Xenograftedマウスモデルを作製した。増殖が認められた移植片を継代移植するとともに、原発巣の組織との比較をHE染色により行った。

B－２：倫理面への配慮

本研究では、非小細胞肺癌組織を用いて研究を行う。組織を用いる際は、法令に基づいて設置された大阪府立成人病センター倫理審査委員会において承認を受け、その利用方法を遵守する。また、患者個人に対し主治医もしくはインフォームド・コンセント担当者が説明を行ない、遺伝子解析研究に対する署名、捺印を文書にて頂いている。臨床情報に関しては、大阪府立成人病センター内で匿名化され、その扱いに関しては、センター内の規定に従って扱う。

実験動物に関しては、動物実験等の実施に関する基本指針に基づき設置された大阪府立成人病センター内の動物実験委員会の規定に基づき実験を行う。

C．研究結果

全 60 症例中 27 症例（45%）で移植モデルが確立された。その内訳は、扁平上皮癌が 13 例（13／24）、小細胞癌が 2 例（2／2）、多形細胞癌が 4 例（4／4）、腺癌が 8 例（8／30）であった。確立した移植マウスは継代可能であり、症例数としては問題ないと考えている。HE 染色を行い、組織学的に検討したところ、腺癌は腺腔構造を有し、また扁平上皮癌は層状構造と部分的な壊死部分を含んでおり、外科切除標本と比べて相同性が観察された。

扁平上皮癌の外科切除腫瘍及び移植マウスから得られた腫瘍とではよく似た組織像であった（図1）。

また、肺腺癌の外科切除腫瘍及び移植マウスから得られた腫瘍もよく似た組織像であった（図 2）。一方、肺腺癌の培養細胞株 PC14 をマウスに移植した腫瘍では、外科切除腫瘍とは異なる組織像を示していた（図 2-C）。また、小細胞癌及び転移性大腸癌でも外科切除腫瘍と同様の組織像を示していた（図 3, 4）。また、腺癌の症例においては、移植マウスにおける腫瘍で組織内の組織像の多様性が認められた（図 5）。さらに、腎臓及び肝臓への浸潤像も確認された（図 6）。

Nude マウスと SCID マウスとの比較では、移植マウスの作製効率に差は認められなかった（表 1）。また、年齢や術後の移植までの時間に関しても作製効率に差は認められなかった（表 1）。

次に、免疫組織化学染色による解析を行った。Ki-67、p53 の染色像は外科摘出サンプルと移植マウスとでほぼ一致していた（図 7）。また、1 サンプルのみの解析ではあるが、CD56、Synapto、Chr-A、PAS に関しても一致していた（表 2）。

さらに、シスプラチン耐性を獲得した組織像の観察では、原発巣とのはっきりとした組織的な違いはみられなかった（図 8）。

D．考察・結論

外科切除腫瘍を直接マウスに移植するモデルマウスを確立した。培養細胞系よりも腫瘍組織の組織像や腫瘍細胞の多様性を保持していることが確認された。また、扁平上皮癌に比べ、腺癌の成功率が悪かったが、Nude マウス及び SCID マウスともに同じ傾向であり、腫瘍細胞の性質による違いであると考えられる。

これらのマウスモデルを用いた抗癌剤評価が可能であることが確かめられたため（総括研究報告参照）、分子標的薬の投与を行い、腫瘍縮小効果及び皮疹関連遺伝子の遺伝子発現を解析する。また、得られたシスプラチン耐性組織の発現解析や遺伝子変異探索を行い、耐性メカニズムを解明していきたい。

E. 研究発表

1. 論文発表

(1) Surgical treatment for patients with solitary metastasis in the mediastinal lymph node from renal cell carcinoma. Kanzaki R, Higashiyama M, Okami J, Kodama K. Interact Cardiovasc Thorac Surg. 2009 Apr;8(4):485-7. Epub 2009 Jan 5.

(2) Innate immune therapy with a Bacillus Calmette-Guérin cell wall skeleton after radical surgery for non-small cell lung cancer: a case-control study. Kodama K, Higashiyama M, Takami K, Oda K, Okami J, Maeda J, Akazawa T, Matsumoto M, Seya T, Wada M, Toyoshima K. Surg Today. 2009;39(3):194-200. Epub 2009 Mar 12.

