Table 3. Adverse events ($n = 54$)

	Grade				Grades 1–4	Grades 3–4
	1	2	3	4	%	%
Hematological toxicity						
Leucocytes	3	19	31	1	100	59
Neutrophils	2	9	24	19	100	80
Hemoglobin	11	29	8	0	89	15
Platelets	15	23	12	0	83	22
Non-hematological toxicity						
Bilirubin	15	9	3	0	50	6
AST	23	6	2	0	57	4
ALT	20	11	4	0	65	7
Creatinine	7	0	0	0	13	0
Nausea	19	11	3	—	61	6
Vomiting	11	5	1	0	32	2
Anorexia	18	11	9	0	70	17
Stomatitis	20	10	1	0	57	2
Diarrhea	12	5	0	0	32	0
Constipation	2	0	1	0	6	2
Ileus	—	0	1	0	2	2
Colitis	—	0	1	0	2	2
Fatigue	22	14	3	0	72	6
Fever	15	5	0	0	37	0
Alopecia	13	2	—	—	28	0
Rash	13	17	4	0	63	7
Pigmentation changes	27	7	—	—	63	0
Hand-foot skin reaction	3	0	0	0	6	0
Infection without neutropenia	2	2	2	0	11	4
Febrile neutropenia	—	—	1	0	2	2
CNS cerebrovascular ischemia	—	—	1	1	4	4

AST, aspartate aminotransferase; ALT, alanine aminotransferase.

To date, several Phase II studies testing the gemcitabine plus S-1 combination as first-line therapy for advanced pancreatic cancer have been published (Table 4) (15–18). One study was conducted in Japan and the remaining studies were in Korea. Although various schedules of gemcitabine and S-1 administration were used, the regimens adopted in all studies including this study were similar: gemcitabine at a dose of 1000–1250 mg administered on days 1 and 8 or 8 and 15 and S-1 at a dose of 60–80 mg/m^2/day on days 1–14 of a 21-day cycle. The incidences and severity of toxicities reported in these trials, especially hematological toxicities, have varied widely among the studies. Interestingly, hematological toxicities were more frequently observed in the two Japanese studies, including this study, than the Korean studies. It is well known that the toxicity profile of S-1 differs between Asians and Caucasians (19); Goh and coworkers (20) carried out a study to compare S-1 pharmacokinetics and CYP2A6 activity among Asian and Caucasian patients, and reported that Asian patients had lower 5-FU exposure and lower CYP2A6 activity compared with Caucasian patients. However, the reasons for the discrepancies between the Japanese and Korean studies remain unclear.

In this trial, GS therapy produced a promising efficacy with a response rate of 44.4%. The efficacy of GS therapy reported in the recent studies as well as this study has been consistent (Table 4), with response rates of 27.3–38%, median time to tumor progression of 4.6–5.43 months and median overall survival of 7.89–12.5 months. Recently, the results of a randomized Phase II study comparing GS therapy with gemcitabine alone were reported (21). In that study, 106 patients were randomly assigned at a 1:1 ratio to either the GS group or the gemcitabine-alone group. Patients assigned to GS therapy received gemcitabine at a dose of 1000 mg/m^2 on days 1 and 15 and S-1 at a dose of 40 mg/m^2 twice daily on days 1–14, every 4 weeks. The objective response rate was 18.9% in the GS group and 9.4% in the gemcitabine group. Patients in the GS group demonstrated significantly longer PFS than those in the gemcitabine group [median PFS, 5.4 versus 3.6 months; hazard ratio = 0.64 (95% CI: 0.42–0.97); $P = 0.036$], while overall survival did not differ significantly between the two groups [median

Table 4. Phase II studies of GS therapy for advanced pancreatic cancer

Author	Gemcitabine (mg/m^2)	S-1 (mg/m^2/day)	Cycle (day)	No. of patients	Metastatic disease (%)	RR (%)	Median TTP/PFS (months)	Median OS (months)	Grade 3/4 neutropenia (%)	Grade 3/4 thrombocytopenia (%)
Nakamura *et al.* (15)	1000 (days 8, 15)	60 (days 1–14)	21	33	100	48	5.4	12.5	55	15
Lee *et al.* (16)	1250 (days 1, 8)	80 (days 1–14)	21	32	90.6	44	4.92	7.89	28.1	15.6
Kim *et al.* (17)	1000 (days 8, 15)	60 (days 1–14)	21	22	86.3	27.3	4.6	8.5	18.2	4.5
Oh *et al.* (18)	1000 (days 1, 8)	80 (days 1–14)	21	38	84	29	5.43	8.4	39.5	2.6
Current study	1000 (days 1, 8)	80 (days 1–14)	21	55	100	44.4	5.9	10.1	80	22

RR, response rate; TTP, time to progression; PFS, progression-free survival; OS, overall survival.

overall survival, 14.1 versus 8.7 months; hazard ratio = 0.69 (95% CI: 0.43–1.08); $P = 0.105$].

Since it is speculated that combination chemotherapy with S-1 and gemcitabine might be superior to monotherapy with gemcitabine from the results of the recent trials, a Phase III trial was planned to confirm the efficacy of GS therapy (ClinicalTrials.gov, NCT00498225). The Phase III study known as 'GEST' is a randomized controlled study involving three arms: gemcitabine monotherapy as a control arm, S-1 monotherapy and GS therapy. The trial was designed to evaluate overall survival as the primary endpoint, non-inferiority of S-1 to gemcitabine and superiority of GS therapy over gemcitabine. The enrollment of 750 patients was planned and has already been completed and the final analysis of the results will be reported in the near future.

In conclusion, the current Phase II study demonstrated encouraging antitumor activity following GS therapy with good overall survival in patients with metastatic pancreatic cancer. The clinical benefits of GS therapy are now investigated in the GEST trial.

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Conflict of interest statement

None declared.

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Appendix

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Possibility of immunotherapy for biliary tract cancer: how do we prove efficacy? Introduction to a current ongoing phase I and randomized phase II study to evaluate the efficacy and safety of adding Wilms tumor 1 peptide vaccine to gemcitabine and cisplatin for the treatment of advanced biliary tract cancer (WT-BT trial)

Takuji Okusaka · Makoto Ueno · Tosiya Sato · Yuji Heike

Abstract
Background/purpose In biliary tract cancer, few clinical studies evaluating immunotherapy have been reported. A phase I and randomized phase II study with Wilms tumor 1 (WT1) peptide vaccine plus gemcitabine and cisplatin (GC) for chemo-naïve patients with unresectable or recurrent biliary tract cancer was started, because the overexpression of WT1 is seen in the majority of patients with this disease, encouraging the potential of WT1-based immunotherapy. This trial was registered at the UMIN Clinical Trials Registry as UMIN 000004886.
Methods and results The aim of this trial is to evaluate the efficacy and safety of the regimen and to determine whether the regimen should be compared with the current standard regimen, GC, in a subsequent phase III trial for patients with unresectable or recurrent biliary tract cancer. Six patients in the phase I study and a total of 100 patients in the phase II study will be accrued over a 2-year period. The patients in the phase II study will be randomized at a 2:1 ratio to receive GC either with or without WT1 peptide vaccine. The primary endpoint of the phase II study is the 1-year overall survival rate.
Conclusions This is the first randomized trial to evaluate the use of immunotherapy in patients with advanced biliary tract cancer.

Keywords Biliary tract cancer · Immunotherapy · Chemotherapy · Wilms tumor 1 (WT1) peptide vaccine · Randomized trial

Introduction

Systemic chemotherapy is usually indicated for patients with unresectable advanced biliary tract cancer or for those who have relapsed after operation; however, no standard treatments with solid evidence of a survival benefit have been established for such patients [1]. Although gemcitabine (GEM) alone was regarded as the de-facto standard regimen for advanced biliary cancer until recently, gemcitabine plus cisplatin (GC) has become the new standard regimen, based on the results of the ABC-02 trial [2], which showed a significant survival advantage for the GC combination over GEM alone. Even with the establishment of a standard therapy for this disease, the prognosis of these patients remains dismal: their median survival period is only around 10 months [2, 3]. Therefore, a clear need exists for new, effective, treatments for the management of biliary tract cancer (Fig. 1).

Recent progress in understanding the basic aspects of immunology has led to the development of immune-based therapies for various types of cancers. The identification of

