensity per 10000 cells by flow cytometer (BD LSRFortessa Flow Cytometer; BD Biosciences, San Jose, CA, USA), which excites at 640 nm with emissions in the range of 670±14 nm.

Talaporfin-mediated PDT *in vitro*

ESCC cells were placed into 96-well plates at a concentration of 1×10^4 cells per well, and incubated with talaporfin sodium (0–100 μg/mL) for 24 h, Cells were washed twice with PBS, immersed in fresh medium without talaporfin sodium, and then they were subjected to laser irradiation (wavelength, 664 nm; laser power, 15 mW/cm^2; total amount of irradiation, 10 J/cm^2) [18]. Phase-contrast images were acquired using a Nikon Eclipse TE300 microscope (Nikon Instruments Inc., Tokyo, Japan). Cell viability at 48 h after t-PDT was assessed using the Cell Proliferation Reagent WST1 assay (Roche Applied Science, Penzberg, Germany).

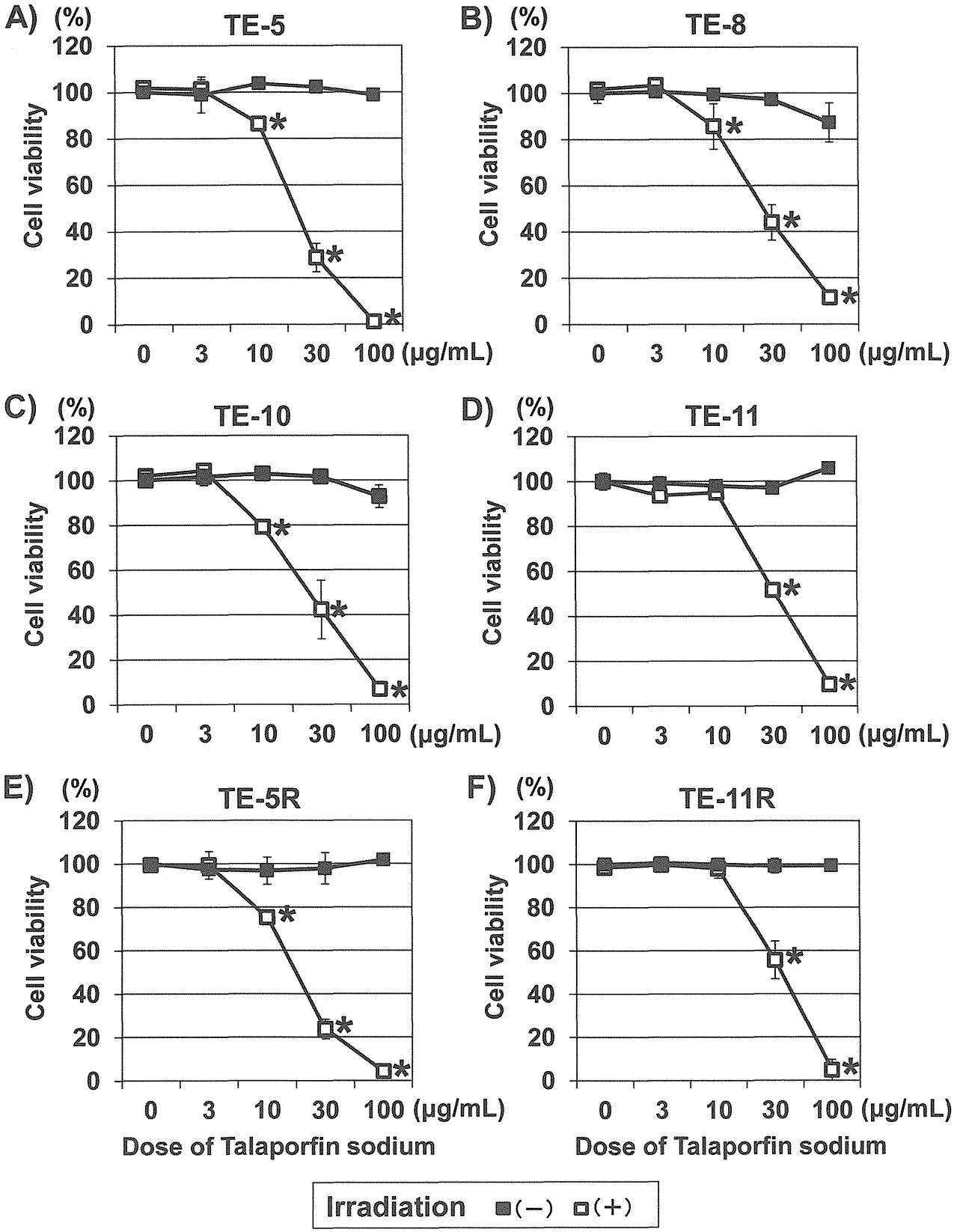
A)
TE-5
B)
TE-8
C)
TE-10
D)
TE-11
E)
TE-5R
F)
TE-11R
(%)
Cell viability
120
100
80
60
40
20
0
0 3 10 30 100 (μg/mL)
Dose of Talaporfin sodium
Irradiation (−) (+)