(3) Malignant pleural mesothelioma with long-term tumor disappearance of a local relapse after surgery: a case report. Higashiyama M, Oda K, Okami J, Maeda J, Kodama K, Imamura F. J Med Case Reports. 2009 Mar 27;3:6800.

(4) Pulmonary resection in patients aged 80 years or over with clinical stage I non-small cell lung cancer: prognostic factors for overall survival and risk factors for postoperative complications. Okami J, Higashiyama M, Asamura H, Goya T, Koshiishi Y, Sohara Y, Eguchi K, Mori M, Nakanishi Y, Tsuchiya R, Miyaoka E; Japanese Joint Committee of Lung Cancer Registry. J Thorac Oncol. 2009 Oct;4(10):1247-53.

(5) Solitary pulmonary metastasis of mucoepidermoid carcinoma of the palate 43 years after the initial treatment. Okami J, Tomita Y, Higashiyama M, Kodama K. Interact Cardiovasc Thorac Surg. 2009 Oct;9(4):728-9. Epub 2009 Jul 22.

(6) A case report of large thymic hyperplasia associated with hyperthyroidism. Takami K, Omiya H, Higashiyama M, Maeda J, Okami J, Oda K, Tsujinaka T, Kodama K. Ann Thorac Cardiovasc Surg. 2009 Dec;15(6):404-7.

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Taniguchi, Jiro Okami, Ken Kodama, Masahiko Higashiyama, kikuya kato. Detection of EGFR Gene T790M mutation in Non-Small Cell Lung Cancer using an improved version of the BEAMing technology. 第68回日本癌学会総会 2009年10月1日 横浜F. 知的財産権の出願・登録状況

1. 特許取得
特になし

2. 実用新案登録
特になし

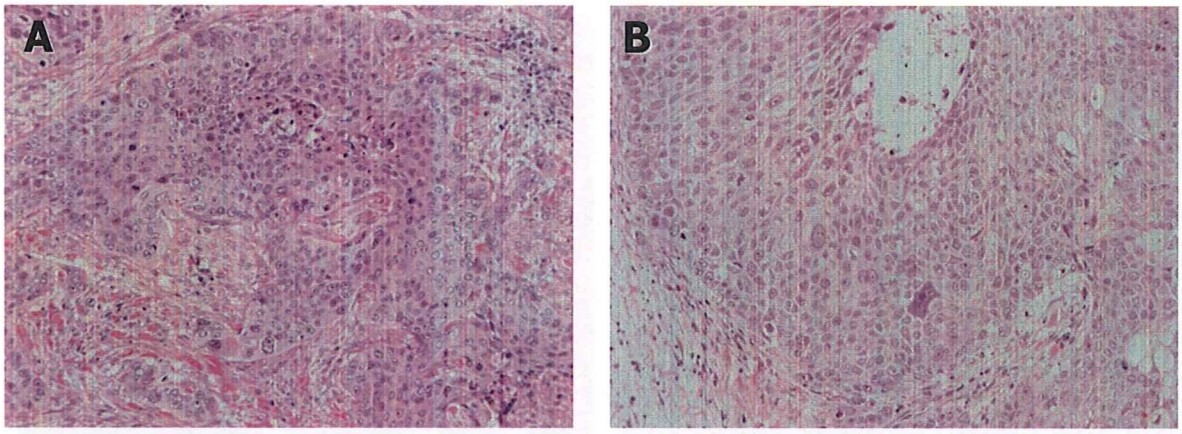


Figure 1. Squamous cell carcinoma histology of surgical (A) and xenograft (B) samples.

