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腱内応力分布からみた肩腱板全層断裂の進展機序：Crescent-shaped tear と L-shaped tear の比較

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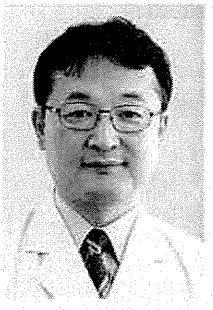

はじめに

肩腱板全層断裂は，しばしば経時的に拡大していくことが知られている．また，様々な形態の断裂がみられることから，断裂型によって進展様式が異なる可能性も考えられる．本研究では，代表的な腱板全層断裂の形態として，crescent-shaped tear および L-shaped tear の3次元有限要素モデルを作成し，両者の応力分布を比較検討することを目的とした．

対象と方法

正常ヒト屍体肩（69歳男性）の CT スキャンを撮像し，DICOM データとして出力した．これを有限要素解析用ソフトウエア・Mechanical Finder (Extended Edition, version 6.1, 計算力学研究センター，東京）上に取り込み，上腕骨頭と腱板の3次元有限要素モデルを作成した．モデル上で，棘上筋腱骨付着部に直径 1 cm，2 cm，3 cm の半円形の欠損を作成し，crescent-shaped tear を再現した．同様に，腱板骨付着部付近に幅 1 cm，2 cm，3 cm の欠損を作成し，その一端から腱の近位方向（内側）に向かって欠損幅の 50% の長さの切り込みを入れ，L-shaped tear を再現した．腱板組織の物理特性については，過去の文献値をもとにヤング率を 305.5 MPa，ポアソン比を 0.497 と仮定した[1]．上腕骨骨幹部を全方向に拘束し，肩下垂位における外転運動を模して，棘上筋腱に 50 N，肩甲下筋に 23 N，棘下筋腱に 63 N の引っ張り荷重をかけた．各腱にかける荷重値は，過去の報告をもとに，以下の式を用いて算出した．

各腱にかける荷重 (N) =

% maximal RVC　PCSA (cm^2) × 46.1 (N/cm^2)

このうち，% maximal RVC (the ratio compared to the maximal isometric reference voluntary contraction) については，Kronberg らが報告した筋電図による肩周囲筋の筋活動評価のデータを基に算出した[2]．PCSA はそれぞれの筋の physiological cross-sectional area であり，Itoi らの報告に基づいて棘上筋 5.7 cm^2，棘下筋 16.3 cm^2，肩甲下筋 13.7 cm^2 とした[3]．また，定数 46.1 (N/cm^2) は Ikai らの報告に準じて設定した[4]．

作成した6個のモデルに対して弾性解析を行い，腱内部における von Mises 相当応力の分布を比較検討した．

結　果

応力分布は，断裂型によって一定の傾向を示していた．Crescent-shaped tear では，相当応力は腱板断裂の前後縁から前方と後方に広がっていた（図 1-a）．一方，L-shaped tear では，断裂部の前後縁だけでなく，内側の切れ込み部分にも応力集中がみられていた（図 1-b）．さらに，各断裂型における断裂部周囲の最大相当応力値は，断裂サイズの拡大とともに増大していくことが明らかになった（表 1）．

考　察

腱板断裂は経時的に拡大していくことが知られているが，その生体力学的な進展機序はまだ十分明らかにされていない．今回の解析から，crescent-shaped tear では，肩の外転運動に伴って，断裂部の前後縁から前後方向に応力が分布することが明らかになった．すなわち，断裂は前後方向に向けて，いわゆる zipper phenomenon に従って拡大していくと推測された[5]．こうした結果は，これまでの研究結果と矛盾しないもので

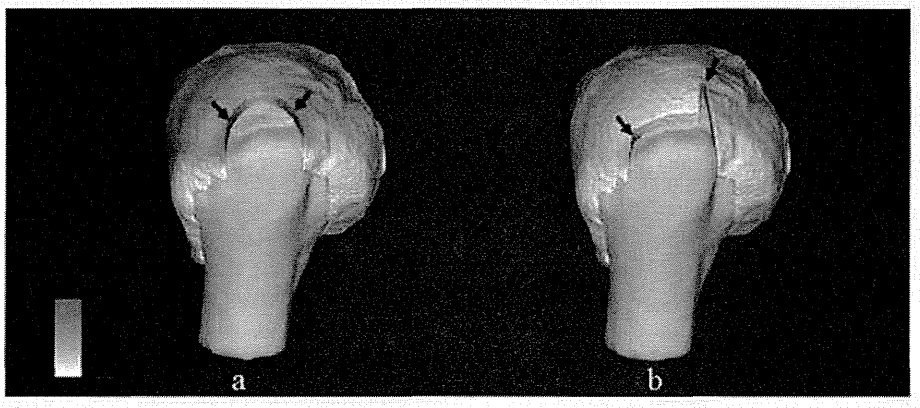

図 1-a, b. 2 cm 断裂モデルにおける腱内応力分布（a：crescent-shaped tear, b：L-shaped tear）
Crescent-shaped tear モデル（a）では，断裂の前後縁に高い応力集中が存在し（矢印），そこから前後方向に応力が分布している．一方，L-shaped tear モデル（b）では，断裂の前後縁だけでなく，内側の切れ込み部分にも応力集中がみられる（矢印）．

表 1. 断裂幅ごとの最大相当応力値（MPa）

	1 cm	2 cm	3 cm
Crescent-shaped tear	1.60	2.16	2.99
L-shaped tear	0.53	0.95	2.80

あった[6,7]．また，L-shaped tear では，切れ込み部分にも応力集中がみられたことから，前後方向だけでなく，内側方向にも進展してゆく可能性があると考えられた．

さらに，最大相当応力値が断裂サイズの拡大とともに増大していくことから，断裂サイズの拡大に伴って，断裂の進展も加速されていく可能性があると考えられた．言い換えれば，小断裂の拡大には比較的長期の時間経過を要するが，大断裂は比較的短期間にさらに大きくなるということになる．このことから，臨床の場で腱板全層断裂に対して保存治療を行う場合，超音波や MRI などで断裂サイズを定期的に確認することが重要であり，もし拡大傾向がみられた場合は，それ以上の拡大を避けるために，早期に手術治療に移行することが望ましいと考えた．

結　論

腱板全層断裂の進展様式は断裂型によって異なっており，crescent-shaped tear では主に前後方向に，L-shaped tear では前後だけでなく内側方向にも進展していくと推測された．また，こうした断裂の進展は，断裂サイズの拡大に伴って加速されていく可能性があると考えられた．

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A Strategy for Supraclavicular Lymph Node Dissection Using Recurrent Laryngeal Nerve Lymph Node Status in Thoracic Esophageal Squamous Cell Carcinoma

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Background. The desirability of supraclavicular lymph node (LN) dissection, which is the cervical part of three-field LN dissection, has been discussed for a long time. In this study, we examine the pattern of supraclavicular LN metastasis in esophageal cancer, with a particular focus on the correlation between recurrent laryngeal nerve (RLN) LN and supraclavicular LN metastasis.

Methods. In all, 220 cases of R0 resected T1 to T3 squamous cell carcinomas were retrospectively examined. All of these patients underwent bilateral RLN LNs dissection; none received cancer treatment before surgery.

Results. Of 21 upper esophageal cancer cases, 33.3% of the patients had metastasis in the supraclavicular LN. Every patient in whom supraclavicular LN metastasis developed had metastasis in the RLN LN. Of 141 cases of middle esophageal cancer, 19.1% had metastasis in the supraclavicular LN. Among the patients whose RLN LN metastasized, 38.3% had metastasis in the supraclavicular LN. A similar correlation between RLN LN and supraclavicular LN metastasis was observed in lower esophageal cancer cases, especially in T3 cases. When considering cancers of the esophagus and patients who had metastasis in the supraclavicular LN, our data demonstrated that RLN LN metastasis did not always lead to metastasis on the same side of the supraclavicular LN.

Conclusions. The status of the RLN LN can be an indicator of supraclavicular LN dissection in upper esophageal cancer patients and advanced cases of middle and lower esophageal cancer patients. Bilateral supraclavicular LN dissection should be recommended even when only unilateral RLN LN metastasis occurs.

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The lymph nodes (LNs) around the recurrent laryngeal nerve (RLN) are well known as frequent metastatic sites of squamous cell carcinoma of the esophagus, and their dissection has been reported as beneficial [1–4]. The cervical area of this LN is located adjacent to the cervical esophagus, which is described as paraesophageal LN in the International Union Against Cancer (Union Internationale Contre le Cancer [UICC]) seventh edition [5]. Although this LN had been included in the regional LN in the UICC seventh edition, supraclavicular (SC) LNs whose dissection also has been reported as improving survival [6–9] were excluded from this category. The desirability of SC LN dissection, which is the cervical part of three-field LN dissection, has been debated for a considerable time during the history of esophageal cancer surgery, and the issue still remains controversial [10–12]. One argument against this dissection is based on the SC LN metastasis being relatively rare in operable patients. Therefore, to justify such dissection, it is imperative to determine which patients will benefit from this procedure.

According to the previous reports, RLN LNs can be a predictor for performing SC LN dissection [13–16]. This idea seems reasonable because lymphatic channels connect RLN LN to SC LN [17]. Conversely, because there are long longitudinal lymphatic extensions in esophageal submucosa, early esophageal cancer may metastasize directly to SC LN even from the lower esophagus [18, 19]. Consequently, tumor depth must be considered to obtain exact data regarding the pattern of SC LN metastasis. In this study, we retrospectively analyzed the correlation between SC LN and RLN LN metastasis, adding the variables of location and depth of the tumor. Additionally, to establish the strategy for SC LN dissection, the correlation of laterality in SC LN and upper RLN LN metastasis was examined.