Figure 2. Cytotoxic effect of t-PDT on ESCC cells. Cell viability at 48 h after t-PDT was assessed using the WST-1 assay. t-PDT induced a talaporfin sodium dose-dependent cell death in ESCC cells (white square in the figures) regardless of differentiation grade or 5-FU resistance. (A) TE-5 (derived from poorly differentiated ESCC), (B) TE-8 (derived from moderately differentiated ESCC), (C) TE-10 (derived from highly differentiated ESCC), (D) TE-11 (derived from moderately differentiated ESCC), (E) TE-5R (5-FU-resistant cells derived from parental TE-5 cells) and (F) TE-11R (5-FU-resistant cells derived from TE-11 cells). A viability of 100% was defined as the amount of absorption at 450 nm found in untreated (non-irradiated and absence of treatment with talaporfin sodium) cells. Each point represents the mean ± S.D. from experiments conducted at least in triplicate. *$P<0.01$ vs untreated (non-irradiated and absence of treatment with talaporfin sodium) cells ($n=3$).
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Annexin V/Propidium iodide double-staining flow cytometry

The FITC Annexin V Apoptosis Detection Kit I (BD Biosciences) was used to assess cell apoptosis induced by t-PDT. Cells (TE-11R) were harvested at 4 h after treatment with talaporfin sodium with or without subsequent irradiation, and were stained with annexin V–FITC and propidium iodide (PI). These cells were analysed with flow cytometer (BD FACSCanto II Flow Cytometer; BD Biosciences). Unstained cells were used as negative controls. Data collected were analysed using the BD FACSDiva software (BD Biosciences). Cells were discriminated into four groups: viable cells (annexin V–/PI–), necrotic dead cells (annexin V–/PI+), early apoptotic cells (annexin V+/PI–) and late apoptotic cells (annexin V+/PI+) [20].

Measurement of intracellular ROS levels

The generation of intracellular ROS during t-PDT was measured using an OxiSelect Intracellular ROS assay kit (Cell Biolabs, Inc., San Diego, CA, USA), which uses the oxidation-sensitive fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). This assay was performed by adding DCFH-DA to TE-11R cells 4 h after t-PDT and quantifying intracellular ROS levels by detecting oxidized fluorescent 2′,7′-dichlorodihydrofluorescein (DCF) using a fluorometric plate reader (ARVO X5; PerkinElmer, Waltham, MA, USA) at 480/530 nm.

DNA double-strand breakage assay

Phosphorylation of the histone H2A variant (γ-H2AX) is a marker of DNA double-strand breaks, which is the gravest form of