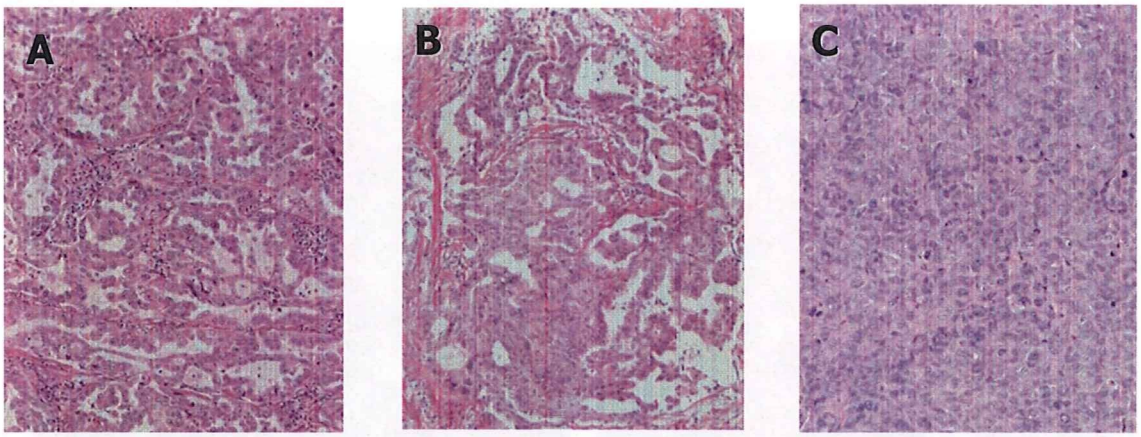


Figure 2. Adenocarcinoma carcinoma histology of surgical (A), xenograft (B), and xenograft tumor of established cell line (PC-14) samples(C).

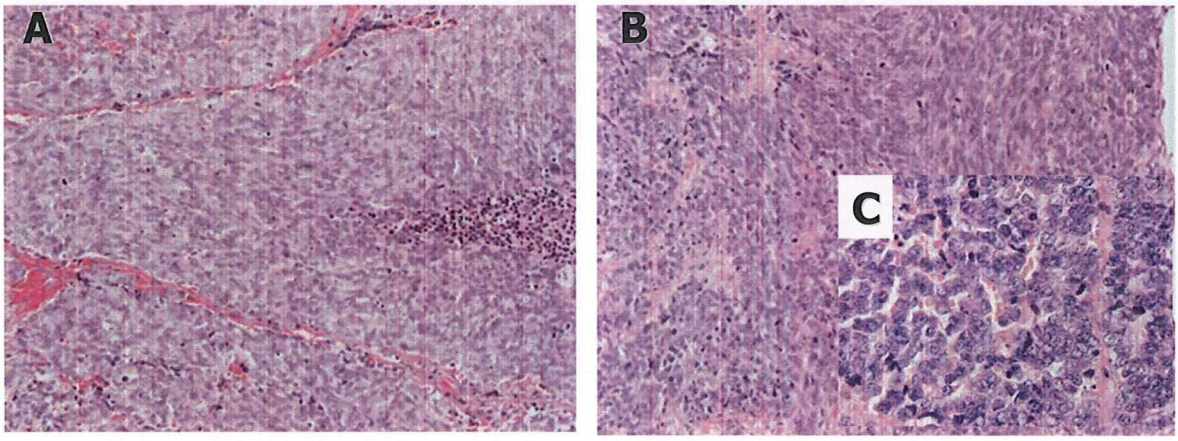


Figure 3. Small cell lung carcinoma histology of surgical (A) and xenograft (B) (C) samples.

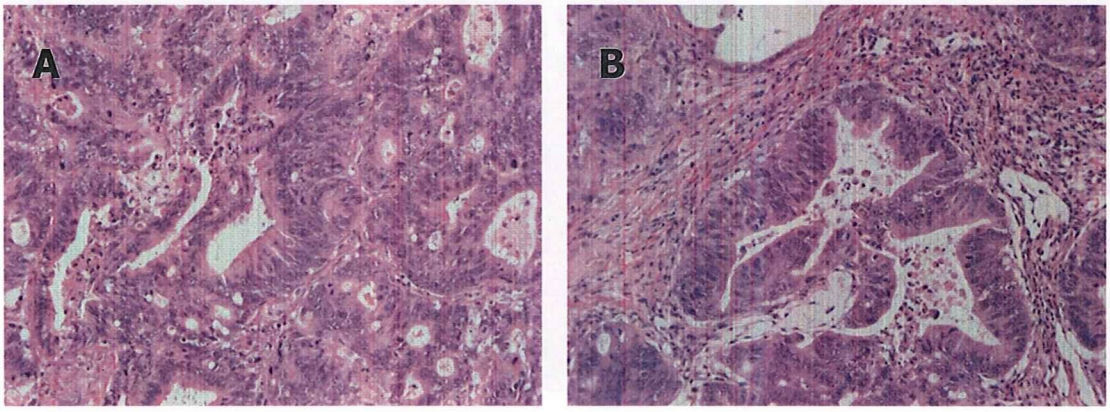


Figure 4. Metastatic colon cancer histology of surgical (A) and xenograft (B) samples.