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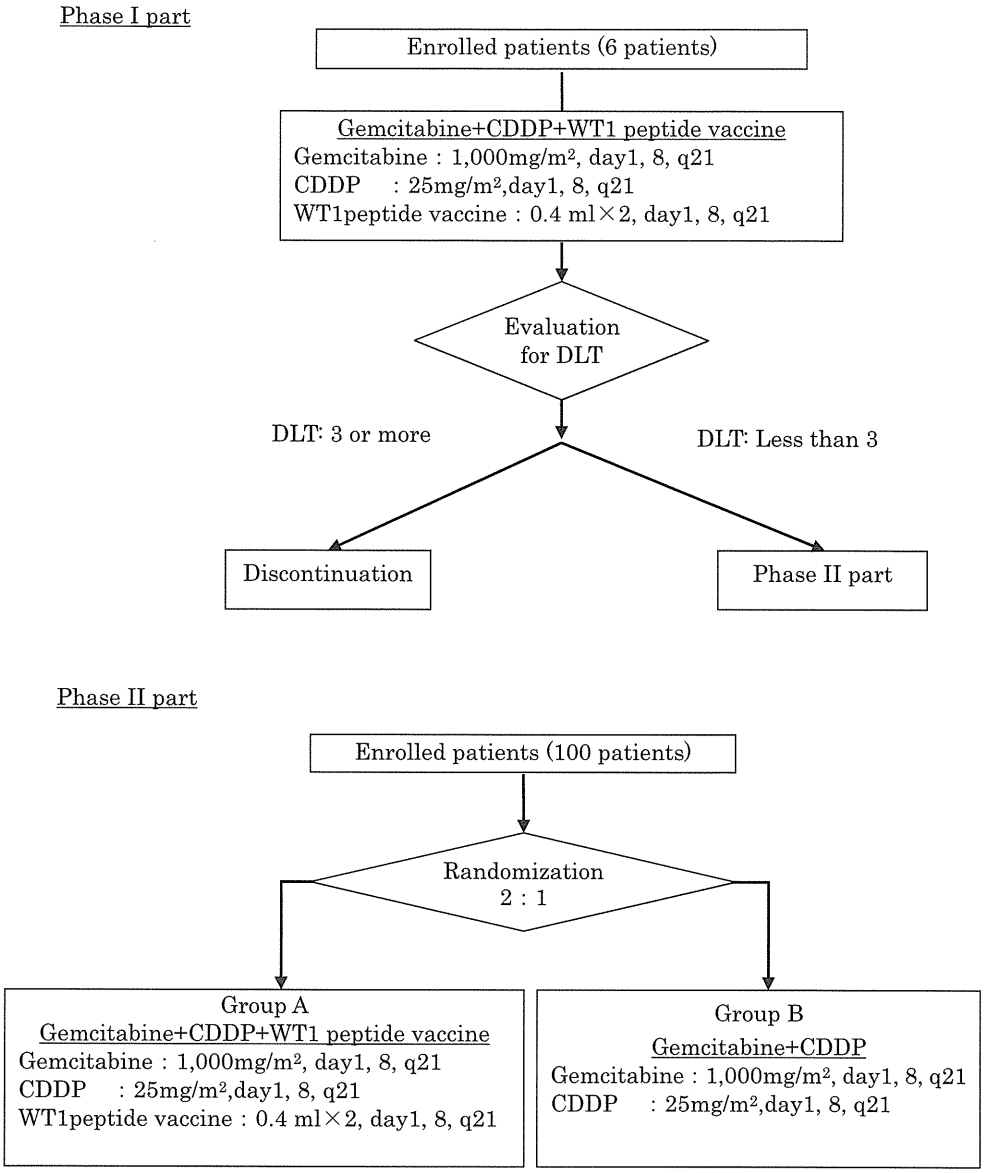

Fig. 1 Study design. *DLT* dose-limiting toxicity, *CDDP* cisplatin, *WT 1* Wilms tumor 1

various cancer antigens has facilitated many clinical trials of cancer vaccines that are expected to become new treatment strategies. Recently, sipuleucel-T immunotherapy for metastatic, asymptomatic hormone-refractory prostate cancer [4] and immunotherapy with ipilimumab for metastatic melanoma [5] have produced statistically significant improvements in survival, and both of these treatments have been approved by the United States Food and Drug Administration. Sipuleucel-T stimulates T-cell immunity against prostatic acid phosphatase, and ipilimumab blocks the potentiation of cytotoxic T-lymphocyte-associated antigen 4 and the antitumor T-cell response. Unfortunately, few preclinical studies examining biliary tract cancer have shown promising immune responses similar to those induced by sipuleucel-T against prostate cancer or those induced by ipilimumab against melanoma, and few clinical studies of immunotherapy for biliary tract cancer have been reported because of the rarity of this disease and the poor physical conditions of most patients at the time of the initial diagnosis. However, GEM has been reported not to suppress immunological cells, but to increase the population of dendritic cells that serve as antigen-presenting cells [6, 7]. Therefore, we conducted a phase I trial of Wilms tumor 1 (WT1) peptide vaccine and GEM combination therapy in patients with advanced pancreatic or biliary tract cancer [8]. Although the aim of that study was to assess the safety of the combination of WT1 peptide vaccine and GEM in a small population, it also showed that the WT1 peptide vaccine was safe enough to be employed in patients with advanced pancreatic or biliary tract cancer in combination with GEM, and

that the efficacy of the combination therapy seemed to be promising, as outlined below.

We recently initiated a phase I and randomized phase II study to evaluate the efficacy and safety of adding the WT1 peptide vaccine to GC in advanced biliary tract cancer (WT-BT trial), since GC has become the new standard and because the WT1 peptide vaccine is an attractive candidate as a partner for chemotherapy to improve survival in patients with advanced biliary tract cancer. WT1 protein is overexpressed in various types of cancer cells, including biliary tract cancer cells [9], and it was ranked as the No. 1 antigen in the cancer antigen prioritization project of the National Cancer Institute [10].

To our knowledge, this is the first randomized clinical trial to evaluate immunotherapy for biliary tract cancer. The study complied with the Declaration of Helsinki. Informed consent was obtained from all the patients, and the protocol was approved by the ethics committees at all participating institutions. This trial was registered at the UMIN Clinical Trials Registry as UMIN 000004886 (http://www.umin.ac.jp/ctr/index.htm). The study was initiated in January 2011.

1. The results of a phase I trial of WT1 peptide vaccine and GEM combination therapy in patients with advanced pancreatic or biliary tract cancer

An open-labeled, dose-escalation phase 1 trial of WT1 vaccine and GEM combination therapy for patients with advanced pancreatic cancer or biliary tract cancer was performed. The primary endpoint was the evaluation of the toxicity, safety, and optimal immunological dose of the vaccine. Human leukocyte antigen (HLA)-A 0201, HLA-A 0206, and/or HLA-A 2402-positive patients with inoperable advanced pancreatic or biliary tract cancer who had not previously been treated with GEM were eligible for this study. Six doses of GEM and 4 doses of WT1 peptide (1 or 3 mg) emulsified in Montanide adjuvant were administered over 2 months. Twenty-five patients (13 male and 12 female) were enrolled. Nine patients had inoperable advanced pancreatic cancer, 8 had gallbladder cancer, 4 had intrahepatic, and 4 had extrahepatic bile duct cancer. The adverse events were comparable to those seen with GEM alone. Delayed-type hypersensitivity test was positive after vaccination in 2 patients, and WT1-specific T cells in peptide-stimulated culture were detected by tetramer assay in 59% (13 of 22) of the patients. The disease control rate at 2 months was 89% for pancreatic cancer and 50% for biliary tract cancer. With a median follow-up time of 259 days, the median survival time for patients with biliary tract cancer was 288 days, and that for patients with pancreatic cancer was 259 days. Although objective clinical efficacy was not apparent, the safety of the WT1 vaccine and GEM combination therapy was confirmed in this study.

2. An ongoing phase I and randomized phase II study to evaluate the efficacy and safety of adding WT1 peptide vaccine to GC in advanced biliary tract cancer (WT-BT trial).

Protocol summary of the WT-BT trial

Study setting

The study is a multi-institutional open-label phase I and randomized phase II trial.

Objectives and endpoints

The aim of this phase I/II study is to determine the recommended dosage of WT1 peptide vaccine when used in combination with GC chemotherapy and to clarify the safety and efficacy of GC plus WT1 peptide vaccine when administered at the recommended dose, in comparison with GC alone.

In the phase I study, we will investigate the frequency of the dose-limiting toxicity (DLT). The criteria for a DLT will include: Grade 4 neutropenia for 8 or more consecutive days, Grade 3 neutropenia accompanied by a fever ($\geq$37.5°C), Grade 4 thrombocytopenia or the need for a transfusion, a Grade 4 aspartate transaminase (AST)/alanine transaminase (ALT) elevation or a Grade 3 AST/ALT elevation for 8 or more consecutive days, Grade 3 or 4 nonhematological toxicity (except for rash, hyperglycemia, gamma-GTP elevation, and any temporary events not affecting the protocol treatment), Grade 3 or 4 local skin inflammation at the vaccine injection sites, or Grade 1 or greater interstitial pneumonia.

In the phase II study, the primary endpoint will be the 1-year overall survival rate for all eligible patients. Overall survival will be defined as the number of days from randomization until death from any cause, and the data will be censored as of the last follow-up day on which the patient was alive. The secondary endpoints will be progression-free survival, response rate, median survival time, 2-year overall survival rate, percentage of adverse events, percentage of serious adverse events, and immunological responses (multimer assay and delayed-type hypersensitivity).

Eligibility criteria

Inclusion criteria

For inclusion in the study, patients are required to fulfill all the following criteria:

1. Clinically diagnosed with biliary tract cancer, including intrahepatic cholangiocarcinoma, extrahepatic cholangiocarcinoma, gallbladder cancer, and ampulla of Vater cancer.
2. Recurrent or unresectable biliary tract cancer.
3. Histologically proven papillary adenocarcinoma, tubular adenocarcinoma, or adenosquamous carcinoma for patients with extrahepatic cholangiocarcinoma, gallbladder cancer, and ampulla of Vater cancer; histologically proven adenocarcinoma for patients with intrahepatic cholangiocarcinoma.
4. Without central nervous system metastasis.
5. Without moderate or greater ascites/pleural effusion.
6. No previous therapy for biliary tract cancer.
7. No previous operation, chemotherapy, or radiotherapy for any other malignancies within the past 5 years.
8. No previous chemotherapy containing gemcitabine or cisplatin for any other malignancies.
9. An Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1.
10. Sufficient oral intake.
11. Age of 20–80 years.
12. Adequate organ functions.
13. HLA of A2402, A0201, or A0206.
14. Written informed consent.

Exclusion criteria

Patients will be excluded if they meet any of the following criteria:

1. Simultaneous or metachronous (within the past 5 years) double cancers, with the exception of intramucosal tumors curable with local therapy.
2. Pregnant or lactating women or women of childbearing potential and men who wish to father children.
3. Psychosis.
4. Patients requiring systemic steroid medication.
5. Interstitial pneumonia or fibroid lung disease.
6. Active bacterial or fungous infection.
7. Severe complications.
8. Drug allergies to drugs containing iodine compounds and/or gadolinium.
9. Inadequate physical condition, as diagnosed by the primary physician.

Randomization in the phase II study

After the fulfillment of the eligibility criteria has been confirmed, patient registration for both the phase I and II studies will be made by faxing the Data Center. Eligible patients in the phase II study will be stratified according to HLA (A2402/A02XX) and then randomized at the Data Center at a 2:1 ratio, using a minimization method and balancing the study arms according to institution, primary tumor (gallbladder cancer/other than gallbladder cancer), and history of surgical resection for the primary tumor (recurrent/advanced) to receive GC either with or without the WT1 peptide vaccine.