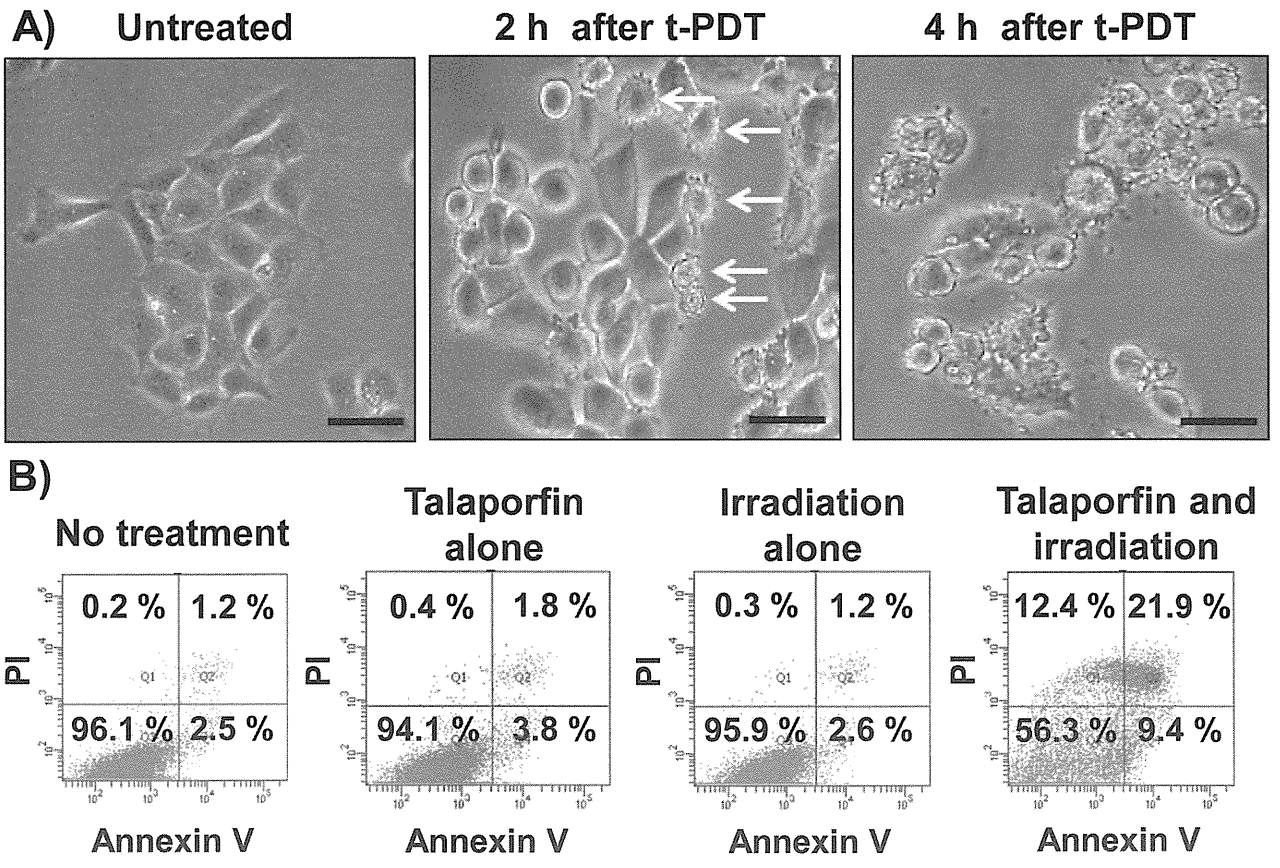

Figure 3. Induction of apoptosis in TE-11R cells treated with t-PDT. (A) TE-11R cells were pretreated with talaporfin sodium (30 μg/mL) for 24 h, and then irradiated (10 J/cm^2). Phase-contrast images were taken at 2 or 4 h after t-PDT. The images of untreated cells are also shown. Arrows indicate perinuclear vacuolization and cell shrinkage suggesting apoptosis. Scale bar, 100 μm. (B) Flow cytometric analysis of apoptosis in TE-11R cells treated with or without talaporfin sodium (30 μg/mL) for 24 h with or without subsequent laser irradiation (10 J/cm^2). Cells were stained with FITC-labelled annexin V and propidium iodide (PI) 4 h after irradiation. A representative experiment out of three is shown.
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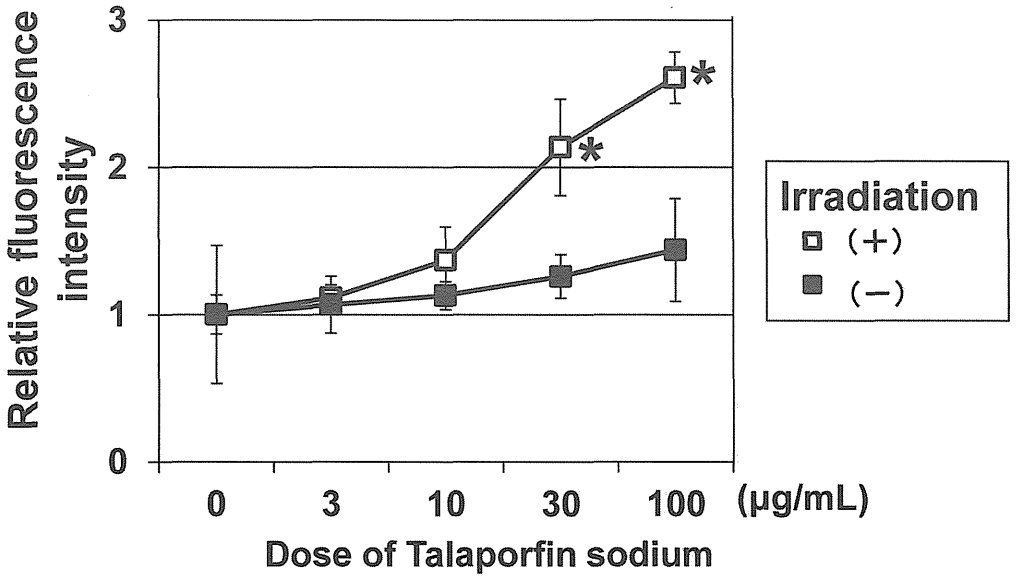

Figure 4. Generation of ROS in TE-11R cells treated with t-PDT. (A) TE-11R cells were treated with the indicated concentrations of talaporfin sodium for 24 h and received irradiation subsequently. Intracellular ROS levels at 4 h after irradiation treatment were determined by DCF assay. The intracellular ROS level was significantly increased by t-PDT in a talaporfin sodium dose-dependent manner. * $P<0.01$ vs untreated (non-irradiated and absence of treatment with talaporfin sodium) cells (n = 3).
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DNA damage [21]. We investigated the DNA double-strand breaks induced during t-PDT using the OxiSelect DNA Double-Strand Break Staining Kit (Cell Biolabs, Inc.). Cells (TE-11R) were treated with the indicated talaporfin sodium with or without subsequent irradiation, and were stained with an anti-phospho-histone antibody 24 h after treatment. DNA double-strand breaks labeled with FITC-conjugated secondary antibody were assessed using a fluorescence microscope (BZ-9000 BIOREVO, Keyence Corp., Osaka, Japan).