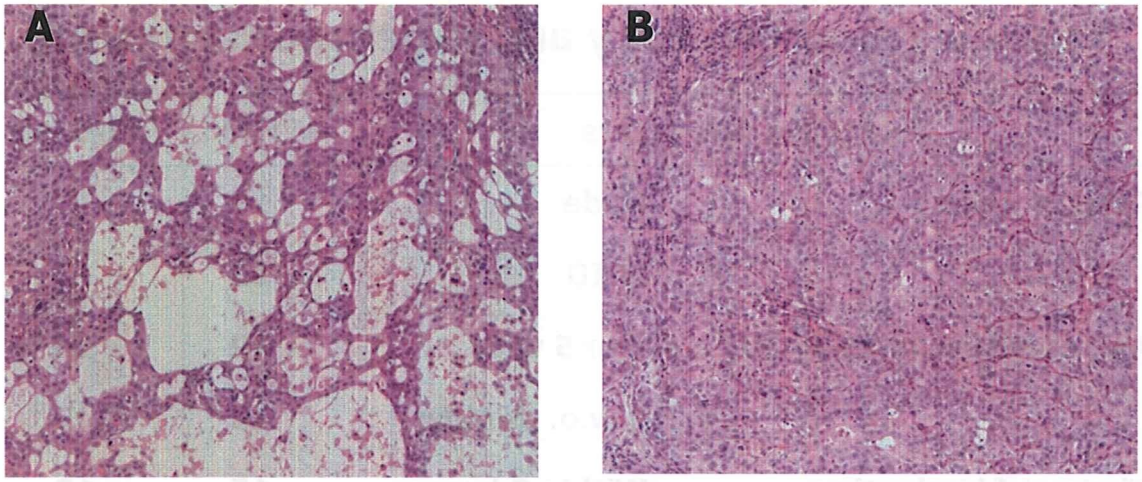


Figure 5. Histological heterogeneity of a xenograft tumor. Histological heterogeneity is often observed in human lung adenocarcinoma. This xenograft tumor derived from lung adenocarcinoma showed glandular pattern (A) and less differentiated cobble stone pattern (B).

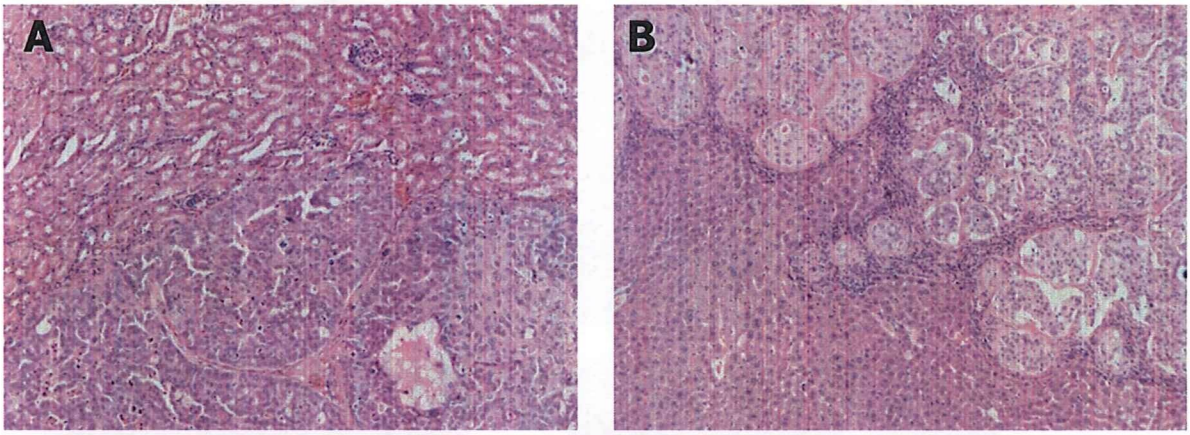


Figure 6. Direct invasion into adjacent organs of xenograft tumors. Invasion is one of the hall-marks of malignant tumor. When cancer cells were implanted into peri-renal pocket (backside of abdominal cavity), xenograft tumor grew in abdominal cavity and invaded into adjacent organ including kidney (A), liver (B), and ovary.