Patients and Methods

From January 1986 to 2011, 926 patients with esophageal cancer underwent esophagectomy in our single institution. Among them, we selected patients according to the

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following criteria to obtain the exact data of RLN LN state and exclude residual cancer cases: (1) T1 to T3 squamous cell carcinoma cases; (2) underwent the McKeown procedure; (3) a bilateral RLN LN dissection was performed; (4) an R0 resection was done; (5) the surgical margin was pathologically negative; (6) there was no treatment for cancer before surgery because the status of LN metastasis could have been modified by that presurgical treatment; and (7) a follow-up examination had been done more than 1 year after the surgery, except when the patient died of cancer within 1 year of the surgery. In total, 220 cases met these criteria. The present retrospective study protocol was reviewed and approved by the Tohoku University Institutional Review Board.

Surgical Procedure

All 220 patients underwent esophagectomy with either fifth intercostal thoracotomy or thoracoscopic procedure by left lateral decubitus position. Thoracic nodes including paraesophageal LN, bilateral recurrent laryngeal nerve nodes, subcarinal nodes, and bilateral pulmonary hilar nodes were dissected in all the cases. Through upper midline laparotomy or laparoscopic procedure, the paracardiac node, celiac nodes, and nodes along the left gastric artery were dissected, and the stomach tube was made. Through cervical collar incision, the cervical paraesophageal nodes, including cervical RLN LNs, were dissected bilaterally. Bilateral SC LN dissection was performed on 84 patients who were suspected of having metastatic SC LN before the surgery. After the stomach tube was pulled up to the neck through a posterior mediastinal route or a retrosternal route, an anastomosis between the stomach tube and the esophagus was carried out using Gambee techniques.

Diagnosis

All specimens were pathologically diagnosed at the Tohoku University Department of Pathology. The UICC seventh edition was used for TNM classification, and pathology results determined the T and N scale. In this study, SC LN is defined as the cervical LN located at the lateral side of the jugular vein, the cranial side of the clavicles, caudal side of the cricoid cartilage level, and the dorsal side of the sternocleidomastoid muscle. All patients were regularly followed up at intervals of 4 to 6 months for the first 5 years postoperatively. A computed tomography scan and upper endoscopy were performed at every follow-up to rule out the possibility of disease recurrence. A positron emission tomography scan was used to confirm cancer recurrence. When metastasis was detected in the SC area of patients who had not undergone SC LN dissection, we also included those cases in the SC LN metastasis group.

Postoperative Morbidity and Adjuvant Therapy

The vocal cord was examined in all of the cases by using a bronchoscope when the intubation tube was extubated. If any dysmotility was found in the vocal cord, we defined these cases as RLN palsy. When the diaphragm did not show any movement through radiographic examination, we defined these cases as phrenic nerve palsy. Anastomosis leakage was confirmed in all of the cases either by using endoscopy or contrasted radiography. Chyle leakage was diagnosed by detecting a large amount of milky white effusion from either the thoracic cavity or the neck.

Postoperative adjuvant therapy was administered to patients whose performance status was 0 or 1. Patients who had more than three metastatic LN were the target of adjuvant chemotherapy or radiotherapy or both. Patients whose condition did not permit adjuvant chemoradiotherapy, or who refused additional therapy, were followed up closely in our outpatient clinic.

Statistics

All two-by-two tables were analyzed by Fisher's exact test. Disease-specific survivals were analyzed using Kaplan-Meier methods including only cancer death, and overall survivals were analyzed including all causes of death. The statistical significance of differences was compared by the log rank test. These analyses were performed electronically with the use of statistical software (JMP, version 9; SAS Institute, Cary, NC).

Results

The clinical and pathologic characteristics of the study population are summarized in Table 1. Nineteen patients had cancer metastasis in the SC LN at the time of surgery and 22 patients had metastasis in the SC LN after surgery.

Table 1. Summary of 220 Patients

Characteristics	Number of Patients
Total cases	220
Age, years	42–83 (average 63.5)
Sex	
Male	186
Female	34
T classification	
T1	78
T2	26
T3	116
N classification	
N0	95
N1	65
N2	36
N3	24
M classification	
M0	201
M1 (SC LN)	19
Recurrence at cervical LN	22
Tumor location	
Upper	21
Middle	141
Lower	58

LN = lymph node; SC = supraclavicular.

Table 2. Frequency of Recurrent Laryngeal Nerve Lymph Node and Supraclavicular Lymph Node Metastasis in Each T Stage

Type of Cancer	Total	T1	T2	T3
Upper esophageal cancer				
RLN LN metastasis/total cases	14/21 (66.7%)	5/8 (62.5%)	2/3 (66.7%)	7/10 (70.0%)
SC LN metastasis/total cases	7/21 (33.3%)	2/8 (25.0%)	2/3 (66.7%)	3/10 (30.0%)
Middle esophageal cancer				
RLN LN metastasis/total cases	47/141 (33.3%)	6/50 (12.0%)	10/17 (58.8%)	31/74 (41.9%)
SC LN metastasis/total cases	27/141 (19.1%)	4/50 (8.0%)	4/17 (23.5%)	19/74 (25.7%)
Lower esophageal cancer				
RLN LN metastasis/total cases	12/58 (20.7%)	4/20 (20.0%)	2/6 (33.3%)	6/32 (18.8%)
SC LN metastasis/total cases	7/58 (12.1%)	2/20 (10.0%)	1/6 (16.7%)	4/32 (12.5%)

LN = lymph node; RLN = recurrent laryngeal nerve; SC = supraclavicular.

Among 22 cases, 3 cases had lung metastasis and 2 cases had liver metastasis at the same time that SC LN metastasis had occurred. In upper esophageal cancer cases, 66.7% of the patients had RLN LN metastasis and 33.3% had SC LN metastasis (Table 2). Among all patients with metastasis in the RLN LN, 7 of 14 patients had metastasis to the SC LN (Table 3). Among patients who had SC LN metastasis, all had metastasis in the RLN LN. With regard to cancers of the middle esophagus, 33.3% of the patients had RLN LN metastasis and 19.1% had SC LN metastasis (Table 2). Among these middle esophageal cancer patients with metastasis in the RLN LN, 18 of 47 patients (38.3%) also had metastasis to the SC LN, and this ratio rose with the depth of tumor invasion (Table 3). Fisher's exact test demonstrated that patients with T3 tumor and RLN LN metastasis have a statistically high risk of SC LN metastasis. In cancer of the lower esophagus, however, the rates of RLN LN metastasis (20.7%) and of SC LN metastasis (12.1%) were not as high as the rates observed in middle esophageal cancer (Table 2). However, among patients whose cancers had metastasized from their lower esophagus to RLN LN, 3 of 12 patients (25%) had metastasis to the SC LN (Table 3). This trend was apparent in T3 cases and was similar to what we observed in middle esophageal cancers. Conversely, RLN LN status in T1 and T2 cases did not contribute to the information of SC LN metastasis.

To investigate laterality between RLN LN and SC LN metastasis, the patients whose cancers had metastasized in the unilateral side of the RLN LN were identified, and laterality of SC LN metastasis was analyzed (Fig 1). With upper esophageal cancers, unilateral RLN LN metastasis tends to develop on the same side of the SC LN metastasis. However, unilateral RLN LN metastasis did not always develop on the same side of the SC LN when there was metastasis in cancers of the middle and lower esophagus.

The 84 patients who underwent SC LN dissection were used to evaluate the efficiency of this procedure (Table 4). Among these 84 patients, 19 had SC LN metastasis by pathology evaluation, only 3 patients had cancer recurrence in the cervical LN, and 16 had no recurrence at the neck (Table 5). Interestingly, the adjuvant therapy did not contribute to suppression of the cervical LN recurrence in patients who had metastasis in SC LN.

Table 3. Correlation Between Recurrent Laryngeal Nerve Lymph Node Metastasis and Supraclavicular Lymph Node Metastasis in Each T Stage

Type of Cancer	Total RLN−	Total RLN+	T1 RLN−	T1 RLN+	T2 RLN−	T2 RLN+	T3 RLN−	T3 RLN+
Upper EC								
SC−	7	7	3	3	1	0	3	4
SC+	0	7	0	2	0	2	0	3
p Value[a]		0.046		0.464		0.333		0.475
Middle EC								
SC−	85	29	41	5	6	7	38	17
SC+	9	18	3	1	1	3	5	14
p Value[a]		< 0.001		0.411		0.603		0.002
Lower EC								
SC−	42	9	14	4	3	2	25	3
SC+	4	3	2	0	1	0	1	3
p Value[a]		0.0147		1.000		1.000		0.015

[a] Fisher's exact test.

EC = esophageal cancer; RLN = recurrent laryngeal lymph node; SC = supraclavicular.

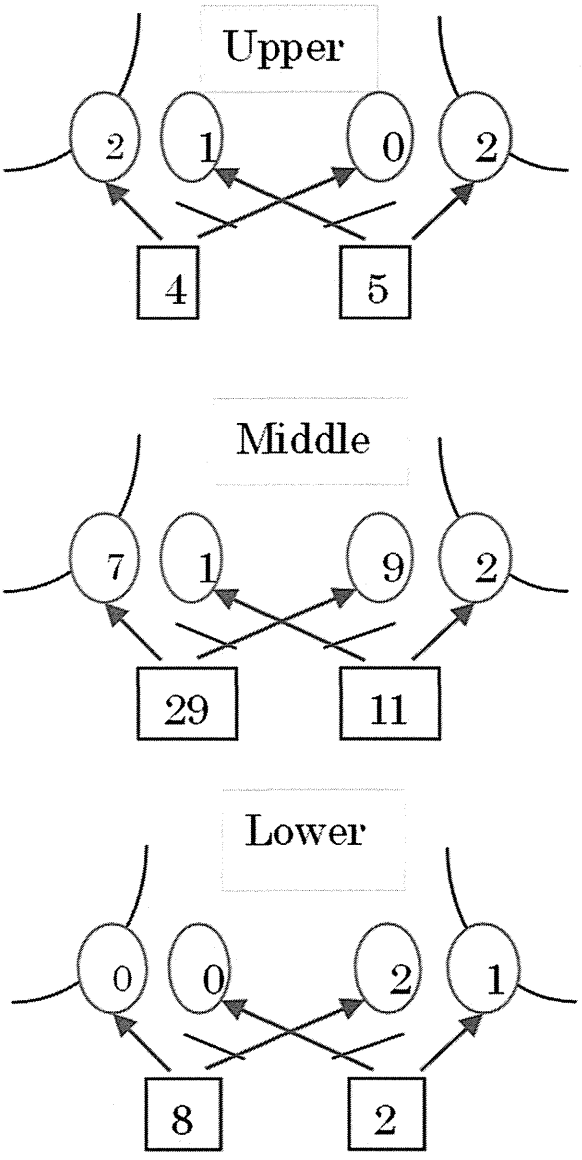

Fig 1. Laterality of recurrent laryngeal nerve (RLN) lymph node (LN) and supraclavicular (SC) LN metastasis in each tumor location. Every case had only unilateral RLN LN metastasis. Boxes shows the number of RLN LN metastasis, and ovals show the number of SC LN metastasis in each side.