Soft agar colony-formation assays

The inhibitory effect of anchorage-independent cell growth after t-PDT was examined by soft agar colony-formation assays. Briefly, 2.5×10^4 cells of TE-11R cells were suspended in 0.67% agarose containing media with or without talaporfin sodium (30 μg/mL), and overlaid on top of 1% agarose containing the medium per well. Subsequently, the gel was laser irradiated 24 h after the gel formation, and cells were grown for 2 weeks. Colonies were stained with 0.02% Giemsa Stain Solution (Muto Pure Chemicals Co., Ltd., Tokyo, Japan), and the number and the size of colonies per high-power field were measured using a Nikon Eclipse TE300 microscope.

Xenograft transplantation and t-PDT *in vivo*

All experiments conformed to the relevant regulatory standards and were approved by the Institutional Animal Care and Use Committee of the Bozo Research Center (Approval number: APS13003, APS13006) (Tokyo, Japan). Mice were bred and housed in a temperature- and light-controlled facility with unlimited access to food and water. Xenograft transplantation using ESCC cells was performed as described previously [22]. Briefly, 10×10^6 TE-11R cells were suspended in 50% matrigel (BD Biosciences), followed by their subcutaneous implantation into the dorsal skin of NOD/SCID male mice (7 weeks of age; CLEA Japan, Inc., Tokyo, Japan). Xenografted tumors were used for t-PDT when they had reached a tumor volume of about 50–150 mm^3 at 35 days after the injection. Tumors were free of evident necrosis at the time of treatment. The optimal doses of talaporfin sodium and laser irradiation were as reported previously [19]. In brief, the indicated concentration of talaporfin sodium (0–10 mg/kg) was administered intravenously via the tail vein of NOD/SCID mice, and tumors were irradiated using a semiconductor laser irradiator at a light dose of 100 J/cm^2 and a wavelength of 664 nm 2 h after the injection of talaporfin sodium. The tumor volume was monitored for 21 days. Mice were painlessly sacrificed under the appropriate anesthesia with carbon dioxide inhalation and the cervical dislocation.

Histological analyses and immunostainings

The tumors were resected, fixed with 4% buffered paraformaldehyde solution, embedded in paraffin, and sectioned into 4-μm thickness. For the histological evaluation, the sections were stained with hematoxylin and eosin (H&E). For the immunohistochemistry, the sections were immunostained as previously described [23]. In brief, the sections were incubated with the primary antibody, a mouse monoclonal antibody Ki67 antigen (NCL-Ki67-MM1, Novocastra Laboratories, UK), at 4°C overnight, after which the secondary antibodies were added. Negative controls were prepared with isotype IgG.

Statistical analyses

Statistical analyses were performed using the SPSS statistics software (version 17; SPSS Inc., Chicago, IL, USA). Data from triplicate experiments are presented as the mean ± standard deviation (S.D.) and were analysed by a 2-tailed paired *t*-test. Two-way repeated-measures ANOVA with a post-hoc Bonferroni correction was used for multiple comparisons with a control group. $P<0.05$ was considered statistically significant.