Treatment methods

For the patients in the phase I study, the GC and WT1 vaccine will be administered according to the following schedule: cisplatin (25 mg per m^2 of body-surface area) followed by gemcitabine (1000 mg per m^2) administered intravenously on days 1 and 8 every 3 weeks, with the vaccine (3 mg per body) injected subcutaneously alternating between 2 areas on the unilateral axillary fossa and inguen on days 1 and 8.

For both arms in the phase II study, GC will be administered according to the same dose and schedule as those used in the phase I study, but the vaccine will be administered only for the GC plus WT1 peptide vaccine arm.

The protocol treatments will be continued until disease progression, unacceptable toxicity, or patient refusal, although cisplatin will be continued for only a maximum of 24 weeks.

Follow-up

Enhanced abdominal computed tomography (CT)/magnetic resonance imaging, chest CT/X-rays, and tumor marker levels (carcinoembryonic antigen [CEA] and carbohydrate antigen [CA] 19-9) will be evaluated at least every 6 weeks during the protocol treatment. Patients will be seen on days 1 and 8 of every cycle for a physical examination to monitor their symptoms and the possible toxic effects of treatment. Adverse events will be graded according to the Common Terminology Criteria for Adverse Events (CTCAE) version 4.0.

Study design and statistical analysis

In the phase I study, six patients will be recruited to determine whether a WT1 peptide vaccine dose of 3 mg per body can be recommended for use in combination with GC. A dose of 3 mg per body is the recommended dose for the WT1 peptide vaccine when used in combination with GEM alone, as determined in the previous phase I study. If treatment-related DLTs occur in no more than two of the six patients, transition to the phase II study will be

permissible with the approval of the independent data monitoring committee. If DLTs occur in three or more patients, transition to the phase II study will be terminated.

In the phase II study, 100 patients will be allocated to either of the two arms to evaluate the safety and efficacy of GC plus WT1 peptide vaccine, in comparison with GC alone. The sample size was determined based on the feasibility of the study after considering the research period, the number of participating institutions, and the available financial resources. A total of 66 patients in the GC plus WT1 peptide vaccine arm would enable the 1-year overall survival rate to be estimated with an accuracy of ±10%.

Interim analysis and monitoring

We do not plan to perform an interim analysis in this study. In-house monitoring will be performed every 6 months by the Data Center to evaluate the study progress and to improve the quality of the study.

Discussion

So far, no consensus exists regarding the "best criteria" for evaluating the effectiveness of cancer immunotherapy. Evidence of therapeutic activity may be difficult to obtain in early-phase trials using standard endpoints such as the antitumor response according to the Response Evaluation Criteria in Solid Tumors (RECIST), because most cancer immunotherapies are not expected to result in notable tumor shrinkage. Recently published FDA guidance suggests that the development of a cancer vaccine may present different considerations for clinical trial design than the development of a traditional cytotoxic drug or biological product for the treatment of cancer (http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/default.htm).

We retrieved clinical trials using immunotherapy for biliary tract cancer through PubMed (http://www.ncbi.nlm.nih.gov/pubmed) and ClinicalTrial.gov (http://clinicaltrials.gov/), although no reports or ongoing studies were found in this category, except for two trials: our previous phase I study examining GEM plus the WT1 peptide vaccine [8], and another study (phase II) examining chemoradioimmunotherapy, with interleukin 2 and 13-cis-retinoic acid being used for the immunotherapy [11]. Both studies conducted for pancreatic or biliary tract cancer showed some promise for a survival advantage, although the reported evidence was immature. We initiated the current phase I and randomized phase II studies to evaluate the efficacy and safety of adding the WT1 peptide vaccine to GC for the treatment of advanced biliary tract cancer. These studies are only the initial step in the development of immunotherapy for this disease, although we hope that the trial may provide useful data for assessing the true activities of this treatment.

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Conflict of interest None.

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臨床試験論文の読み方

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1. はじめに

臨床試験の論文を正しく読むためには，臨床試験の方法論，すなわち臨床試験の計画から結果の解釈までの一連の方法の体系について知る必要がある。本稿では，臨床試験，主にランダム化対照試験（RCT: randomized controlled trial）の標準的方法論について述べる。

2. 医学・医療と技術評価

一般に，医学は普遍性のある真実を追求する科学の一分野である一方，医療は多様性のある個人に対して最適な技術を選択して適用することが要求される場である。技術評価は，主に統計学に基づく科学的方法を駆使して医療技術を相対的に評価し，医学から医療への橋渡しを行う（図1）。

統計学を医学・医療の領域に導入する際には，2つの大きなギャップを認識しておく必要がある。1つは，「決定論」と「非決定論（確率論）」のギャップである。1800年代半ばにクロード・ベルナールが「統計学に立脚している限り，医学は永久に推測科学に止まるであろう」と決定論的な考え方を主張して以来，医学の世界では決定論的な思想が支配的である。もう1つのギャップは，意思決定の主体に関わる問題であり，「対集団の確率」と「対個人の確率」とのギャップである。たとえば，ある医薬品を承認すべきかどうかという判断は，その国の人々という集団に対するベネフィットとリスクのバランスで決定される。その決定は「対集団の確率」に基づく。一方，医療の場で診断や治療を行う際には，個人に対するベネフィットとリスクを評価しなければならない。たとえば，胎児診断を行って，医師が「胎児に異常がある確率は80%」と言ったとき，その80%は集団での頻度であり，この確率は確信度を量的に表現したものである。しかしながら，それを聞いた母親の「子供には異常があるか（100%），ないか（0%）のどちらか」という感覚では，この集団での確率を抵抗なく受け入れられない。このような確率に関する認識のギャップを認識しないで，道具としての統計学だけを医学・医療の領域に導入することは非常に危険である[1]。

臨床試験は，20世紀を代表する英国の統計学者R.A.フィッシャー（1890～1962）が創始した統計的実験（技術的実験とも呼ばれる）の方法論を基礎としている。科学的実験は，人工的に作り出された純粋な条件のもとでの因果関係を確定しようとするのに対して，統計的実験は以下の特徴を有する[2]。

- 実験の場は，現実の応用の場に近い状況に設定される
- 結果の分析には誤差の存在を前提にしなければならない
- いくつかの因子を同時に変化させて結果を見る必要があることがある
- 目的は，何らかの基準によって現実の場において最も良い結果が得られるような条件を求めることである

つまり，臨床試験のプロセス全体―計画を立て，データを収集し，検証・推測を行う―を保証するためには統計的な方法が不可欠である。特に，再現性によって結果を保証することが可能な基礎実験と異なり，同じデザインで繰り返すことが困難な臨床試験においては，プロセスの妥当性から結果を保証するしかない。

3. 臨床試験デザイン

臨床試験に携わる統計家（試験統計家）の役割は，「臨床試験に統計的原則が適切に適用されていることを，臨床試験に携わる他の専門家と共同して保証すること」で

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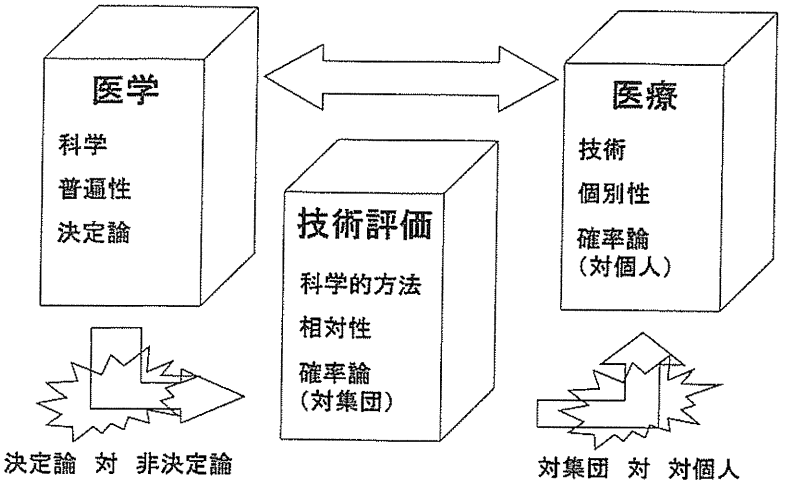


図1 医学・医療と技術評価

ある[3]。臨床試験の性格は検証的試験と探索的試験の2つに大きく分類される。検証的試験とは，事前に定めた仮説を検証するための試験，探索的試験とは検証的でない試験である。しかし実際には，いかなる試験も検証的な側面と探索的な側面の両方を持っており，ある技術を最初にヒトに適用するような試験は探索的な側面が強く，ランダム化対照試験は検証的な側面が強い。重要なことは，試験実施計画書（以下，プロトコル）の作成段階で，それらを区別しておくことである。プロトコルに予定された解析の結果のみが検証的であるとみなされる[3]。一般に，臨床試験を実施する企業や研究者は，得られたデータを様々な手法や様々な部分集団で解析したい誘惑に駆られる。しかしながら，事前に計画していなかった解析から得られた結果を強調しすぎると，消費者リスク（効果のない医療技術が使用されるリスク）—統計的には第Ⅰ種の過誤確率—が増大する。消費者リスクを保証することは，検定の有意水準をある小さな値（例えば，0.05）に設定することに対応している。

プロトコルの核となるのは，「背景と根拠」，「目的」，「対象（適格規準）」，「治療計画」，「エンドポイント（評価項目）」である。これらすべてが確定した段階で，試験統計家が中心となり「試験デザイン」の検討に入る。試験デザインとは，以下を含む。

- 対照の選択…無対照，用量対照，プラセボ対照など
- 統計的仮説…優越性仮説，非劣性仮説など
- 比較の様式…並行群間比較，クロスオーバー，用量漸増など
- ランダム化の有無
- 盲検化の有無
- 中間モニタリングの有無と方法
- 目標症例数の設定

なお，プロトコルに記載すべき項目とその作成要領については，京都大学医学部附属病院 探索医療センター検証部のHP（http://www.kutrc.org）あるいは先端医療振興財団 臨床研究情報センターのHP（http://www.tri-kobe.org）を参照されたい。