Disease-specific survival and overall survival data of these 84 patients with SC LN metastasis is presented in Figure 2. Their 5-year survival rates were 28.7% and 22.7%, which was similar to the rates in a previous report [6]. When compared with the RLN LN metastasized patient (Fig 3), 5-year survival was only approximately 10% lower in cases of metastasis of the SC LN. To analyze the benefit of SC LN dissection among the non RLN LN metastasized and metastasized patients, survival data of both the SC LN dissected and nondissected patients is charted in Figure 4 and Figure 5. Although these data seem to show less benefit in the SC LN dissected group, this group includes more of the advanced and upper esophageal cancer cases, which have a decreased chance of survival.

Table 4. Summary of 84 Patients Who Underwent Supraclavicular Lymph Node Dissection

Characteristics	Number of Patients
Total cases	84
Age, years	45–77 (average 60.9)
Sex	
Male	72
Female	12
T classification	
T1	24
T2	11
T3	49
N classification	
N0	34
N1	23
N2	17
N3	10
M classification	
M0	65
M1	19 (SC lymph node)
Location	
Upper	16
Middle	60
Lower	8
Adjuvant therapy	
None	40
Chemotherapy	18
Radiotherapy	4
Chemoradiotherapy	22

SC = supraclavicular lymph node.

To evaluate the operative risk from SC LN dissection, we analyzed postoperative morbidity by comparing the SC LN dissected group with the nondissected group (Table 6). Overall, 41.3% of the patients had RLN palsy and 11.3% of the patients had anastomosis leakage, which corresponds with a previous report from our institution [20]. Among those patients, Table 6 also shows that patients who underwent SC LN dissection had more risk of RLN palsy and tracheostomy.

Comment

The LN dissection of esophageal cancer patients has long been controversial. Because the frequency of metastasis around the RLN has been well known in thoracic esophageal cancer patients [1–4], there would be no argument about dissecting these LNs. Moreover, because there is no obvious borderline between the thoracic and cervical RLN, we should dissect the LNs around the cervical RLN as well. In the UICC seventh edition, the cervical paraesophageal LN was defined as regional LN. Because the RLN runs near the esophagus at the cervix, dissection of the cervical paraesophageal LNs should include the LN along these nerves naturally. Moreover, disease-specific and overall 5-year survival

Table 5. Evaluation of Cervical Lymph Node Recurrence Among 84 Patients Who Underwent Supraclavicular Lymph Node Dissection

Metastasis LN/Cervical LN Recurrence	No Recurrence	Recurrence	Total
No SC LN metastasis	62 (30)	3 (3)	65 (33)
SC LN metastasis	16 (8)	3 (3)	19 (11)
Total	78 (38)	6 (6)	84 (44)

The numbers in parentheses represent patients who received adjuvant therapy.

LN = lymph node; SC = supraclavicular.

rates for patients who had metastasis at the RLN LN are 38.5% and 33.3%, respectively—eventually leading to the necessity of the dissection of those LNs.

The issue is whether we need to dissect SC LN or not. Although our data demonstrated that the SC LN dissected group showed a high rate of RLN palsy and tracheostomy, this LN is located at the posterior-lateral side of the jugular vein so it should not increase the risk of RLN injury anatomically. The explanation

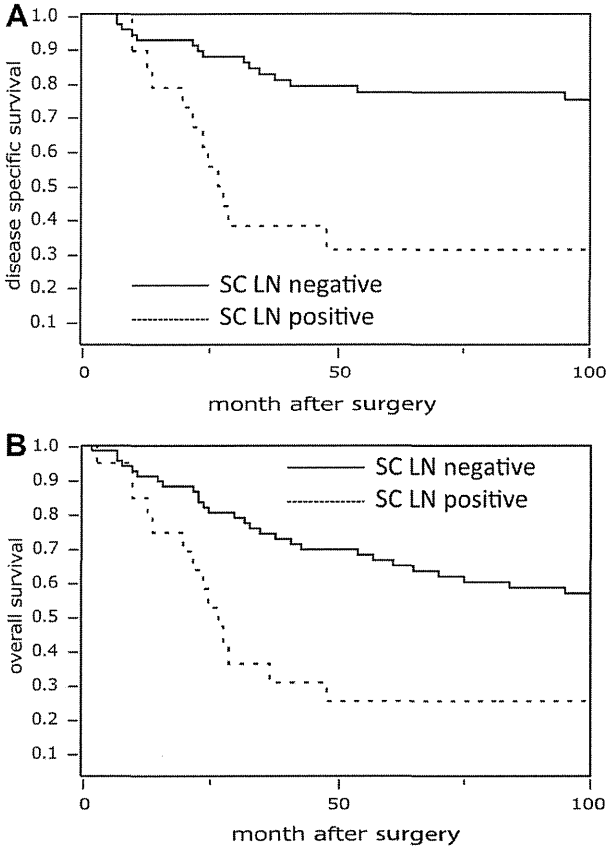

Fig 2. Kaplan-Meyer curves of 84 esophageal squamous cell carcinoma patients who underwent supraclavicular (SC) lymph node (LN) dissection. Comparison of (A) disease-specific survival and (B) overall survival between pathologically SC LN metastasis negative group (solid line) and positive group (dashed line). Five-year survival was 28.7% and 22.7%, respectively, in each positive case.

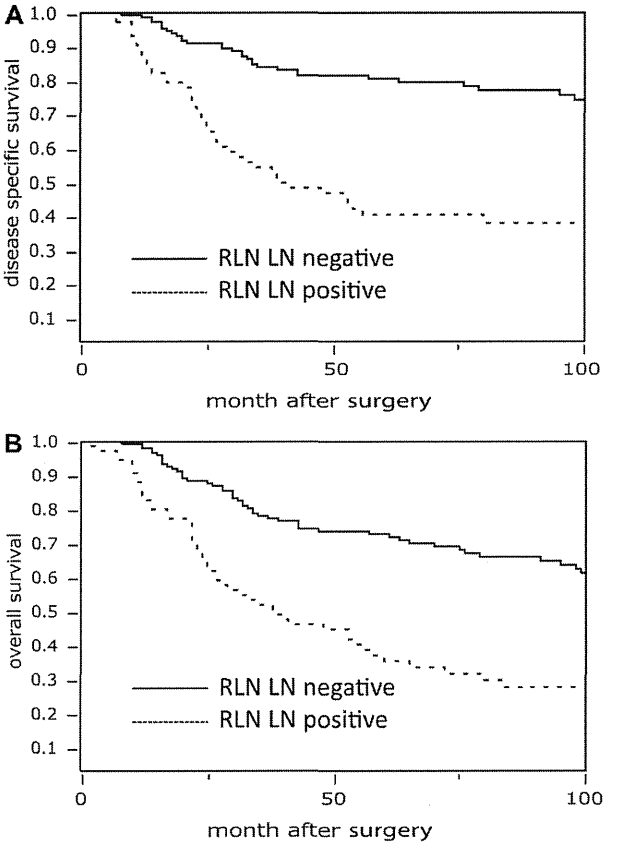

Fig 3. Kaplan-Meyer curves of 220 patients with squamous cell carcinoma of esophagus. Comparison of (A) disease-specific survival and (B) overall survival between pathologically recurrent laryngeal nerve (RLN) lymph node (LN) metastasis negative group (solid line) and positive group (dashed line). Five-year survival was 38.5% and 33.3%, respectively, in each positive case.

of this result is that the SC LN dissected group had a remarkable frequency of RLN LN metastasis, which should naturally raise the risk of RLN paralysis and tracheostomy by dissecting these LNs. Although the thoracic duct and the phrenic nerve are located in the area of SC LN dissection, we experienced only 1 case of chyle leakage and 5 cases of phrenic nerve palsy in SC LN dissected patients; that was not statistically significant compared with non–SC LN dissected patients.

In our study, among 19 patients who had SC LN metastasis at the dissected LN, only 3 had recurrence at the cervical LN, which means that this dissection can control the local recurrence of cervical LN. Also from Kaplan-Meier data of those 19 patients (Fig 2), their survival is better than that of patients who had distant metastasis, according to a previous study [6]. If the SC LN is defined as nonregional LN and excluded from the dissection area for any of these esophageal cancers, not a few cases would lose their opportunity to limit their cancers to the cervical LN and to increase their chances for survival. Therefore, SC LN metastasis should not be

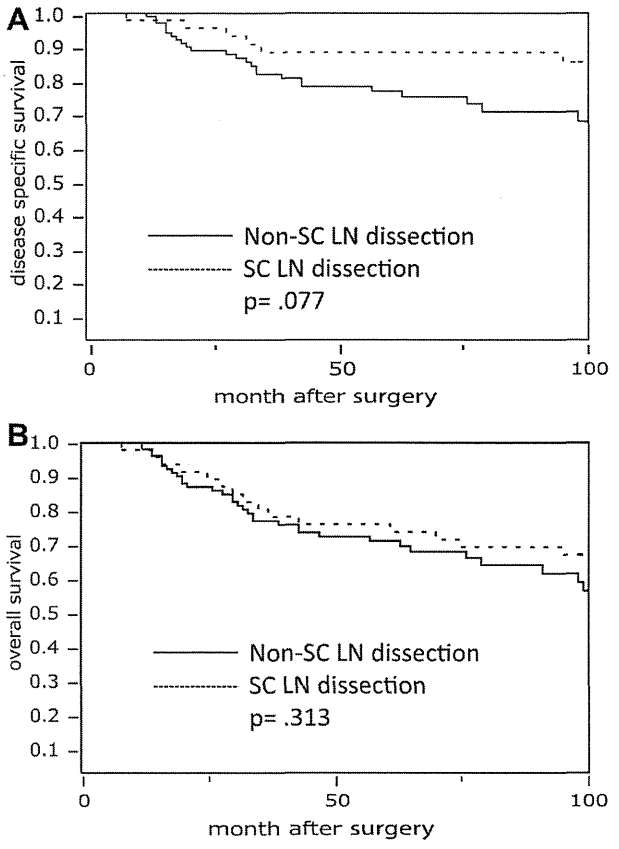

Fig 4. Kaplan-Meyer curves of 147 patients who had no recurrent laryngeal nerve (RLN) lymph node (LN) metastasis. Comparison of (A) disease-specific survival and (B) overall survival between supraclavicular (SC) LN dissected group (dashed line) and non-SC LN dissected group (solid line).

treated as a distant metastasis. Adding the factor of frequency of metastasis, SC LN must be considered as regional LN with respect to upper esophageal cancer, and to some extent, to advanced cases of middle and lower esophageal cancer.