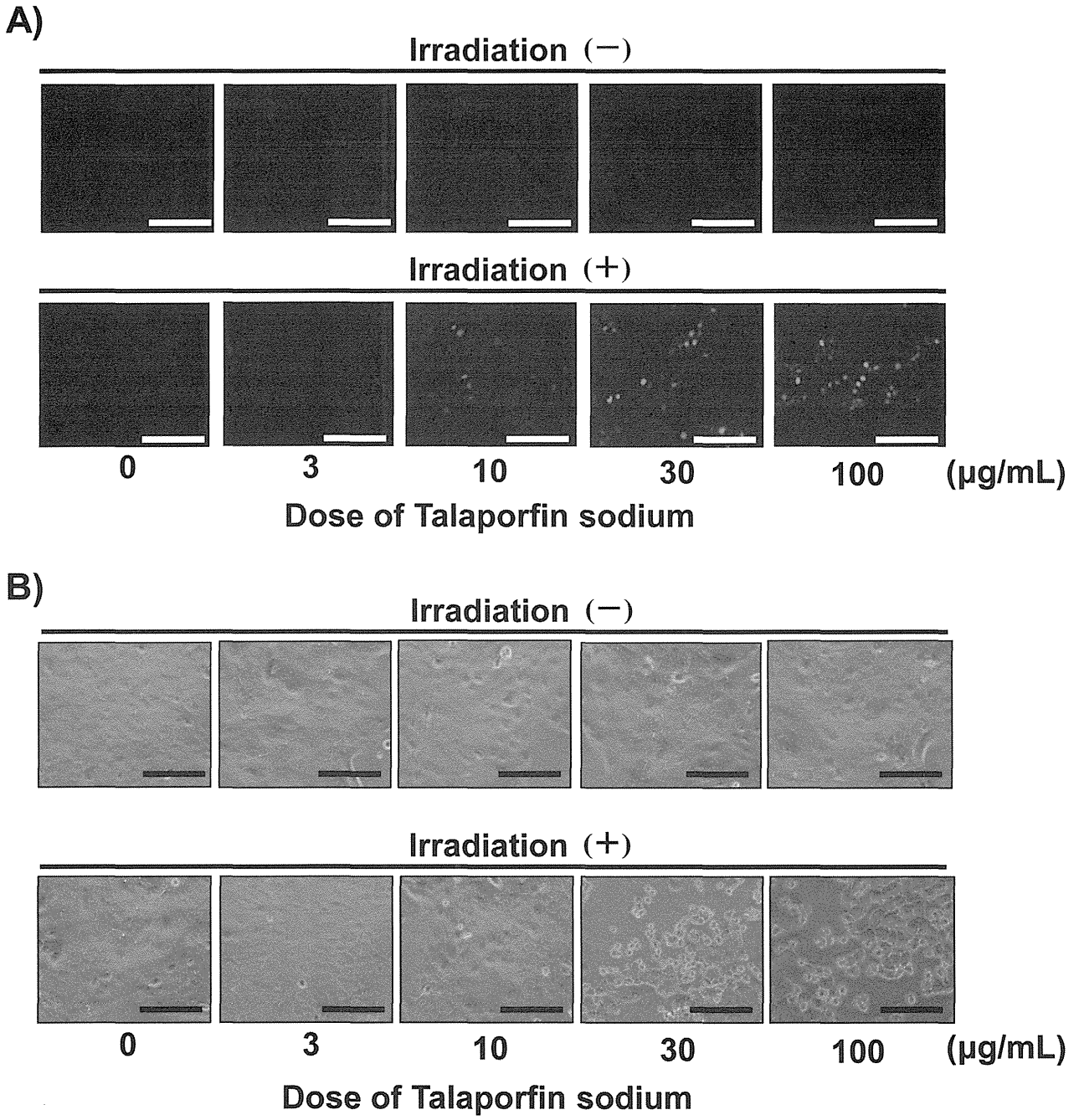

Figure 5. Formation of DNA double-strand breaks in TE-11R cells treated with t-PDT. TE-11R cells were treated with the indicated concentrations of talaporfin sodium for 24 h with or without subsequent laser irradiation (10 J/cm^2). (A) The expression of γ-H2AX was evaluated by fluorescence microscopy at 24 h after the irradiation. Under the non-irradiated conditions, γ-H2AX expression was not observed (upper panels), whereas a talaporfin sodium dose-dependent phosphorylation of γ-H2AX was found in the irradiated groups (lower panels). A representative experiment out of three is shown. Scale bar, 50 μm. (B) Phase contrast image was shown at 24 h after the irradiation. Scale bar, 50 μm.
doi:10.1371/journal.pone.0103126.g005

Results

Measurement of talaporfin sodium in cultured ESCC cells

Uptake of talaporfin sodium by cultured ESCC cells was determined by the fluorescence intensity of talaporfin sodium. As shown in Fig. 1, talaporfin sodium was incorporated in ESCC cells in a dose-dependent manner (Data S1 in File S1). Cell type did not affect the incorporation of talaporfin sodium (Fig.1, Data S1 in File S1).

Cytotoxic effect of t-PDT in ESCC cells

To determine the cytotoxic effect of t-PDT in ESCC cells, we examined cell viability using a WST-1 assay 48 h after t-PDT. As