4. エンドポイント

エンドポイントとは，「試験の目的に関連する仮説を検証するうえで臨床的に意味があり，客観的に評価できる観察・検査項目またはそれらの合成指標」と定義される。例えば，癌の臨床試験の場合，全生存期間，無病生存期間，無増悪生存期間，腫瘍反応などが代表的なエンドポイントである[4]。全生存期間—ある時点からあらゆる原因による死亡までの時間—は，臨床的に最も適切と考えられているが，患者の長期追跡が必要であることや再発・増悪後の治療に影響を受けることなどの欠点を有する。また，QOL（quality of life）は患者の利益を直接的に表す指標であるが，測定の妥当性・信頼性の問題や複雑な統計解析手法が必要になるという短所を持つ（表1）。その臨床試験で評価すべき最も重要な1つのエンドポイントを主要エンドポイント，それ以外を副次エンドポイントと区別し，主要エンドポイントに対して次項で述べる統計的仮説が設定され，それに基づいて目標症例数が計算される。

臨床試験のエンドポイントを変数の型で分類すると，連続型（例：臨床検査値の推移），二値分類型（例：改善の有無，腫瘍反応の有無），順序分類型（例：改善度—かなり改善，改善，不変，悪化），時間—イベント型（例：全生存期間）の4つに大きく分けられる。エンドポイントの定義を事前にプロトコルに明記することは重要である。特に時間—イベント型の場合，その起点と終点，および打ち切り—ある時点までイベントを発生していない状態で観察を打ち切られること—を詳細に定義し

表1 各エンドポイントの長所と短所

エンドポイント	長 所	短 所
腫瘍縮小効果	標準化 多施設試験に適用可 早期の結果	測定精度 困難（中皮腫，腹膜疾患） 患者利益との相関
無増悪期間， 無増悪生存期間	救済療法の影響なし	評価・評価者バイアス 対照要 生存期間の代替性は部分的に評価
全生存期間	臨床的に適切	対照要 治療のクロスオーバー，後治療による影響 長期追跡要
QOL	直接的な患者利益の指標	多重比較の問題 時間集約的な評価 複雑な解析
分子バイオマーカー	予測的であれば患者選択に有用かも 耐性メカニズムに洞察が得られるかも	開発早期には有効性の代替性は未検証
イメージング	効果の早期評価可	反応評価への有用性小 費用と時間の問題 多施設試験の場合の標準化

Dhani N et al. Clin Cancer Res 2009; 15: 1873-1882 より引用

ておく必要がある。通常，生存曲線はカプラン・マイヤー（Kaplan-Meier）の方法で推定される。カプラン・マイヤー曲線を解釈する際の留意点としては，

・打ち切りの発生がイベントの発生と独立という仮定が必要
・X年生存率の推定値には誤差が含まれている
・リスク集合が小さくなると，信頼区間幅は大きくなる

が挙げられる。特に，追跡不能による打ち切りはイベント（例えば，死亡）の発生と独立ではない可能性があり，推定値に大きく影響を与えるので，追跡不能の割合が10%を超えるような臨床試験は一般に質が低い信頼できない臨床試験と評価される。

ある状況では，全生存期間のような臨床イベントに基づくエンドポイント（真のエンドポイント）の代わりとなる評価が簡便なエンドポイント（代替エンドポイント）の利用が必要になる。代替エンドポイントに関する解説およびその統計的評価法については，最近の総説を参照されたい[5]。

5. 統計的仮説

統計的仮説の代表的なものは，優越性仮説と非劣性仮説である。優越性仮説を証明しようとする試験（優越性試験）とは，試験治療の効果が対照治療（活性対照またはプラセボ対照）よりも「臨床的に優れること」を示すことが目的の試験である。一方，非劣性仮説を証明しようとする試験（非劣性試験）とは，試験治療の効果が対照治療よりも「臨床的に劣らないこと」を示すことが目的の試験である。多くの場合は優越性仮説が設定されるが，対照治療（通常，活性対照）に比べて安全性あるいは経済性に優れていることが見込まれる場合に，このような非劣性仮説が許容されることがある。

非劣性試験を計画する際には，非劣性マージン（臨床的に意味のある最小の差：Δ）の決定，データの質などについて十分な注意が必要である。例えば，ハザード比を治療効果の尺度とした臨床試験の場合，優越性試験では，ハザード比の95%信頼区間の上限が1より小さければ，有意水準5%で試験治療が優れると判断される。一方，非劣性試験では，その95%信頼区間の上限が

1+Δ よりも小さければ，有意水準 5%で試験治療は対照治療に Δ 以上は劣らない，と判断される（図 2）。

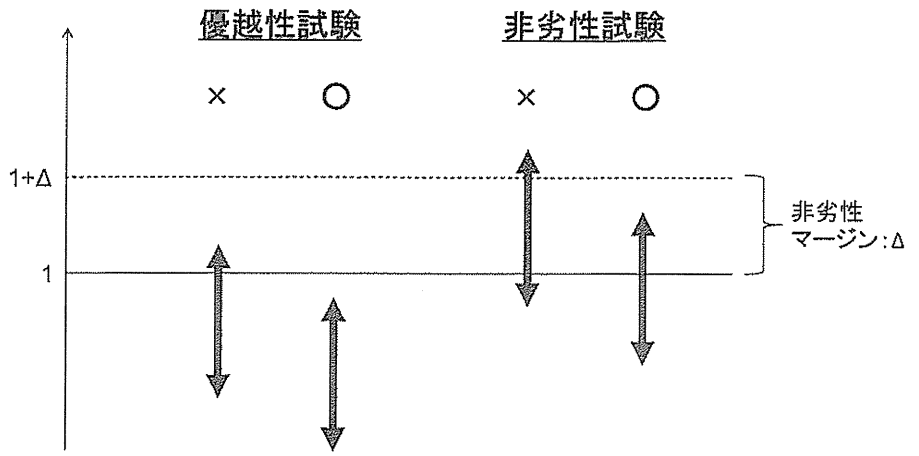
6. ランダム化

臨床試験に被験者を登録する際に重要なことは，被験者の適格性をできるだけ早く正しく確認することである。その作業を客観的かつ公正に行うためには，実施医療機関から独立した登録センターを設置した方がよい。連絡手段としては，以前は電話あるいは Fax 通信が主流であったが，最近では電子的データ収集（EDC: electronic data capturing）システムの利用が主流になりつつある。

ランダム化対照試験の場合には，登録の際にランダム化という操作が必要になる（図 3）。ランダム化を行うことにより，試験群と対照群の比較可能性（内的妥当性とも呼ばれる）が保証される。ランダム化は「実験計画法」を確立した英国の遺伝学者・統計学者 R.A.フィッシャーの偉大な発明の一つである。実験に伴う誤差には以下の 2 種類がある。ちなみに，フィッシャーは臨床試験ではなく，農事実験を行っていた。

- 偶然誤差…測定誤差のようにある確率分布に従うと想定できる誤差であり，繰り返し測定を行えばその大きさについて推定可能な誤差
- 系統誤差…圃場の肥沃度や日当たりの不均一性のように偶然による変動とは見なせない誤差であり，繰り返しには関係なく結果を歪める原因となる誤差

ランダム化の目的は，一言で言うと「系統誤差を偶然誤差に転化すること」である。その意義は，



矢印は、ハザード比（試験薬のハザード率／対照薬のハザード率）の95%信頼区間

図 2　優越性試験と非劣性試験の判断規準

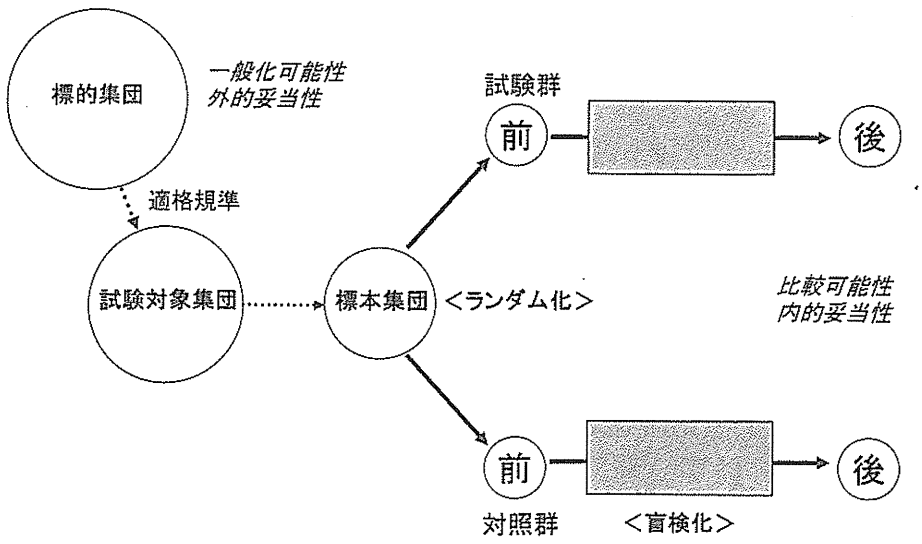


図 3　ランダム化対照試験

・予後因子が既知か未知かにかかわらず，予後因子の分布が類似したグループを作る
・データ解析の際に治療効果の定量的な評価のための正しい統計的根拠を与える

ことである[3]。

臨床試験におけるランダム化の方法は，次の被験者への割付が予見できない程度に複雑にする必要があるが，複雑にしすぎると手続きに誤りが発生しやすくなる。また，予後に影響する因子（予後因子）の分布を治療群間で均等にするためには，それらの因子で層を作り（層別化），その層ごとにランダム化を行う必要がある。臨床試験で利用される代表的なランダム化の手法は，固定型割付―層別置換ブロックランダム化など―と適応型割付／動的割付―最小化法など―に分類される[6]。ランダム化の方法には多くの選択肢があり，試験統計家がそれぞれの利点と欠点を考慮して決定するのが通常である。

7. 統計学的考察

試験統計家がプロトコルに記載すべき統計学的事項として，「目標症例数の設定根拠」，「解析対象集団」，「解析項目・方法」，「中間モニタリング」がある。

1）目標症例数の設定根拠

一般には「標本サイズの設定」と呼ばれる，実験において統計学的に妥当な標本の大きさを決める技法は，臨床試験においては「目標症例数の設定」と呼ばれている。臨床試験の症例数は科学性，倫理性，実施可能性のバランスを考慮して決める必要があり，多すぎても少なすぎてもいけない。