This SC LN dissection has a risk of being an unnecessary surgery because metastasis of the SC LN is less frequent. With that in mind, what is necessary now is to determine which patients will benefit from this LN dissection. From our data, upper esophageal cancer cases had 33.3% of SC LN metastasis. Although this percentage is sufficient to justify performing this LN dissection for every upper esophageal cancer patient, RLN LN status would also provide more information about the necessity of dissecting this LN, because every patient whose SC LN metastasized also had metastasis in the RLN LN. Although middle esophageal cancer cases had a lower frequency of SC LN metastasis, RLN LN metastasis cases tend to have SC LN metastasis as well. As tumor depth increased, this frequency also increased and rose to 45.2% in T3 cases. Also among lower esophageal cancer patients, there was a strong correlation between SC LN and RLN LN metastasis in T3 cases.

The SC LN dissection should be done bilaterally even when the patient has only unilateral RLN LN metastasis. Our data suggest that the stream of lymph duct does not flow unilaterally. Especially metastasis of the right RLN LN tends to go left to SC LN metastasis, rather than left to right in lower esophageal cancer. This phenomenon is not so surprising if we think about the anatomic lymph flow of the thoracic duct. But because the number of left RLN LN metastases was small, further study is needed.

Even though we demonstrated that the local control is possible by SC LN dissection, as shown in Table 5, survival data did not show any benefit for patients who underwent SC LN dissection. We should notice, though, that this group includes more of the advanced and the upper esophageal cancer cases, as shown in Tables 1 and 4. Previous studies indicated that SC LN dissection should provide a better chance of survival [6–9]; however, none of them is a prospective and randomized study, and that is why this issue still remains controversial. To establish a definite answer to this problem, a randomized study is critical because the patient who undergoes SC LN dissection tends to have advanced cancer and LN metastasis. Ideally, randomized studies should be done among the patients who have a high risk of SC LN metastasis, meaning those who have RLN LN metastasis.

From our results, we propose that the dissection of SC LN should be routinely performed in cases of upper esophageal cancer and also in cases of middle and lower

Table 6. Frequency of Recurrent Laryngeal Nerve Lymph Node Metastasis and Postoperative Morbidity in the Supraclavicular Lymph Node Dissected Group and the Non–Supraclavicular Lymph Node Dissected Group

Postoperative Morbidity	SC LN Dissection (n = 84)	Non–SC LN Dissection (n = 136)	*p* Value[a]
RLN LN metastasis	38 (45.2%)	35 (25.7%)	0.003
RLN palsy	43 (51.2%)	48 (35.3%)	0.024
Phrenic nerve palsy	5 (5.9%)	2 (1.5%)	0.101
Anastomosis leakage	5 (6.0%)	20 (14.7%)	0.051
Chyle leakage	1 (1.2%)	1 (0.7%)	0.999
Tracheostomy	14 (16.6%)	9 (6.6%)	0.023
Operation-associated death	1 (1.2%)	0 (0%)	0.382

[a] Fisher's exact test.

LN = lymph node; RLN = recurrent laryngeal nerve; SC = supraclavicular.

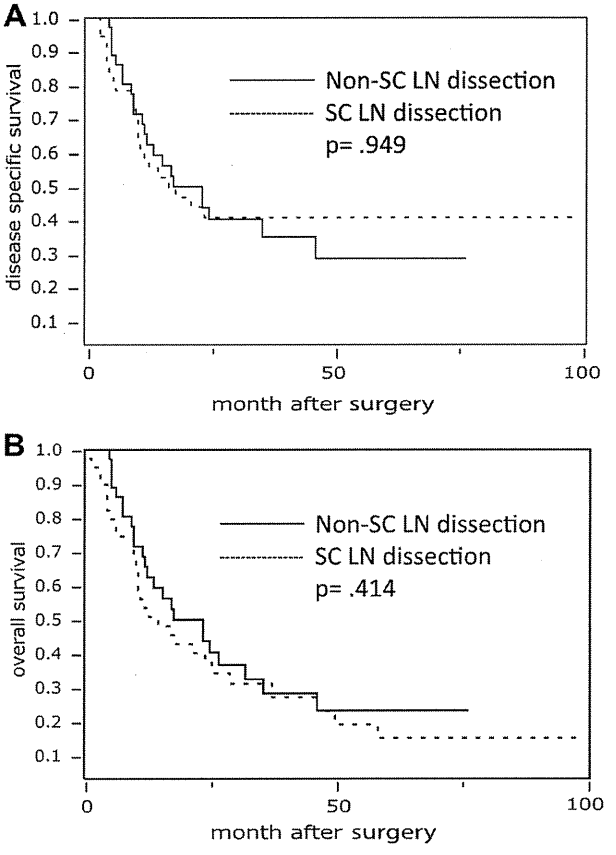

Fig 5. Kaplan-Meyer curves of 73 patients who had recurrent laryngeal nerve (RLN) lymph node (LN) metastasis. Comparison of (A) disease-specific survival and (B) overall survival between supraclavicular (SC) LN dissected group (dashed line) and non-SC LN dissected group (solid line).

advanced esophageal cancer with metastasis of the RLN LN. This SC LN dissection would provide better control of cervical cancer recurrence. However, esophageal cancer patients have different backgrounds such as age, medical history, and other conditions. Therefore we do not think that our suggestion should be the sole criterion for performing SC LN dissection. Rather, these data regarding rates of metastasis when combined with considerations about the condition of the individual patient should assist the surgeon in deciding whether to perform SC LN dissection.

In conclusion, we demonstrated the frequency of SC LN metastasis in each setting of esophageal squamous cell carcinoma. The RLN LN status can be an indicator of SC LN dissection in upper esophageal cancer patients and advanced cases of middle and lower esophageal cancer patients. Bilateral SC LN dissection should be recommended even though there is only unilateral RLN LN metastasis. The SC LN dissection contributed to better local control among the patients who had SC LN metastasis. However, further study is needed to determine the actual benefit of SC LN dissection and to resolve this issue definitively.

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1(OH) Vitamin D3 Supplementation Improves the Sensitivity of the Immune-Response during Peg-IFN/RBV Therapy in Chronic Hepatitis C Patients-Case Controlled Trial

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Abstract

Objective: 1,25(OH)$_2$ vitamin D3 can affect immune cells. However, the mechanism responsible for the favorable effects of 1(OH) vitamin D3, which becomes 1,25(OH)$_2$ vitamin D3 in the liver, is not clear. The aim of this study is to analyze the immunological response of 1(OH) vitamin D3 supplementation in CH-C patients.

Design: Forty-two CH-C patients were treated with 1(OH) vitamin D3/Peg-IFNα/RBV. Forty-two case-matched controls were treated with Peg-IFNα/RBV. The expression of Interferon-stimulated genes (ISGs)-mRNA in the liver biopsy samples and JFH-1 replicating Huh-7 cells were quantified by real-time PCR. Ten kinds of cytokines in the plasma were quantified during treatment by using a suspension beads array. A trans-well co-culture system with peripheral blood mononuclear cells (PBMCs) and Huh-7 cells was used to analyze the effect of 1(OH) vitamin D3. The activities of the Th1 response were compared between subjects treated with 1(OH) vitamin D3/Peg-IFN/RBV and those treated with Peg-IFN/RBV therapy alone.

Results: 1(OH) vitamin D3/Peg-IFN/RBV treatment could induce rapid viral reduction, especially in *IL28B T/T* polymorphism. Several kinds of cytokines including IP-10 were significantly decreased after 4 weeks of 1(OH) vitamin D3 treatment ($p<0.05$). Th1 responses in the subjects treated with 1(OH) vitamin D3/Peg-IFN/RBV were significantly higher than those treated with Peg-IFN/RBV at 12 weeks after Peg-IFN/RBV therapy ($p<0.05$). The expression of ISGs in the patient's liver biopsy samples was significantly lower than in those treated without 1(OH) vitamin D3 ($p<0.05$).

Conclusion: 1(OH) vitamin D3 could improve the sensitivity of Peg-IFN/RBV therapy on HCV-infected hepatocytes by reducing the IP-10 production from PBMCs and ISGs expression in the liver.

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Introduction

Hepatitis C Virus (HCV) is a non-cytopathic virus that causes chronic inflammation, fibrosis and hepatocellular carcinoma (HCC) [1]. Recently, it has been reported that vitamin D3 supplementation could improve the SVR in chronic hepatitis C (CH-C) patients [2,3]. Moreover, the amount of 25-hydoxyvitamin D3 (25(OH) vitamin D3) in the serum could affect the treatment response to pegylated interferon α (Peg-IFN-α)/ribavirin (RBV) therapy and is complementary to interleukin 28B (*IL-28B*) rs1297860 C/T polymorphism in enhancing the correct prediction of the SVR in CH-C [4]. Another group reported that, in patients with genotype 1 HCV persistent infection, the 25(OH) vitamin D serum levels and *IL28B* polymorphism were independently associated with the likelihood of achieving a rapid viral response and SVR after treatment with Peg-IFN/RBV [5].