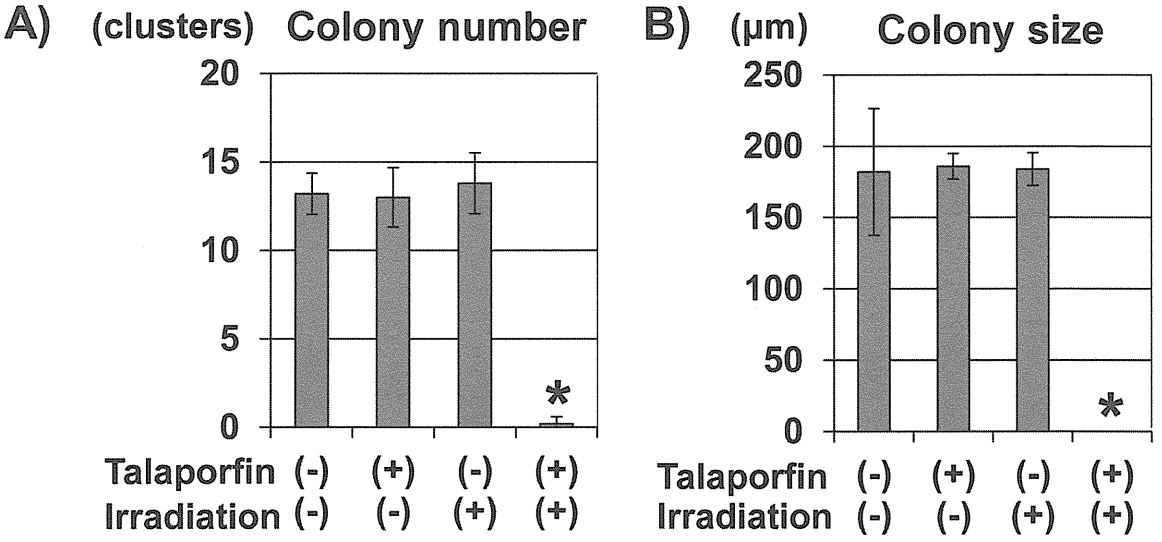

Figure 6. Inhibition of anchorage-independent cell growth due to t-PDT. Soft-agar colony-formation assays demonstrated the activity of anchorage-independent cell growth in TE-11R cells. The histograms show the average number (A) or size (B) of colonies per high-power field. Assays were performed in triplicate. t-PDT blocked colony formation completely, although talaporfin sodium alone or irradiation alone had no effect. *$P<$ 0.01 vs untreated (non-irradiated and absence of treatment with talaporfin sodium) cells (n = 3).
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shown in Fig. 2, neither talaporfin sodium alone nor diode laser alone exhibited cytotoxicity in ESCC cells; however, the combination of talaporfin sodium with subsequent laser irradiation induced an apparent dose-dependent cytotoxicity. Moreover, those cytotoxic effects were almost equally observed in ESCC cells independent of the grade of differentiation (Fig. 2 A–D) and 5-FU sensitivity (Fig. 2E and F) (Data S2 in File S1).

Induction of apoptosis by t-PDT in TE-11R cells

We assessed the morphological changes over time in ESCC cells treated with t-PDT. Fig. 3A shows the phase-contrast images of TE-11R cells treated with t-PDT, which demonstrates that perinuclear vacuolization and cell shrinkage were robustly induced within 2 h of laser irradiation. Moreover, nuclear fragmentation and disruption of the cell membrane were observed 4 h after treatment. Thus, drastic morphological changes that were indicative of apoptosis were observed. Moreover, the number of annexin V-positive cells was increased 4 h after t-PDT, whereas treatment with talaporfin alone or irradiation alone had no effect (Fig. 3B, Data S3 in File S1).

Increased levels of intracellular ROS and induction of DNA double-strand breakage by t-PDT

Next, we examined whether ESCC cells treated with t-PDT show increased levels of intracellular ROS or DNA damage. As shown in Fig. 4, a DCF assay revealed that intracellular ROS levels were significantly elevated by t-PDT in a talaporfin sodium dose-dependent manner. Furthermore, talaporfin sodium induced a dose-dependent phosphorylation of γ-H2AX in TE-11R cells treated with t-PDT, indicating that t-PDT induced DNA double-strand breaks, which is the most serious type of DNA damage (Fig. 5).

t-PDT blocked anchorage-independent cell growth of TE11R cells

We tested whether t-PDT affects the anchorage-independent cell growth of ESCC cells. Consistent with the data showing the cytotoxic effect of t-PDT (Fig. 1), neither talaporfin sodium alone nor diode laser alone influenced colony formation; however, t-PDT completely blocked colony formation (Fig. 6).

t-PDT suppressed the ESCC-xenografted tumors

Lastly, we examined the anti-tumor effect of t-PDT in ESCC-xenografted tumors in NOD/SCID mice. t-PDT successfully suppressed tumor growth *in vivo* in a talaporfin sodium dose-dependent manner (Fig. 7A, Data S4 in File S1). There was no significant change in body weight between the groups. Damage to normal skin was not observed in any of the mice. Significant tumor regrowth was not evident over the 3 weeks that followed t-PDT at the dosage of 10 mg/kg of talaporfin sodium. Histopathological and immunohistochemical examination revealed that the tumors irradiated after the administration of talaporfin sodium were subjected to the potent tissue injury, which was accompanied with completely abolished Ki67 staining (Fig. 7B).