目標症例数は，主要エンドポイントに関する情報に基づいて計算される。その時点における情報を最大限利用するものの，一時的な仮定に基づく概算であることに注意が必要である。例えば，ある癌の補助化学療法の臨床試験において，標準治療を受ける患者の5年生存率を推定することだけを考えても，利用できる情報にはかなり大きなばらつきがある。これらの前提条件を慎重に検討した上で，通常は仮説検定という方法に基づき，帰無仮説，対立仮説，検定統計量，有意水準，第Ⅰ種の過誤，第Ⅱ種の過誤または検出力が臨床的および統計的な観点から設定され，さらに試験の性格―探索的または検証的―を考慮して目標症例数が決定される。

2）解析対象集団

解析対象集団は，ITT (intention-to-treat) の原則に従って定義すべきである。これは，「被験者が実際に受けた治療ではなく，被験者を治療しようとした意図（intention to treat）に基づいて評価する」という原則である[3]。従って，登録されたすべての被験者を解析対象とすることが原則であるが，登録後に判明した不適格例，試験治療を全く受けなかった例を対象から除くことは一般に許容される。いずれにしても，有効性あるいは安全性に関する主要な解析対象集団の定義をプロトコルに明記し，報告時には解析対象から除外した例数とその理由を明記する必要がある。

検討したい部分集団（subgroup または subset）をプロトコルに明記しておくことも重要である。部分集団解析の問題は，

・多数の検定を実施することによる，第Ⅰ種の過誤確率の上昇
・各部分集団の大きさが小さくなることによる，第Ⅱ種の過誤確率の上昇

の2つが複雑に影響して解釈が困難になることである。1つの解決策として，交互作用がない―各部分集団において治療効果が一様である―という仮説の検定を行うことにより第Ⅰ種の過誤確率を一定以下に保つことができる。

表2　変数の型別の標準的な統計解析手法

目的	連続変数	分類変数	時間－イベント変数
分布の記述	ヒストグラム，箱ヒゲ図，散布図	ヒストグラム，分割表	生存曲線（Kaplan-Meier 法）
要約統計量	平均，分散，中央値，パーセント点，相関係数	頻度，一致度，相関係数	x 年生存割合，中央生存期間
検定（単純）	t 検定，分散分析，Wilcoxon 検定	χ^2 検定，Fisher 正確検定	logrank 検定
検定（層別）	分散分析	Mantel-Haenszel 検定	層別 logrank 検定
回帰モデル	分散分析，重回帰分析	logistic 回帰分析	Cox 回帰分析

3）解析項目・方法

臨床試験で利用される標準的な統計解析手法について表2に示す。ランダム化対照試験では，ランダム化によって比較可能性が保証されているので，観察研究のように複雑な回帰モデルを用いて交絡因子を調整する必要はない。

4）中間モニタリング

中間モニタリング（中間解析，中間評価とも呼ばれる）の目的は，

・試験治療の優越性が疑いなく立証された場合
・適切な試験治療の差を示す見込みがないことが判明した場合
・許容できない有害事象が明らかになった場合

に試験を早期に中止することである[3]。中止規則の設定には多くの統計的方法が開発されてきているが，実際に臨床試験を中止すべきかどうかという判断は純粋に統計的な問題ではなく，臨床的のみならず社会的な影響も考慮する必要がある。そのような判断を公正に行う場として，中間モニタリングを実施する際には，当該臨床試験に関与しない第三者からなる独立データモニタリング委員会（効果安全性評価委員会とも呼ばれる）を設置しなければならない。

8. おわりに

1938年，R.A.フィッシャーは次のように述べている；「同じだけの時間と労力をかけたとしてもデータ収集の過程，または実験計画を厳密に検討しているか否かによって，得られる収穫は10倍から12倍にもなる。実験終了後に統計学者に相談を持ちかけるのは，統計学者に，単に死後診察を行って下さいと頼むようなものである。統計学者はおそらく何が原因で実験が失敗したかという実験の死因について意見を述べてくれるだけであろう」。

何度も同じ実験を繰り返すことが可能な基礎実験と異なり，臨床試験を同一のデザインで繰り返すことは許されない。また，一定の証拠を得ることのできないようなデザインに基づく臨床試験は非科学的かつ非倫理的である。臨床試験の質はデザインで決まるということが本稿から少しでも伝われば幸いである。

著者のCOI（conflicts of interest）開示：本論文発表内容に関連して特に申告なし

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Runx3 Is Required for Full Activation of Regulatory T Cells To Prevent Colitis-Associated Tumor Formation

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Runx3 Is Required for Full Activation of Regulatory T Cells To Prevent Colitis-Associated Tumor Formation

Manabu Sugai,*,[1] Koji Aoki,†,[1] Motomi Osato,‡,§,¶,‖ Yukiko Nambu,*,# Kosei Ito,** Makoto M. Taketo,† and Akira Shimizu*

Inflammation is increasingly recognized as an essential component of tumorigenesis, which is promoted and suppressed by various T cell subsets acting in different ways. It was shown previously in *Runx3*-deficient mice that differentiation of CD8 T and NK cells is perturbed. In this study, we show that *Runx3* is also required for proper differentiation and function of regulatory T cells. In *Runx3*-deficient mice, T cells were unable to inhibit inflammation and to suppress tumor development. As expected, recombination activating gene 2-deficient mice bearing *Runx3*-deficient lymphocytes spontaneously developed colon tumors. However, tumor formation was completely blocked by transfer of either regulatory T cells or CD8 T cells derived from wild-type mice to mutant mice or by housing mutant mice in a specific pathogen-free condition. These results indicate that *Runx3*-deficient lymphocytes and microorganisms act together to induce inflammation and consequently induce the development of colon tumors. *The Journal of Immunology*, 2011, 186: 6515–6520.

Colitis-associated cancer is the most serious complication of inflammatory bowel disease (IBD) (1). Accumulating evidence indicates that immune responses have positive and negative roles in tumor formation (2) and maintenance; chronic inflammatory disease increases the risk of cancer development (3–5), whereas the suppression of immune responses against tumors by enhancing regulatory T cell (Treg) activity allows tumor cells to survive (6). Thus, to maintain a tumor-free status, it is important for the host to respond appropriately during the course of inflammation and tumor formation. TGF-β1 is essential for the maintenance of inflammatory homeostasis, and the loss of the TGF-β1 signaling pathway results in severe inflammation and malignant tumor formation (7), especially in adenomatous polyposis coli (*Apc*) mutant mice (8). T cells are known to be among the targets of the TGF-β1 signaling pathway because T cell-specific deletion of Smad4 results in spontaneous gastrointestinal cancer (9). It has been demonstrated that various CD4 T cell subsets differ in their ability to inhibit or enhance IBD and that these T cell subsets act cooperatively in immune surveillance against tumors. The functional balance of various T cell subsets therefore plays a central role in maintaining the integrity of the epithelial barrier and inhibiting tumor formation in the gastrointestinal tract.

Acting downstream of TGF-β1 signaling, Runx proteins are the interacting and functional partners of R-Smad proteins (10–13). The loss of Runx proteins in lymphocytes can therefore affect the severity of inflammation in the gastrointestinal tract and the incidence of tumor formation. Among the three Runx proteins, Runx3 is involved in the differentiation of immune cells, including CD8 and NK cells (14–17), both of which have cytotoxic activity against tumors. Furthermore, *Runx3*-deficient mice have defects in a subset of dendritic cell (18) and B cell functions (19). Thus, $Runx3^{-/-}$ mice are severely compromised immunologically. The functions of Runx3 are not limited to lymphoid tissues, and this protein is also involved in the regulation of epithelial homeostasis, acting within the epithelium of the gastrointestinal tract. Ito and colleagues (20) have demonstrated that $Runx3^{-/-}$ gastric epithelial cells are resistant to the growth-inhibitory and apoptosis-inducing action of TGF-β1, resulting in hyperplasia of the gastric mucosa. In addition, Runx3 was shown to attenuate β-catenin/T cell factor functions in intestinal tumorigenesis (21). These data indicate that Runx3 is a tumor suppressor acting in the gastrointestinal epithelium. This notion is strongly supported by the analysis of human tumors. Contrary to these results, Groner and colleagues (22) have demonstrated that $Runx3^{-/-}$ mice develop spontaneous IBD and gastric hyperplasia. Because Runx3 was expressed at a high level in lymphoid and myeloid cells, these authors concluded that the colitis and gastric lesions in $Runx3^{-/-}$ mice result from the loss of Runx3 in leukocytes (22). However, both groups performed the experiments in mice that were deficient in Runx3 in all tissues, including epithelium and lymphocytes. Therefore, their conclusion needs further investigation. To determine whether hyperplastic changes in the intestines in $Runx3^{-/-}$ mice are caused by a defect of epithelial cells or blood cells, Ito et al. (21) generated mice whose leukocytes, but not epithelial cells, were $Runx3^{-/-}$ by transplanting bone marrow cells from $Runx3^{-/-}$ mice into irradiated wild-type (WT) mice. These mice showed no symptoms of hyperplasia or dysplasia in the intestines 1 y after

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Abbreviations used in this article: FLC, fetal liver cell; FLT, fetal liver cell transfer; IBD, inflammatory bowel disease; iTreg, inducible regulatory T cell; nTreg, natural regulatory T cell; SPF, specific pathogen-free; Teff, effector T; Treg, regulatory T cell; WT, wild-type.

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transplantation (21). This observation supports the direct involvement of epithelial cells in tumor formation in *Runx3*$^{-/-}$ mice. However, we cannot rule out the involvement of WT-derived radio-resistant lymphocytes in maintaining the health of intestinal epithelial cells. To exclude this possibility, we used recombination activating gene 2 knockout (*Rag2*$^{-/-}$) mice as recipients because Rag2 is a essential factor for lymphocyte generation.