Although several kinds of mechanisms for the favorable effects of vitamin D3 supplementation were reported, the total effect of vitamin D3 supplementation remains unclear [6,7]. One group reported that 25(OH) vitamin D3, but not vitamin D3 or 1, 25 dihydoxyvitamin D3 (1, 25$(OH)_2$ vitamin D3), appeared to inhibit the viral life cycle at the level of infectious HCV assembly [7]. Another group reported that vitamin D3 or 1,25$(OH)_2$ vitamin D3 and IFN-α could synergistically inhibit HCV production by enhancing the IFN signaling pathway [6]. However, the effect of vitamin D3 on the adaptive immune system in CH-C patients has not been reported yet.

It has been reported that vitamin D3, as synthesized in the skin by photolysis from 7-dehydrocholesterol or ingested with food, is transported in the blood to the liver where it is hydoxylated at the C-25-position [8]. Then, it is hydoxylated at the C-1α-position to form the active metabolite 1,25 $(OH)_2$ vitamin D3 in the kidney [9,10]. In this study, we selected 1(OH) vitamin D3, since the local concentration in the liver should be higher than other metabolites of vitamin D3. Moreover, 1 (OH) vitamin D3 is safe and commonly used in worldwide. 1,25 $(OH)_2$ vitamin D3 is known to regulate calcium and phosphorus metabolism in skeletal homeostasis [11]. It has been reported that 1,25$(OH)_2$ vitamin D3 plays an important role as an immune-modulator targeting various immune cells [12–15]. Various kinds of immune cells express not only vitamin D receptors (VDRs) but also vitamin D-activating enzymes, allowing local conversion of inactive vitamin D into 1,25 $(OH)_2$ vitamin D3 within the immune system [16,17]. The active metabolite1,25$(OH)_2$ vitamin D3 could enhance the anti-mycobacterial activity in monocytes by enhancing the chemotactic and phagocytic capacity of macrophages [18]. Moreover, 1,25$(OH)_2$ vitamin D3 might play an important role in the binding and capturing of antigens by dendritic cells (DCs) at the initiation of the immune response [19]. On the other hand, some groups reported that 1, 25$(OH)_2$ vitamin D3 could inhibit the differentiation and maturation of DCs [19,20]. In addition to monocyte-derived cells, $CD3^+$ T cells, $CD19^+$ B cells, natural killer cells (NK cells) could be directly and/or indirectly affected by 1, 25$(OH)_2$ vitamin D3[12,17,21–24]. It has been reported that 1, 25$(OH)_2$ vitamin D3 could contribute to the suppression of the immune response in autoimmune diseases [14,15,25]. More Recently, the expression of specific VDRs in liver cells and reduced expression of VDRs in CH-C patients have been reported [26]. In addition, an inverse relationship between the liver VDR expression and inflammation severity has been found [26]. However, the effects of 1, 25$(OH)_2$ vitamin D3 for the adaptive immune system in the condition of CH-C patients and during treatment with peg-interferon α and ribavirin (Peg-IFN/RBV) are still unclear. Therefore, it is urgent to analyze the effect of 1, 25$(OH)_2$ vitamin D3 on the adaptive immune responses that could contribute to the outcome of Peg-IFN/RBV therapy.

Materials and Methods

Study Design and Patients

Multi-centers that belong to the Tohoku-liver-study-group (TLG) were involved in this study. Dr. Abu-Mouch et al. reported that the SVR rate of Peg-IFN/RBV plus Vitamin D treatment group was 86% in the AASLD 2009 annual meeting [27]. On the

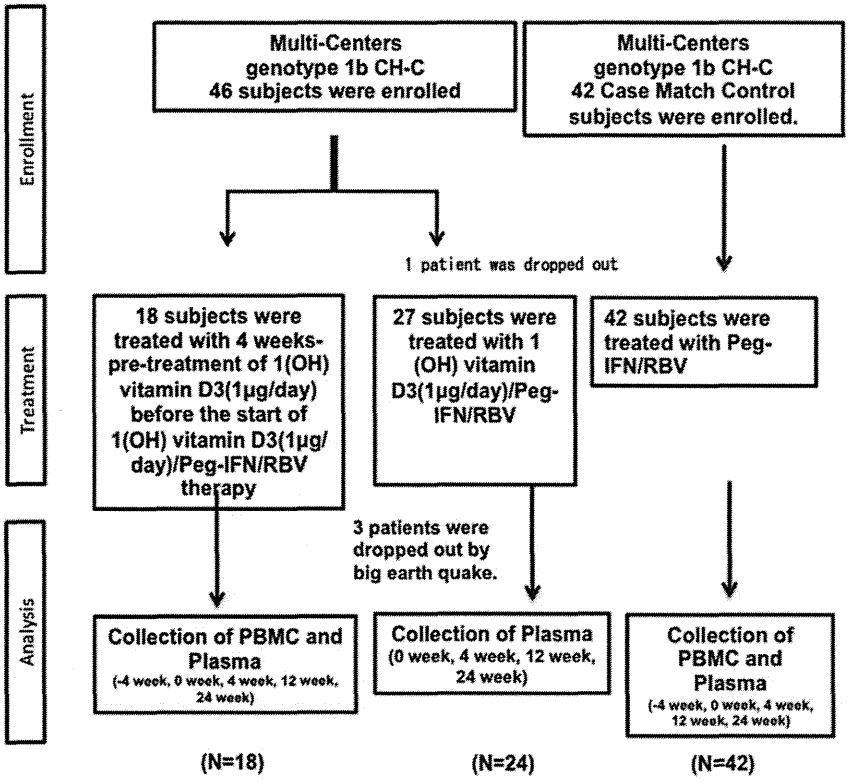

Figure 1. Enrollment of CH-C patients. 46 patients with genotype 1b and high viral loads were enrolled in this study. In total, 4 patients were dropped from this study.
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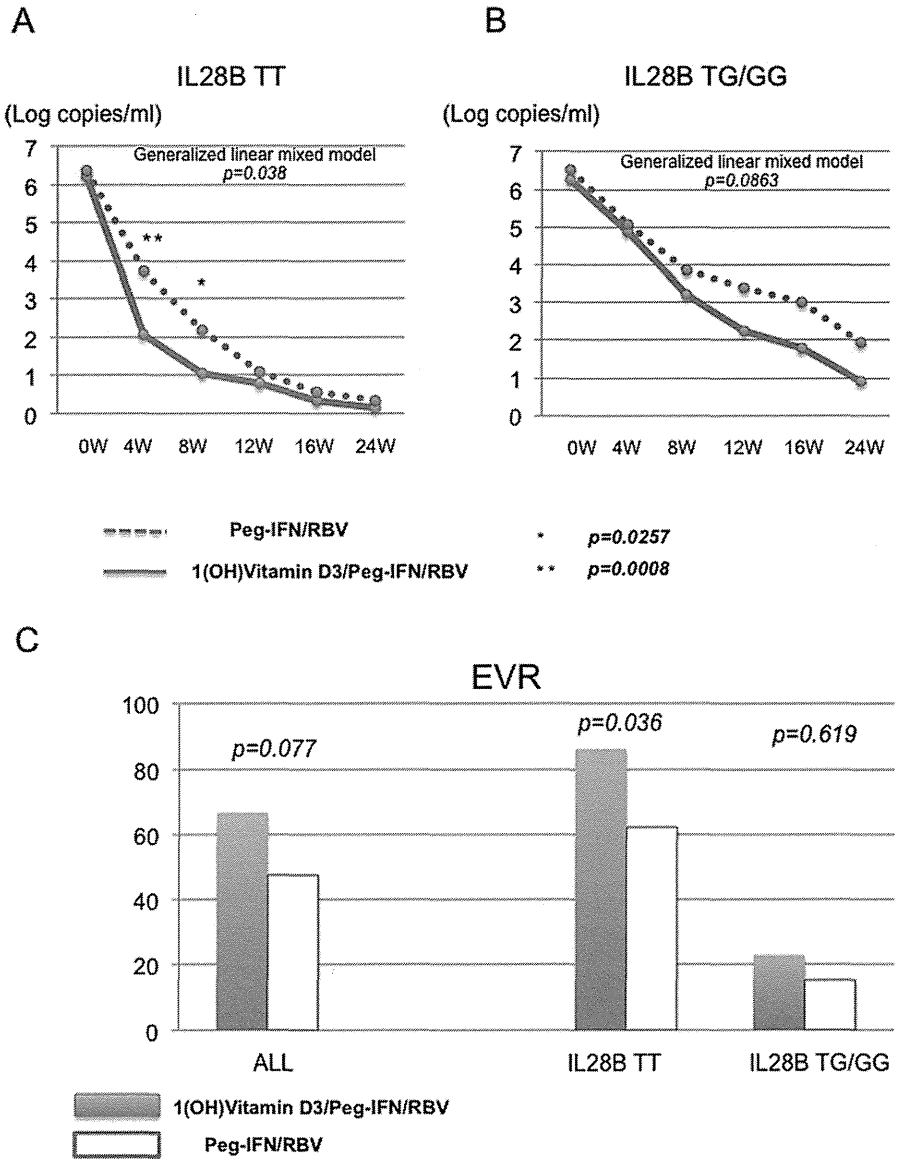