Discussion

In this study, we demonstrated that t-PDT induced potent cytotoxicity in ESCC cells independent of their differentiation grade or 5-FU sensitivity. Apoptotic cells were induced within 4 h after t-PDT and were accompanied by increased levels of intracellular ROS and DNA double-strand breaks. Moreover, t-PDT suppressed anchorage-independent cell growth in ESCC cells *in vitro*, and, most importantly, showed a potent anti-tumor effect in ESCC cells *in vivo*.

ESCCs are heterogeneous tumors with highly differentiated cell nests known as central keratinization (i.e., keratin pearl) and/or poorly differentiated cell nests [22]. Histological grade regarding ESCC differentiation is associated with functional malignant potentials, such as invasion and metastasis [24,25],_ENREF_28 and with poor prognosis [24]. In this study, t-PDT yielded potent cytotoxicity in ESCC cells derived from both highly differentiated and poorly differentiated histological grade. Moreover, it exhibited a valid cytotoxic

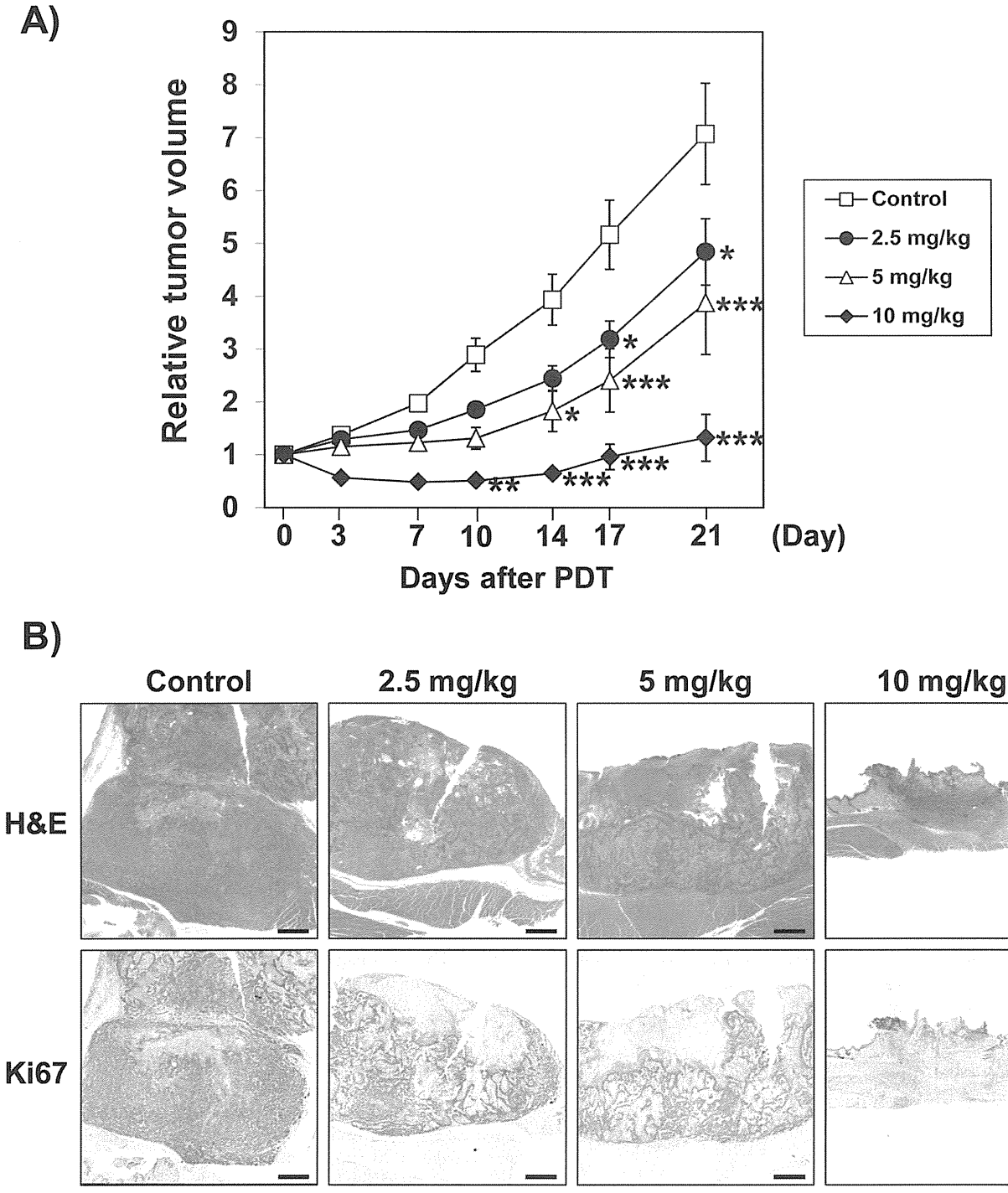

Figure 7. t-PDT suppress tumor formation *in vivo*. (A) Tumor response in mice treated with t-PDT at the indicated doses of talaporfin sodium. t-PDT was performed when tumor volume reached about 50–150 mm^3. Each point represents the average response rate (relative tumor volume on tumor size in day 0) of seven mice. Talaporfin sodium dose-dependent tumor reduction was shown in xenografted ESCC tumors treated with t-PDT. $^*P<0.01$, $^{**}P<0.001$, $^{***}P<0.0001$ vs the control groups at the indicated time points (n = 7). (B) Hematoxylin and eosin and immunohistochemical (Ki67) staining. Scale bar, 1mm. Ki67 positive cells indicate the viable and proliferative ESCC cells.
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effect in 5-FU-resistant ESCC cells, among which TE-11R cells showed an undifferentiated and proliferative phenotype as well as resistance to 5-FU (unpublished data). Thus, a cytotoxic effect of t-PDT in ESCC cells can be expected, regardless of histological grade or the presence or absence of resistance to 5-FU.

The anti-tumor effect of t-PDT has been reported as being mediated by multiple cell death pathways, such as apoptosis or necrosis, depending on the treatment intensity and/or tumor properties [26]. In this study, apoptotic morphological changes were found within 4 h after t-PDT. Annexin V-positive cells were consistently induced by t-PDT. However, PI-positive cells indicating necrosis were also detected in our experiment; thus, both apoptotic and necrotic pathways appear to be involved in the cytotoxicity observed in ESCC cells. Although the specific effectors that discriminate these various cell death pathways were not identified in this study, we presented clear evidence of the potent cytotoxicity and anti-tumor effect of t-PDT in ESCC cells based on *in vitro* and *in vivo* studies, respectively.