In this article, we examined whether the loss of functioning Runx3 in lymphocytes contributes to these phenotypes. We found that loss of Runx3 in T cells resulted in suppression of Treg function and that this suppression was the primary cause of colitis but not gastritis observed in *Runx3*$^{-/-}$ mice. In addition, we assessed tumor formation in the colon. All mice bearing *Runx3*$^{-/-}$ lymphocytes, but not WT mice, developed tumors in the large intestine or cecum when they were housed in a conventional mouse facility. However, tumor formation was completely blocked by housing them in a specific pathogen-free (SPF) condition, indicating that microorganisms are involved in this process. Furthermore, no tumor formation was observed when CD8 T cells or Tregs of WT origin were transferred into mutant mice. These results, in addition to previous observations, suggest that Runx3 is a suppressor of gastrointestinal tract tumors acting in lymphocytes and epithelial cells.

Materials and Methods

Mice

WT, *Runx3*$^{+/-}$, and *Rag2*$^{-/-}$mice with the C57BL/6 genetic background were maintained in an SPF mouse facility. For some experiments, mice that received transfers of fetal liver cells (FLCs) were maintained in a conventional mouse facility. Procedures involving animals and their care were conducted according to the guidelines for animal treatment of the Institute of Laboratory Animals, Kyoto University.

Fetal liver transfer

Single-cell suspensions of 2×10^6 to 4×10^6 whole fetal liver mononuclear cells harvested from *Runx3*$^{-/-}$and WT embryos at E14.5 were injected intravenously into sublethally irradiated (4 Gy) male *Rag2*$^{-/-}$ recipient mice. Mice were sacrificed at least 10 wk after transplantation, and cell compartments were analyzed by flow cytometry or used for in vitro culture. All *Rag2*$^{-/-}$mice, which were used for a series of experiments comparing the effects of Runx3, were age-matched, and FLCs from littermates were used.

Histologic analysis

The colon and cecum were removed from mice after euthanasia and dissected free from the anus to a point distal to the cecum and the small intestine. Contents were removed and cleaned with PBS prior to fixation in 4% paraformaldehyde and routine paraffin embedding. Sections were then cut and stained with H&E.

Cell preparation

Naive CD4 T, CD8 T, and Tregs were prepared by magnetic cell sorting using appropriate isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany). For some experiments, a CD62L$^+$CD44$^-$CD25$^-$CD4$^+$ population was isolated to provide naive CD4 T cells by flow cytometry with a FACSAria (Becton Dickinson, Mountain View, CA).

Cell cultures

In all experiments, the percentages of CD62L$^+$CD4$^+$ T cells or CD4$^+$CD25$^+$ T cells were >95%. Naive CD4$^+$ T cells were activated with plate-bound anti-CD3 (5 μg/ml), soluble anti-CD28 (1 μg/ml), anti–IFN-γ (10 μg/ml), and anti–IL-4 (10 μg/ml) in the presence or absence of TGF-β1 (3 ng/ml).

Flow cytometric analysis

The following Abs were used for staining: FITC anti-mouse Foxp3 (eBioscience, San Diego, CA); biotin anti-mouse CD25 (BD Pharmingen, San Diego, CA); allophycocyanin anti-mouse CD4 (BD Pharmingen); PE anti-mouse CD8 (BD Pharmingen); and PerCP–streptavidin (Molecular Probes, Eugene, OR). All analyses were performed with FACSCalibur or FACSAria (Becton Dickinson).

Retroviral infection

Retrovirus was produced by transfecting the ecotropic Plat-E packaging cell line (23) with pMSCV-IRES-hCD4 retroviral vector (24). After stimulation with plate-bound anti-CD3 and soluble anti-CD28 for 24 h, purified naive CD4 T cells were spin-infected with the virus-containing supernatant in the presence of 4 mg/ml polybrene for 1.5 h at 2500 rpm and 32°C and cultured for 48 h.

After removing virus-containing supernatants, cells were reactivated with plate-bound anti-CD3 (5 μg/ml), soluble anti-CD28 (1 μg/ml), anti–IFN-γ (10 μg/ml), and anti–IL-4 (10 μg/ml) in the presence or absence of TGF-β1 (3 ng/ml).

RT-PCR and real-time PCR

Total RNAs were extracted from cultured T cells or sorted cultured T cells using TRIzol (Life Technologies-BRL, Gaithersburg, MD). Oligonucleotide-primed cDNAs were prepared with reverse transcriptase. For semiquantitation, 50 ng cDNA was serially diluted and subjected to PCR amplification. All PCR products were resolved electrophoretically in 2% agarose gel and visualized by ethidium bromide staining. For real-time PCR, RT-PCR was performed using 2× SYBER PCR Master Mix (Qiagen). Specific primer pairs used for real-time PCR were as follows: Runx3 forward (5′-ACCACGAGCCACTTCAGCAG-3′) and Runx3 reverse (5′-CGATGGTGTGGCGCTGTA-3′); Foxp3 forward (5′-GCATGTTCGCCTACTTCAGAAA-3′) and Foxp3 reverse (5′-CCACTCGCACAAAGCACTTG-3′); granzyme B forward (5′-CTCCACGTGCTTTCACCAAA-3′) and granzyme B reverse (5′-AGGATCCATGTTGCTTCTGTAGTTAG-3′); Hprt forward (5′-GTTGGATACAGGCCAGACTTTGTTG-3′) and Hprt reverse (5′-GATTCAACTTGCGCTCATCTTAGGC-3′). Relative expression of mRNA was normalized to Hprt mRNA levels within each sample.

Results

Runx3 *deficiency in lymphocytes leads to the development of spontaneous colitis but not gastritis*

To determine whether Runx3 expression in lymphocytes affects the homeostasis of gastrointestinal epithelial cells, FLCs from

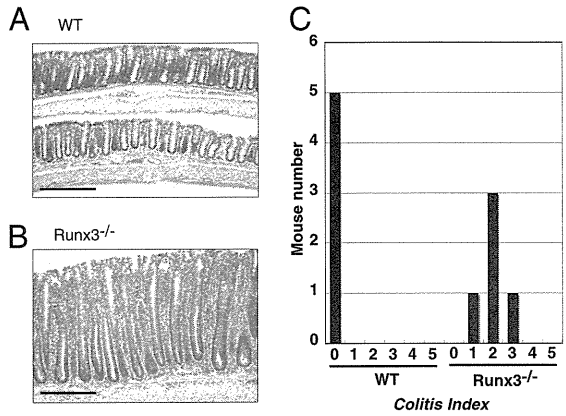

FIGURE 1. Representative photographs of the colon of *Rag2*$^{-/-}$ mice 10 wk after transfer of FLCs from WT and *Runx3*$^{-/-}$ mice. Samples taken from Lymph-*Runx3*$^{-/-}$ or Lymph-WT mice were fixed, sectioned, and stained. Specimens were stained with H&E. Scale bars, 400 μm. *A*, Normal appearance of the colon in a mouse that received WT FLCs. *B*, Moderate colitis in a mouse that received Runx3$^{-/-}$ FLC. *C*, Colitis grades are shown as the colitis index. Colitis was scored from 0 to 5 in a blinded fashion. Histologic features were graded as follows: 0, no inflammation; 1, minimal scattered mucosal inflammatory cell infiltrates, with or without minimal epithelial hyperplasia; 2, mild scattered to diffuse inflammatory cell infiltrates, sometimes extending into the submucosa and associated with erosions, with minimal to mild epithelial hyperplasia and minimal to mild mucin depletion from goblet cells; 3, mild to moderate inflammatory cell infiltrates, often associated with ulceration, with moderate epithelial hyperplasia and mucin depletion; 4, marked inflammatory cell infiltrates, often associated with ulceration, with marked epithelial hyperplasia and mucin depletion; 5, marked transmural inflammation with severe ulceration and loss of intestinal glands. Runx3$^{-/-}$, Lymph-Runx3$^{-/-}$ mice; WT, Lymph-WT mice.

Runx3$^{-/-}$embryos and control littermates were transferred into irradiated *Rag2*$^{-/-}$ mice, and inflammatory status was assessed 10 wk after FLC transplantation ("Lymph-*Runx3*$^{-/-}$" or "Lymph-WT" mice; Runx3$^{-/-}$ and WT indicate the genotypes of transferred FLCs). As shown in Fig. 1*A*, 1*B*, Lymph-*Runx3*$^{-/-}$ mice exhibited spontaneous colitis, but the colitis was not as severe as previously described (22) (Fig. 1*C*). To assess whether the severity of colitis depended on the time course of the disease, we examined the colitis index (25) over a more extended period. The colitis index did not change during the course of our observations from 10 to 65 wk after fetal liver cell transfer (FLT) (Fig. 1*C* and data not shown). These results indicate that Runx3 deficiency in lymphocytes is not sufficient to induce severe colitis, as previously demonstrated (22), at least in this experimental setting. In addition, no Lymph-Runx3$^{-/-}$ mice had gastric hyperplasia. Accordingly, functions of Runx3 in other cell types, but not in lymphocytes, also contribute to the inflammatory status in the gastrointestinal tract.