Figure 2. Comparison of viral dynamics and treatment response. Viral dynamics of subjects with IL28B T/T major homo polymorphism are shown (A). Viral dynamics of subjects with IL28B T/G or G/G minor polymorphism are shown (B). Blue lines indicate viral dynamics of subjects treated with 1(OH) Vitamin D3/Peg-IFN/RBV. Dotted lines indicate viral dynamics of subjects treated with Peg-IFN/RBV. *p<0.05 **p<0.01 The rates of early virological response in the patients treated with 1(OH) vitamin D3/Peg-IFN/RBV and Peg-IFN/RBV are shown (C).
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other hand, the SVR rate of Peg-IFN/RBV treatment group was 42%. Considering the uncertainty, we speculated that the EVR rate might be 90% of the EVR rate in the Peg-IFN/RBV plus Vitamin D treatment group because the reported EVR rate in this study was remarkably high. Based on the results of this study, we enrolled about 80 patients including control patients: there was 10% loss in the proportion of patients during the 48 weeks therapy ($\alpha = 0.05$, statistical power 90%) (EVR rate 77% vs 42%). The alfa level was two-sided. Forty-six CH-C (Genotype 1b) patients were enrolled in this study (Fig. 1). Forty-two matched historical controls treated with Peg-IFN-α/RBV therapy were analyzed. The inclusion criteria were as follows: age between 20 and 75 years, high viral load (>5.0 log copies/mL) by real time PCR analysis of HCV-RNA, absolute white blood cell count >2,000/ml, neutrophil count >1,000/ml, platelet count >90,000/ml, and hemoglobin concentration >11 g/dL in laboratory tests. The exclusion criteria were as follows: other liver diseases, including autoimmune hepatitis and alcoholic hepatitis, decompensated liver cirrhosis, liver failure, severe renal disorders, abnormal thyroid function, poorly controlled diabetes, poorly controlled hypertension, medication with immune-modulators, interstitial pneumonia and severe depression. Permission for the study was obtained from the Ethics Committee at Tohoku University Graduate School of Medicine (permission no. 2010-114) (UMIN000003694). The date of the protocol fixation was 10th June 2010. The anticipated trial start date was 11th June 2010. Patients in the 1(OH) vitamin D3/Peg-IFN/RBV group were treated from June 2010 to June 2012. Patients in the Peg-IFN/RBV group were treated from March

2009 to June 2012. Liver biopsy samples of the historical control were from previous studies (Permission no. 2009-166) (UMIN000002326), (Permission no. 2009-209), and (Permission no. 2010-404). Written informed consent of the control subjects treated with Peg-IFN/RBV treatment was obtained in the previous study and in the present study (Permission no. 2009-166) (UMIN000002326), (Permission no. 2009-209), and (Permission no. 2010-404). Written informed consent was obtained from all the participants enrolled in the 1(OH) vitamin D3/Peg-IFN/RBV treatment group. Participants were monitored for a year. At each assessment, patients were evaluated by hematological test, biochemical laboratory tests, immunological test and virological tests. Liver histology was analyzed at the start of Peg-IFN/RBV therapy using the METAVIR score.

Detection of IL-28B Polymorphism

Genomic DNA was isolated from peripheral blood mononuclear cells (PBMCs) using an automated DNA isolation kit. Then, the polymorphism of *IL28B* (rs8099917) was analyzed using real-time polymerase chain reaction (PCR) (TaqMan SNP Genotyping Assay, Applied Biosystems, CA, USA). Detection of the *IL28B* polymorphism was approved by the Ethics Committee at Tohoku University Graduated School of Medicine (permission no. 2010-323).

Isolation of Peripheral Blood Mononuclear Cells (PBMCs), $CD4^+$ Cells and Cell Culture

PBMCs were isolated from fresh heparinized blood by means of Ficoll-Hypaque density gradient centrifugation (Amersham Bioscience, Uppsala, Sweden). Primary $CD4^+$ cells were isolated using magnetic beads (Dynal). PBMCs were used to analyze the effect of the metabolite of α-calcidol(1(OH) vitamin D3) without direct cell to cell contact in an Huh-7 cells-transwell system. PBMCs and Huh-7 cells were cultured with serum-free complete medium that were previously made by our group [28]. A thousand times higher amount of 1(OH) vitamin D3 was used to analyze the effect of 1,25 $(OH)_2$ vitamin D3, which comes from the lower part of chamber, since the Huh-7 cells have several enzymes that could convert 1(OH) vitamin D3 to 1,25 $(OH)_2$ vitamin D3. The supernatant was harvested at 48 hours after the addition of 1(OH) vitamin D3 or 1,25 $(OH)_2$ vitamin D3.

Flow Cytometry Analysis

PBMCs were stained with CD3-pacific-blue, CD4-PE/Cy7, CD25-PE, CD127-APC, CD183 (CXCR3)-APC/Cy7, CD195 (CCR5)-FITC, Viaplobe and isotype control antibodies (BD pharmingen, San Jose, CA, USA) for 15 min on ice to analyze the frequency of CD3+CD4+CXCR3+CCR5+ cells (Th1) and CD3+CD4+CD25+CD127− (Tregs) by FACSCanto-II (BD). The FCS files 3.0 were analyzed by Flowjo 7.60 software.

Multiplex Beads Suspension Array

The culture supernatant of PBMCs treated with the active vitamin D3 metabolite (1,25 $(OH)_2$ vitamin D3) and the plasma obtained from CH-C patients treated with or without alfa-calcidol (1(OH) vitamin D3) were sequentially analyzed by suspension beads array (BIO-RAD Laboratories, Tokyo, Japan). Suspension beads array was performed following the manufacturer's instruction. Briefly, the supernatant was incubated with first-antibody binding magnetic beads. Then, the detection antibody and PE

Table 1. Clinical characteristics of subjects enrolled in this study.

	PEG-IFNα/RBV	PEG-IFNα/RBV+VD3	PEG-IFNα/RBV+VD3	
	(n = 42)	(n = 42)	With Pre-VD3	Without Pre-VD3
			(n = 18)	(n = 24)
Gender(M/F)	19/23	15/27	6/12	9/15
Age	58.3(35–72)	59.1(29–71)	58.6(29–71)	58.5(43–71)
Body Weight	58.4	58.1(41.2–81)	56.4(41.2–81)	59.4(43–78)
History of IFN(+/−)	13/29	13/29	7/11	6/18
IL-28B(TT/TG,GG)	29/13	29/13	10/8	19/5
Sampling Point (week)	0W	All 0W	−4W	0W
HCV-RNA	6.3(5.1–7.2)	6.3(5.2–7.4)	6.3(5.2–7.1)	6.4(5.3–7.4)
ALT	68.5 (15–234)	66.4(16–242)	47.9(22–108)	78(16–242)
AST	55.2(16–161)	58.1(21–251)	45.3(22–112)	66.1(21–251)
WBC	5045(3050–7800)	5165(2400–9300)	5055(3100–9300)	5530(2400–8130)
RBC	441.3(355–522)	441.5(375–567)	450(375–567)	446(383–515)
PLT	16.6(9.4–29.4)	16.7(9.3–27.6)	16.6(9.3–27.6)	16.7(9.3–23.9)
Nue	2845(1750–5020)	2911(1190–7160)	2792(1190–7160)	3476(1533–5070)
Hb	13.8(11.8–15.9)	13.6(12–16.3)	13.7(12–15.2)	14.1(12.6–16.3)
Serum Ca	9.3(8.5–9.8)	9.2(8.6–10.1)	9.4(8.9–10.1)	9.2(8.6–10)
Insulin	9.4(6.8–20.2)	9.5(1.6–25.5)	9(4.76–20.8)	9.6(1.6–25.5)
T-cho	170.6(118–214)	172.4(116–227)	168.2(116–226)	173.7(119–227)
TG	108.5(55.6–210)	106.4(37–427)	118.9(37–259)	103.2(51–427)

HCV-RNA(log copies/ml), ALT(U/l), AST(U/l), WBC(counts/μl), RBC(x10^3counts/μl), PLT(x10^4counts/μl), Neut(counts/μl), Hb (g/dl), Serum Ca (mg/dl), Insulin (μU/ml), T-cho (mg/dl), TG (mg/dl).

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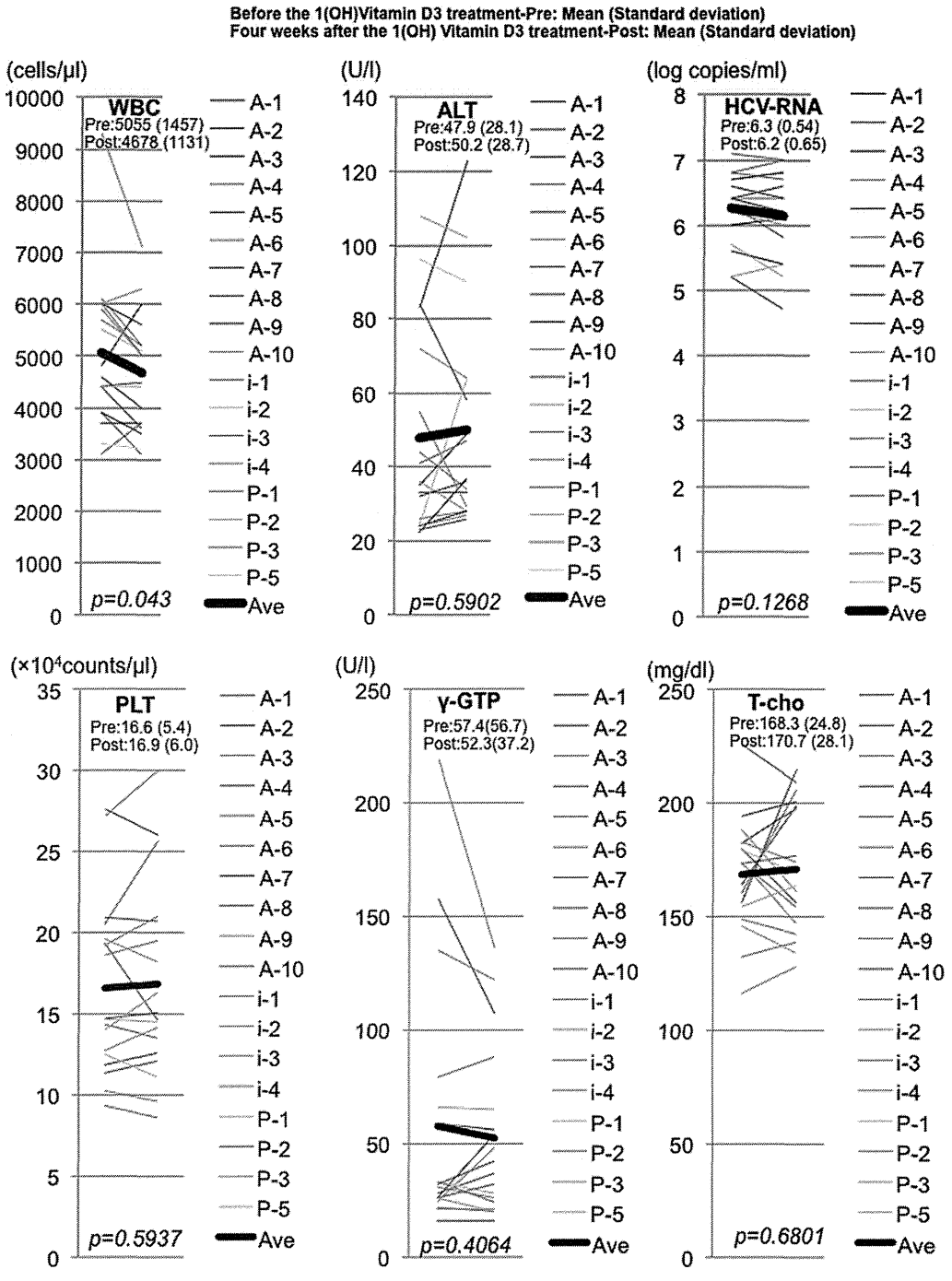