We demonstrated that t-PDT induced an increase of intracellular ROS levels, as well as DNA double-strand breaks in ESCC cells. Our data are consistent with previous reports that PDT-induced cytotoxicities are mediated by the generation of ROS [27]_ENREF_33 or DNA double-strand breaks [28]. Thus, the induction of those factors is suggested to be related to the promotion of cell death, which leads to the active tumoricidal response of t-PDT in ESCC cells.

Anchorage-independent cell growth or tumor formation in xenograft transplantation is the hall-mark of transformed cells, which is the most well-established *in vitro* or *in vivo* assay to detect the malignant transformation of the cells [29–31]. In the present study, we demonstrated that t-PDT efficiently inhibited anchorage-independent cell growth, as well as tumorigenicity in ESCC cells. These results suggest that t-PDT successfully eliminates transformed cells with highly malignant potentials.

Taken together, our results demonstrate that t-PDT had a direct anti-tumor effect in ESCC cells. Although previous studies have revealed the inhibitory effects of some ROS inhibitors [32] or caspase-specific inhibitors [33] on the action of t-PDT, the anti-tumor effects of t-PDT cannot be explained only by a direct cytotoxic action through ROS generation or apoptosis, because secondary vascular effects through endothelial damage are also closely associated with the anti-tumor mechanisms of t-PDT *in vivo* [26,34]. Those indirect anti-tumor effects of t-PDT in ESCC cells warrant elucidation in further studies.

In conclusion, we demonstrated the promising effect of t-PDT in ESCC via experimental preclinical studies. We are currently evaluating the clinical efficacy of t-PDT as a salvage treatment for patients with local failure after definitive chemoradiotherapy. We expect that t-PDT will be a useful therapy to improve the prognosis of patients with localized ESCC in the future.

Supporting Information

File S1 Supporting data. Data S1, The fluorescence intensity of talaporfin sodium in cultured ESCC cells. (A) TE-5, (B) TE-8, (C) TE-10, (D) TE-11, (E) TE-5R, (F) TE-11R, Comp-APC-A: Compensated-Allophycocyanin (APC)-Area. **Data S2, Cytotoxic effect of t-PDT on ESCC cells.** The number of viable cells after t-PDT was expressed as a percentage of unirradiated control cells. (A) TE-5, (B) TE-8, (C) TE-10, (D) TE-11, (E) TE-5R, (F) TE-11R. **Data S3, Flow cytometric analysis with Annexin V/Propidium iodide double-staining.** Annexin V and Propidium iodide (PI) is detected by FITC and phycoerythrin (PE), respectively. **Data S4, Xenograft tumor size.** Each table represents the tumor volume (upper table) and the response rate (lower table).
(XLSX)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: SO MM. Performed the experiments: SO OK MT YN. Analyzed the data: HK AS. Contributed reagents/materials/analysis tools: TH SM. Contributed to the writing of the manuscript: SO TC MM.

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