Runx3 is required for proper Treg function

Because Tregs are important in preventing an excessive inflammatory reaction in the gastrointestinal tract, we examined Foxp3-expressing natural Tregs (nTregs) in the thymus, spleen, and lymph nodes of Lymph-*Runx3*$^{-/-}$ mice. The percentages and numbers of Foxp3$^+$ cells were essentially the same in the different genotypes (Fig. 2*A* and data not shown), indicating that nTreg

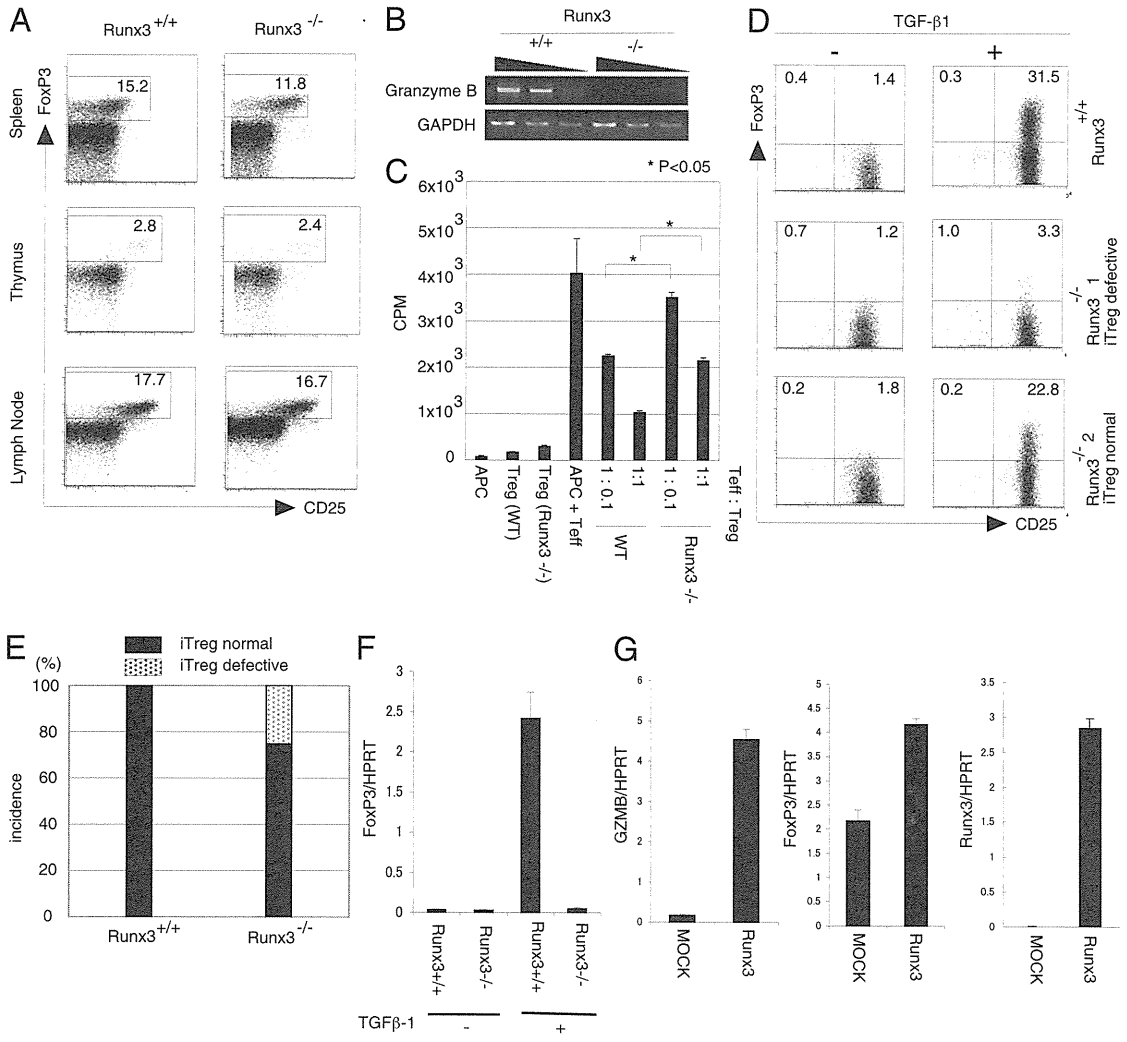

FIGURE 2. Runx3 is required for Treg function. *A*, FACS analysis of spleen cells, thymocytes, and lymph node cells from Lymph-*Runx3*$^{-/-}$ (*Runx3*$^{-/-}$) and Lymph-WT (*Runx3*$^{+/+}$) mice. All plots are gated on CD4$^+$CD8$^-$ cells. Numbers indicate the percentages of Foxp3$^+$ cells. Data are representative of six independent experiments. *B*, CD4$^+$CD25$^+$ cells were cultured with anti-CD3 and IL-2 for 48 h. RNA was prepared, and expression of granzyme B was determined by RT-PCR. GAPDH was used as an internal control for RT-PCR. Data are representative of three independent experiments. Fivefold serial dilutions of cDNAs were amplified for the indicated transcripts. *C*, Suppression assays. CD4$^+$CD25$^-$ responder T cells (2×10^4 cells) were placed in 96-well round-bottom plates with CD4$^+$CD25$^+$ cells and APCs (2×10^4 cells, T cell-depleted spleen cell populations irradiated with 2000 rad) at 37°C in 7% CO_2 and were stimulated for 72 h with mAb to CD3 (1 μg/ml). After 72 h of incubation, cultures were pulsed with [^{3}H]thymidine (1 mCi) 6 h before collection. Teff cells from WT mice were cultured with APC plus anti-CD3 with the indicated ratios of Tregs derived from Lymph-*Runx3*$^{-/-}$ or Lymph-WT mice. The *p* values were calculated by Student *t* test. $*p < 0.05$. *D*, Foxp3 staining of CD4$^+$ T cells after culture with the indicated agents. Numbers shown in the *upper-right corners* indicate the percentages of CD4$^+$Foxp3$^+$ cells. Data are representative of 12 independent experiments. *E*, In each experiment shown in *D*, cells were classified into competent and defective groups with respect to iTreg differentiation. "iTreg normal" (black bar), iTreg competent group in which >20% cells expressed Foxp3; "iTreg defective" (dotted bar), <5% cells expressed Foxp3. *F*, Expression of Foxp3 was determined by real-time PCR using RNA from cultures shown in *D* (*Runx3*$^{+/+}$ and *Runx3*$^{-/-}$-1, iTreg defective). Hprt was used for normalization. *G*, Real-time PCR analysis of granzyme B (GZMB) and Foxp3 expression using cDNAs from retroviral expression of Runx3 in *Runx3*$^{-/-}$ T cells. Hprt was used for normalization. *Runx3*$^{+/+}$, Lymph-WT mice or cells derived from these mice; *Runx3*$^{-/-}$, Lymph-*Runx3*$^{-/-}$ mice or cells derived from these mice.

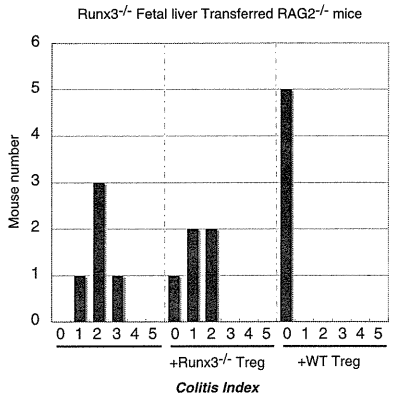

FIGURE 3. Colitis in Lymph-*Runx3*$^{-/-}$ mice is rescued by cotransfer of WT Tregs. Colitis indexes of Lymph-*Runx3*$^{-/-}$ mice were examined 10 wk after transfer of FLT with or without cotransfer of WT or *Runx3*$^{-/-}$ Tregs. Colitis index criteria are described in the legend of Fig. 1.

differentiation in vivo is normal in Lymph-*Runx3*$^{-/-}$ mice. We then examined the expression of essential factors required for Treg function and found that expression of granzyme B (26) was specifically inhibited by the loss of Runx3 (Fig. 2*B*). Because granzyme B and Foxp3 are required for proper nTreg functions, nTregs from Lymph-*Runx3*$^{-/-}$ mice were assessed for suppressive activity in vitro. As shown in Fig. 2*C*, nTregs from Lymph-WT mice

A

FL Genotype	Co-transferred cells	Facility	Tumor	Incidence (%)	
Wild type		SPF	0 / 6	0	
Runx3-/-		SPF	0 / 6	0	
Wild type		Conventional	0 / 6	0	*
Runx3-/-		Conventional	6 / 6	100	*
Runx3-/-	+/+ Regulatory T cells	Conventional	0 / 6	0	*
Runx3-/-	+/+ CD8$^+$T cells	Conventional	0 / 6	0	

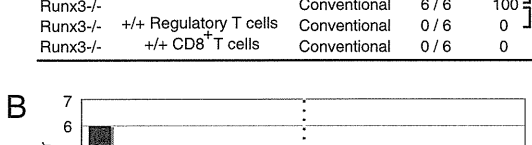

FIGURE 4. Microorganisms increase the severity of colitis and result in tumor formation in Lymph-*Runx3*$^{-/-}$ mice. *A* and *B*, Tumor incidence and the colitis index were examined around 65 wk after FLT transfer. *A*, Tumor incidence. Tumor incidences are shown as X/Y, where X is the number of tumor-bearing mice and Y is the number of mice examined. Significance of differences between groups were estimated by Fisher's exact test with Bonferroni correction. *$p = 0.0022$. *B*, Colitis index after 65 wk of housing in a conventional mouse facility. Colitis index criteria are described in the legend of Fig. 1. The SPF facility was free from the following pathogens: 1, *Citrobacter rodentium*; 2, *Corynebacterium kutscheri*; 3, *Mycoplasma pulmonia*; 4, *Pasteurella pneumotropica*; 5, *Salmonella* spp.; 6, *Pseudomonas aeruginosa*; 7, *Clostridium piliforme*; 8, ectromelia virus; 9, LCM virus; 10, mouse hepatitis virus; 11, Sendai virus; 12, ectoparasites; 13, intestinal protozoa; 14, pinworm; 15, *Pneumocystis carinii*; 16, *Helicobacter hepaticus*; 17, *Helicobacter bilis*. The conventional facility was free from the following pathogens: 1, *Citrobacter rodentium*; 2, *Corynebacterium kutscheri*; 3, *Mycoplasma pulmonia*; 4, *Pasteurella pneumotropica*; 5, *Salmonella* spp.; 6, *Pseudomonas aeruginosa*; 7, *Clostridium piliforme*; 8, mouse hepatitis virus; 9, Sendai virus; 10, intestinal protozoa; 11, dermatophytes; 12, *Staphylococcus aureus*. Co-transferred cells, cells cotransferred with FLCs; FL Genotype, genotypes of the FLCs transferred into *Rag2*$^{-/-}$ mice; *Runx3*$^{-/-}$, Lymph-*Runx3*$^{-/-}$ mice; Wild type, Lymph-WT mice.