Figure 3. Comparison of hematological and biochemical analysis between before and after 4-week 1(OH) vitamin D3 treatment. Representative hematological, biochemical and virological data are shown. WBC indicates white blood cell count. ALT indicates alanine transaminase. HCV-RNA indicates titer of hepatits C virus RNA. PLT indicates platelet count. γ-GTP indicates gamma-glutamyl traspeptidase. T-cho indicates total cholesterol. The data at pre- and post-4weeks administration of 1(OH) vitamin D3 without Peg-IFN/RBV are shown. Black lines indicate the average of each analysis.
doi:10.1371/journal.pone.0063672.g003

conjugated streptavidin were reacted after the appropriate washing steps. Finally, the reaction plates were analyzed by Bio-plex 200 system.

Real-time Polymerase Chain Reaction

RNA was isolated using a Qiagen RNeasy mini kit (Valencia, CA) and the yields were determined by absorption spectroscopy using a Nano-Drop (NanoDrop Products, Wilmington, DE). After the extraction of total RNA and the reverse transcription (RT)

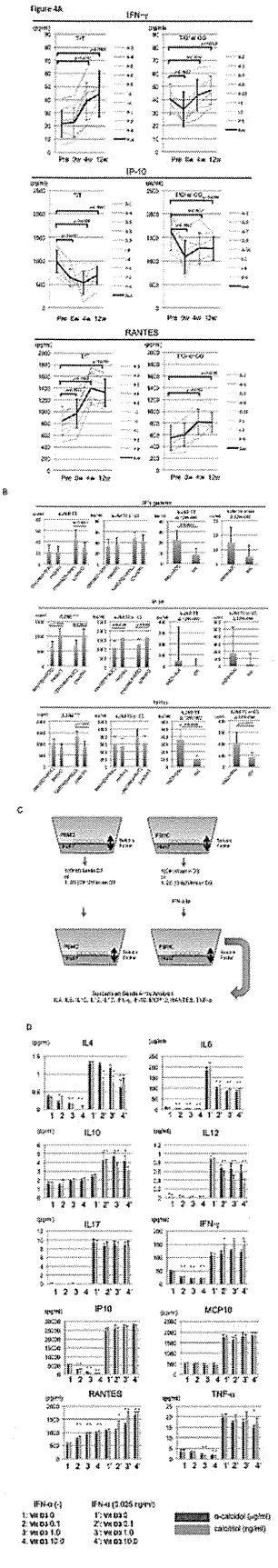

Figure 4. Cytokine profiles in the *ex vivo* and *in vitro* samples treated with vitamin D3. Sequential data of quantification of 3 cytokines (IFN-γ, IP-10 and RANTES) during 1(OH) vitamin D3 pre-treatment (pre vs 0w), 1(OH) vitamin D3/Peg-IFN/RBV therapy are shown (A). Dotted lines indicate the data of each subject. Black lines indicate the averaged data. Error bars indicate standard deviation. The data from IL28B (T/T) subjects or IL28B (T/G or G/G) subjects are shown in the independent graphs (A). Comparisons of the amounts of 3 cytokines (IFN-γ, IP-10 and RANTES) between the 1(OH) vitamin D3/PEG-IFN/RBV group (VitD3+standard of care (SOC)) and Peg-IFN/RBV group (SOC) at 0 weeks and 12 weeks after the start of Peg-IFN/RBV treatment are shown (B). Analysis of the changes in the amounts of the 3 cytokines (IFNγ, IP-10 and RANTES) during 12 weeks treatment of Peg-IFN/RBV is shown. Schema of *in vitro*-analysis of co-culture is shown (B). alfa-calcidol: 1(OH)vitamin D3 and calcitriol: 1,25(OH)vitamin D3 were used to analyze the cytokine production *in vitro*. Black bars indicate the data from samples treated with alfa-calcidol. Gray bars indicate the data from samples treated with calcitriol. *$p<0.05$.
doi:10.1371/journal.pone.0063672.g004

procedure, real-time polymerase chain reaction (PCR) using a TaqMan Chemistry System was carried out. The ready-made set of primers and probe for the amplification of IFN-γ, T-bet, Mx1 (ID Hs00895608), IFI44 (ID Hs00197427), IFIT1 (ID Hs01911452) and glyceraldehyde 3-phsphate-dehydrogenase (GAPDH) were purchased from Perkin-Elmer Applied Biosystems (Carlsbad, CA, USA). The relative amount of target mRNA was obtained by using the comparative threshold (CT) cycle method.

The Quantification of ISGs mRNA in Hepatocyte Cell Culture

Huh-7 cells were treated with ethanol (control), 1(OH) vitamin D3 (1.0 μM) or 1,25(OH)2 vitamin D3 (1.0 μM) after transfection of poly IC (Sigma-Aldrich, St. Louis, MO) or in vitro transcribed JFH-1 full-length RNA. Cells were harvested 30 hour after transfection, and the expression levels of Mx, IFI44 and IFIT1 mRNA were assessed by real-time PCR using TaqMan Gene Expression Master Mix (Applied Biosystems, Carlsbad, CA) and gene-specific primer and probe sets (TaqMan Gene Expression Assay; Applied Biosystems) in accordance with the manufacturer's instructions. The expression levels of genes with or without vitamin D3 treatment were expressed by the log fold increase of untreated Huh-7 cells.

Statistical Analysis

The data in Fig. 2A and B were analyzed using a generalized linear mixed model (Treatment group of 1(OH) vitamin D3/Peg-IFN/RBV and Peg-IFN/RBV were fixed-effect. Duration of treatment was random-effect.) and Student's t test. The data in Fig. 2C were analyzed by χ^2 test. The data in Fig. 3, Fig. 4A and Fig. 5B were analyzed by paired t test. The data in Fig. 4C were analyzed by Dunnett's test. The data in Fig. 5C were analyzed by Tukey's test. The data in Fig. 4B, Fig. 5D and Fig. 6 were analyzed by Student's t test. The cut-off of acceptance of test's results was $p<0.05$ with a confidence interval of 95%. All statistical analyses were carried out using JMP Pro version 10 (SAS Institute Inc., Cary, NC, USA).

Results

Efficacy and Tolerability of 1(OH) Vitamin D3 Combined with Peg-IFN/RBV Therapy

The characteristics of 42 patients treated with 1(OH) vitamin D3 (1 μg/day)/Peg-IFN/RBV therapy are shown in Table 1. The subjects enrolled in this study were 29 to 71 years old. 13 patients