at a 1:1 ratio of effector T (Teff) cells to nTregs suppressed the proliferation of Teff cells by >75%, whereas Tregs from Lymph-*Runx3*$^{-/-}$ mice suppressed Teff cell proliferation by <50%. The reduced suppressive activity of nTregs from Lymph-*Runx3*$^{-/-}$ mice was also observed at other nTreg/Teff cell ratios. Thus, nTreg function is perturbed in Lymph-*Runx3*$^{-/-}$ mice. The suppression of nTreg function may be a mechanism that contributes to the colitis observed in Lymph-*Runx3*$^{-/-}$ mice. We next examined the potential role of Runx3 in inducible regulatory T cell (iTreg) generation by adding TGF-β1 in vitro. The involvement of Runx3 in iTreg differentiation is limited, because in 75% of the experiments, *Runx3*$^{-/-}$ naive CD4 T cells differentiated to iTregs, which is similar to the result obtained for WT cells (*upper* and *lower rows* of Fig. 2*D*, 2*E*). However, in only 25% of the experiments were *Runx3*$^{-/-}$ T cells defective in differentiation to iTregs, as shown in Fig. 2*D* (*middle row*) and Fig. 2*E*. In this case, fewer Foxp3$^+$ cells caused transcriptional inhibition of the Foxp3 gene in *Runx3*-deficient T cells (Fig. 2*F*). The function of Runx3 in Foxp3 transcription was further confirmed by forced expression of Runx3 in *Runx3*$^{-/-}$ CD4 T cells (27–30). Thus, Runx3 proteins stimulate the expression of Foxp3 and granzyme B (Fig. 2*G*). At this time, it is unclear why iTreg differentiation fluctuated in different samples of *Runx3*-deficient cells (*middle* and *lower columns* in Fig. 2*D*, 2*E*). Further experiments will be required to elucidate this important issue.

To determine further the involvement of Tregs in the observed colitis in Lymph-*Runx3*$^{-/-}$ mice, we transferred nTregs together with *Runx3*$^{-/-}$ FLCs into irradiated *Rag2*$^{-/-}$ mice. As shown in Fig. 3, the severity of colitis was assessed 10 wk after FLT transfer. nTregs from Lymph-WT mice completely inhibited colitis; the colitis index was 0 in all mice. In contrast, slight improvements in the colitis index were observed when nTregs from Lymph-*Runx3*$^{-/-}$ mice were used. Taking these results together with the in vitro data, *Runx3*$^{-/-}$ nTregs appear to be

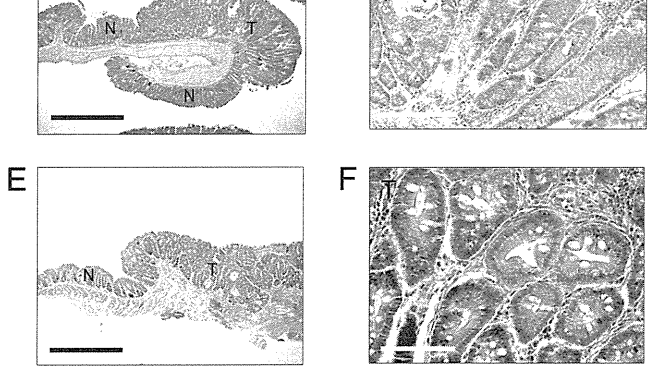

FIGURE 5. Representative tumors of Lymph-Runx3$^{-/-}$ mice in large intestine (*A–D*) and cecum (*E*, *F*). Enlarged pictures of tumor regions are shown at right (*B*, *D*, *F*). Specimens were stained with H&E. Black scale bars, 1 mm (*A*, *C*, *E*); white scale bars, 100 μm (*B*, *D*, *F*). N, normal regions; T, tumors.

functionally defective, which may be a cause of colitis induced in Lymph-*Runx3*$^{-/-}$ mice.

Runx3-*deficient CD8 T cells, Tregs, and microorganisms are required for the development of colitis-associated tumors in lymph*-Runx3$^{-/-}$ *mice*

Our previous experiments (20, 21) indicated that loss of Runx3 in epithelial cells of the gastrointestinal tract causes hyperplasia of the gastric mucosa and the formation of intestinal tumors. In those experiments, we excluded the possibility of the involvement of lymphocytes in colitis and tumor formation by transplanting bone marrow cells from *Runx3*$^{-/-}$ mice into irradiated WT mice (21). However, this procedure is not suitable for assessing the functions of Tregs because ~5% of the lymphocytes survive after irradiation, and the remaining Tregs affect the result. To examine further the possible role of Tregs in colitis, we transferred *Runx3*$^{-/-}$ FLCs into *Rag2*$^{-/-}$ mice, which have no lymphocytes because of a defect in VDJ recombination. As demonstrated earlier, *Runx3*-deficient Tregs are one of the causes of colitis in *Runx3*$^{-/-}$ mice. In our previous report (21), we also demonstrated that small intestinal adenomas developed in *Runx3*$^{+/-}$ mice at around 65 wk of age, with an incidence rate of 54%. We therefore examined the involvement of *Runx3*$^{-/-}$ lymphocytes in intestinal tumor formation using Lymph-*Runx3*$^{-/-}$ mice. Unexpectedly, no tumor formation was observed in these mice. Because the colonic lumen contains abundant commensal bacteria and the composition of this population plays an essential role in colitogenesis, the same assays were performed after housing Lymph-*Runx3*$^{-/-}$ mice in a conventional mouse facility. In this case, all Lymph-*Runx3*$^{-/-}$ mice developed tumors in the large intestine or cecum (Fig. 4*A*; 100% in Lymph-*Runx3*$^{-/-}$ mice compared with 0% in Lymph-WT mice). The colitis index was also increased in Lymph-*Runx3*$^{-/-}$ mice but not in Lymph-WT mice (Fig. 4*B*). A representative tumor in Lymph-*Runx3*$^{-/-}$ mice is shown in Fig. 5. Notably, it contained abundant stromal cells, such as lymphocytes, fibroblasts, macrophages, and smooth muscle fibers, whereas changes in the epithelial cells were milder and different from typical adenomas observed in *Runx3*$^{+/-}$ mice (Fig. 5). These data suggest that loss of Runx3 activity in lymphocytes contributes to the development of IBD and subsequent colon tumorigenesis. However, the mechanisms of tumorigenesis appear different between Lymph-*Runx3*$^{-/-}$ and *Runx3*$^{+/-}$ mice. To examine further the involvement of CD8 T cells and Tregs in the development of intestinal tumors, CD8 T cells or Tregs of WT origin were cotransferred with *Runx3*$^{-/-}$ FLCs. As shown in Fig. 4*A*, tumors were not formed in the colon in either case. These data indicate that loss of Runx3 in Tregs and CD8 T cells also contributes to tumorigenesis, in addition to colitogenic microorganisms in the colon.

Discussion

Our results show that inactivation of Runx3 in lymphocytes can induce colitis and consequently tumorigenesis in the large intestine. Thus, the inflammatory phenotype observed in Lympho-*Runx3*$^{-/-}$ mice is limited to the large intestine and cecum. However, Groner and colleagues (22) demonstrated that *Runx3*$^{-/-}$ mice developed spontaneous IBD and gastric hyperplasia. This difference suggests several possibilities. In comparison with *Runx3*$^{-/-}$ mice (22), Lymph-*Runx3*$^{-/-}$ mice show a weaker inflammatory phenotype. Thus, it is conceivable that other defects, but not lymphocyte defects, caused by the loss of Runx3 contribute to the severity of inflammation. If the gastric mucosa is more resistant to irritating stimuli than the intestinal mucosa, differences in the phenotype can be explained by this difference in inflammatory severity between these mice. Another possibility is that the gastric epithelium requires more Runx3 activity than the intestinal epithelium to maintain epithelial homeostasis; thus, Lymph-*Runx3*$^{-/-}$ mice do not show gastric hyperplasia because Runx3 expression is not perturbed within the gastric epithelium in Lymph-*Runx3*$^{-/-}$ mice. In addition, differences in the targeting strategy will affect the severity of gastrointestinal inflammation, which will change the expression levels of splicing variants of the Runx3 gene (22). The exact mechanisms of these processes are yet to be clarified.

Runx factors may be involved in Treg functions (29–31). Runx1 makes a greater contribution to Treg function than the two other Runx family proteins because Treg-specific deletion of Runx1 results in gastritis (30). Akdis and colleagues (29) found that Runx1 and Runx3 have some functional redundancy in Tregs in the human. In this report, we have shown that *Runx3*-deficient Tregs have a defect in Treg function in vivo and in vitro. In addition, we have found that colitis worsens when Lymph-*Runx3*$^{-/-}$ mice, but not Lymph-WT mice, are housed in a conventional mouse facility. This indicates that commensal microorganisms and Tregs work together to establish a mutual relationship. Taking these observations into account, we propose that both Runx1 and Runx3 are required for the proper functioning of Tregs in becoming tolerant to intestinal bacteria and establishing a good commensal relationship with various bacterial species. However, how to regulate the balance between inhibiting infection and preventing an excessive inflammatory reaction remains to be clarified. In addition, it is unclear why iTreg differentiation fluctuated in different samples of *Runx3*-deficient cells (Fig. 2*D*, *middle* and *lower columns*, 2*E*). It is likely that fluctuations in compensatory expression or activity of other members of the Runx family in *Runx3*$^{-/-}$ T cells affect the capacity of these cells to differentiate into iTregs. This possibility requires investigation in future studies.

Previous reports indicated that Runx3 is required for the proper differentiation and function of CD8 T cells. We therefore examined the involvement of CD8 T cells in tumor formation and found that these cells prevented colitis-associated tumor formation in our experimental setting. In addition to the essential role of CD8 T cells in tumor immunity, recent data indicate that several subsets of CD8 T cells have the capacity to inhibit immune reactions. In this regard, we have not found any obvious anti-inflammatory effect of CD8 T cells. To reveal the exact functions of CD8 T cells in preventing tumor formation in Lymph-*Runx3*$^{-/-}$ mice remains an important issue for investigation.

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Disclosures

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