Figure 5

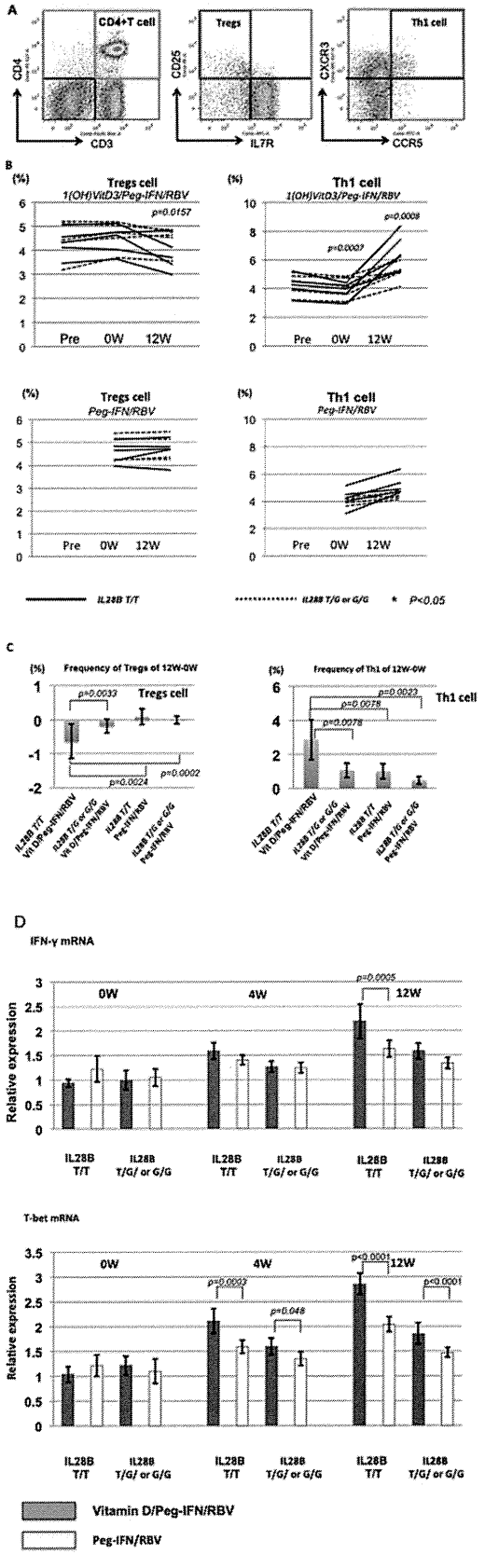

Figure 5. Comparison of Th1 and Tregs between 1(OH) vitamin D3/Peg-IFN/RBV and Peg-IFN/RBV. Representative dot plots of $CD3^+CD4^+CD25^+IL7R^-$ (Tregs) and $CD3^+CD4^+CXCR3^+CCR5^+$ (Th1 cells) are shown. (A) Frequencies of Th1 and Tregs among the 4 groups (IL28B T/T vitamin D3/Peg-IFN/RBV, IL28B T/G or G/G vitamin D3/Peg-IFN/RBV, IL28B T/T Peg-IFN/RBV, and IL28B T/G or G/G Peg-IFN/RBV) are shown. (B) Comparison of the T-bet and IFN-γ mRNA expression between subjects treated with vitamin D3/Peg-IFN/RBV therapy and those treated with Peg-IFN/RBV therapy. Each group included 5 patients. Total mRNA was extracted from isolated $CD4^+$ T cells. The relative expression levels are shown in bar graphs. The statistical analysis was carried out by independent student *t*-test.
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were previously treated with IFN-based therapy and failed to achieve SVR. Another 29 patients were treatment naïve. Case match control subjects treated with Peg-IFN/RBV therapy were enrolled in this study (Fig. 1) (Table 1). All of the enrolled patients had over 5 log copies/ml HCV-RNA and genotype 1b HCV RNA. Thirteen patients had the hetero/minor *IL28B* allele (T/G) (rs8099917) that was reported to be a marker of patients difficult-to-treat with Peg-IFN/RBV therapy [29]. Twenty-nine patients had the major homo *IL28B* allele (T/T) that was reported to be favorable for achieving SVR [29]. Therefore, we compared the viral dynamics between subjects treated with the 1(OH) vitamin D3/Peg-IFN/RBV and subjects receiving the Peg-IFN/RBV with the same IL28B polymorphism (Fig. 2A and B). The titers of HCV-RNA in the *IL28B* (T/T)-HCV patients treated with 1(OH) vitamin D3/Peg-IFN/RBV therapy were significantly lower than those treated with Peg-IFN/RBV at 4 weeks after the start of Peg-IFN/RBV therapy ($p<0.01$). The rate of early virological response in the *IL28B* (T/T) patients treated with 1(OH) vitamin D3/Peg-IFN/RBV was significantly higher than that in those treated with Peg-IFN/RBV alone (Fig. 2C). None of the patients showed side effects from 1(OH) vitamin D3 administration such as hypercalcemia or renal dysfunction, etc. The rate of the sustained virological response (SVR) in the overall patients treated with 1(OH) vitamin D3/Peg-IFN/RBV was 59.45% (45.24% in the overall patients treated with Peg-IFN/RBV) ($p = 0.2059$) The rate of SVR in the *IL28B* (T/T) patients treated with 1(OH) vitamin D3/Peg-IFN/RBV was 73.07% (55.17% in *IL28B* (T/T) patients treated with Peg-IFN/RBV) ($p = 0.1657$). However, this study was conducted to analyze the immunological response during the early phase of Peg-IFN/RBV. The sample size might not be large enough to analyze the SVR rate.

Biological Effect of 1(OH) Vitamin D3 Treatment during Peg-IFN/RBV Therapy

The biochemical and hematological analysis was carried out at 4 weeks before the start of Peg-IFN/RBV therapy and at the start of Peg-IFN/RBV therapy. Of those data, only the absolute counts of white blood cells were significantly decreased after 4 weeks-1(OH) vitamin D3-treatment ($p<0.05$) (Fig. 3). The titers of HCV-RNA were not significantly changed after the 4-week administration of 1(OH) vitamin D3 without Peg-IFN/RBV therapy. Therefore, we examined the immunological effects of 1(OH) vitamin D3. At first, we quantitated 10 cytokines (IL 4, IL 6, IL10, IL12, IL17, IFN-γ, IP-10, MCP-1, RANTES, TNF-α) in the peripheral blood samples during 1(OH) vitamin D3/Peg-IFN/RBV therapy using multiple beads suspension array (Fig. 4A and Fig. S1). Among the *IL28B* T/T polymorphism patients, the amounts of IL4, IP-10 and MCP1 in the peripheral blood serum were significantly reduced after 4-week-1(OH) vitamin D3-treatment. On the other hand, the amounts of IL6, RANTES and TNF-α in the serum were significantly increased after 4-week 1(OH)vitamin D3 treatment. In the *IL28B* T/G or G/G

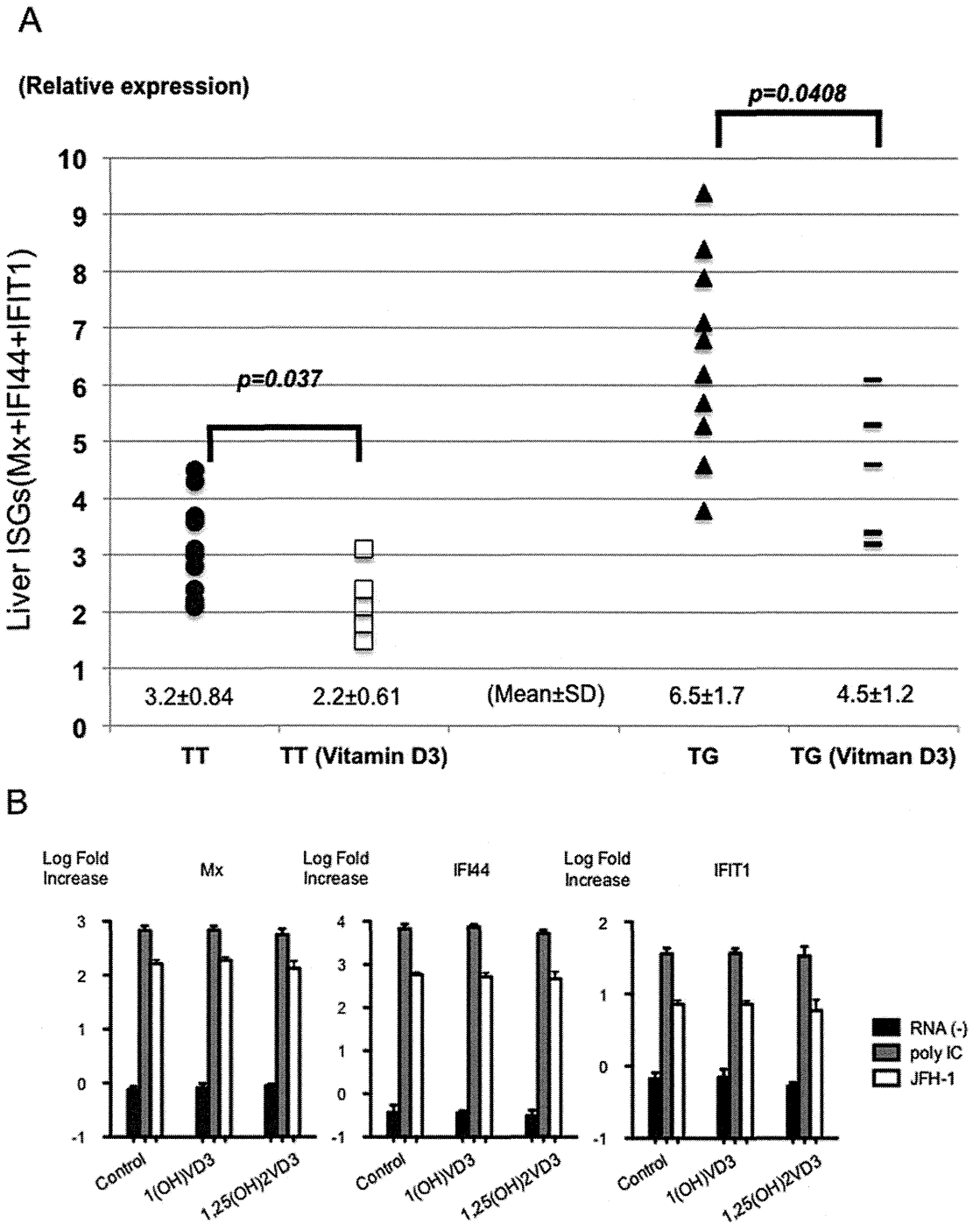

Figure 6. The effect of vitamin D3 on the expression of ISGs mRNA in the liver. The relative amount of target mRNA was obtained by using a comparative threshold cycle (CT) method. The expression levels of Mx, IFI44 or IFIT1 mRNA in an *IL28B T/T* patient treated without 1(OH) vitamin D3 are represented as 1.0 and the relative amounts of target mRNA in the other patients were calculated by the comparative Ct method [42]. Therefore, the standard amount of 3 ISGs (Mx, IFI44 and IFIT1) is 3. The relative amounts of the 3 kinds of ISGs were added and shown in the graph (A). Black circles indicate the data from IL28B (T/T) subjects treated without 1(OH) vitamin D3. White boxes indicate the data from IL28B (T/T) subjects treated with 1(OH) vitamin D3. Black triangles indicate the data from IL28B (T/G or G/G) subjects treated without 1(OH) vitamin D3. Black lines indicate the data from the subjects treated with 1(OH) vitamin D3 (A). The effect of vitamin D3 on the expression of ISGs mRNA in the hepatocyte cell culture are shown (B). Huh-7 cells were treated with ethanol (control), 1(OH) vitamin D3 (1.0 μM) or 1,25(OH)$_2$ vitamin D3 (1.0 μM) after transfection of poly IC (Sigma-Aldrich, St. Louis, MO) or in vitro transcribed JFH-1 full-length RNA. Cells were harvested 30 h after transfection, and the expression levels of Mx, IFI44 and IFIT1 mRNA were assessed by real-time PCR using TaqMan Gene Expression Master Mix (Applied Biosystems, Carlsbad, CA) and gene-specific primer and probe sets (TaqMan Gene Expression Assay; Applied Biosystems) in accordance with the manufacturer's instructions. The expression levels of genes with or without vitamin D3 treatment were expressed by log fold increase of untreated Huh-7 cells.
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