Table 1. Tumorigenecity and experimental factors

Experimental factors		Fail	Success
Type of mouse	Nude	11	7
	SCID	12	13
Age of mouse (weeks old)	4 or 5 w.o.	12	14
	6 w.o. or older	11	6
Timing of implanting	Within 3 hours	15	12
(Hours after surgery)	Longer than 3 hours	8	8

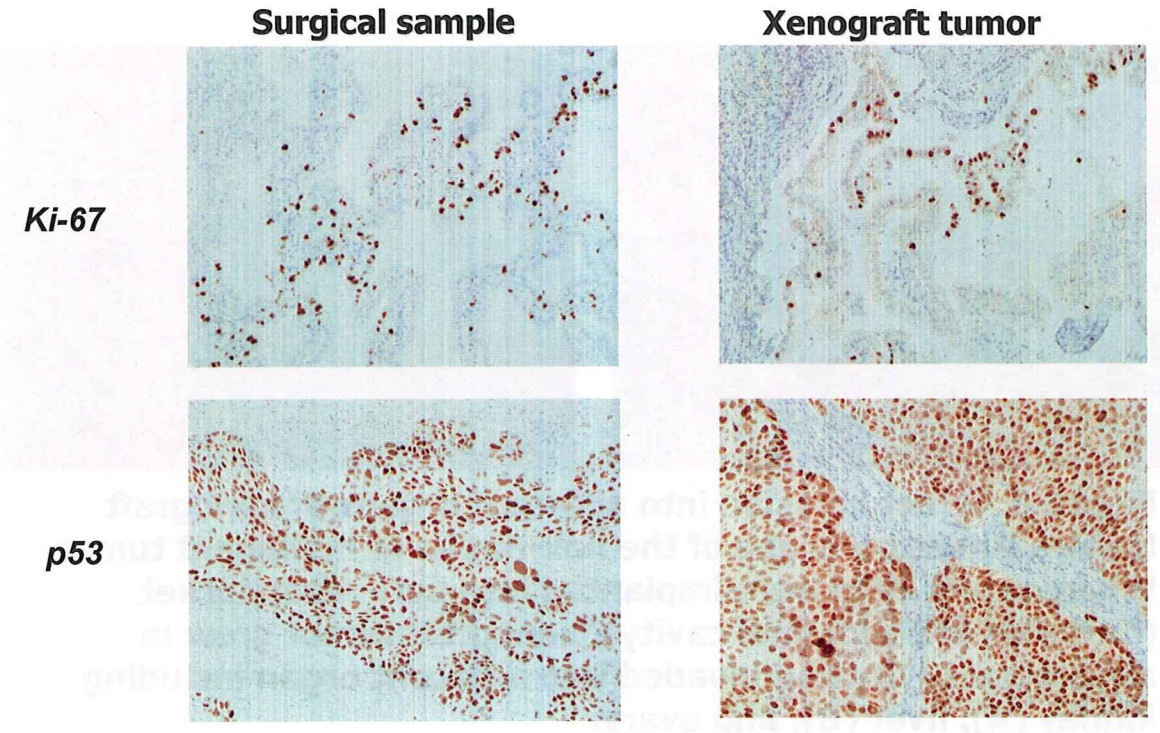


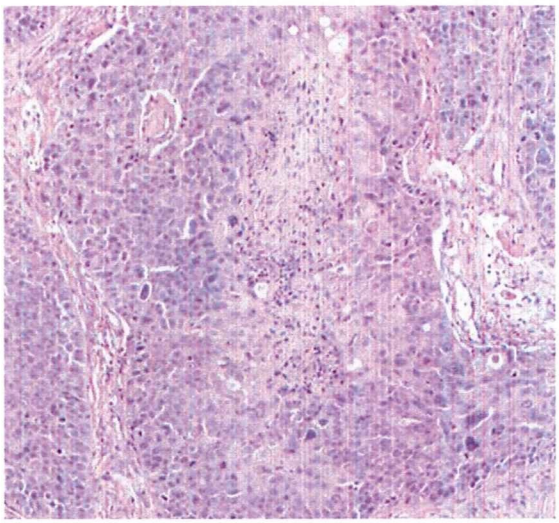
Figure7.Immunohistochemical and mutational analysis

Table2.Immunohistochemical and mutational analysis

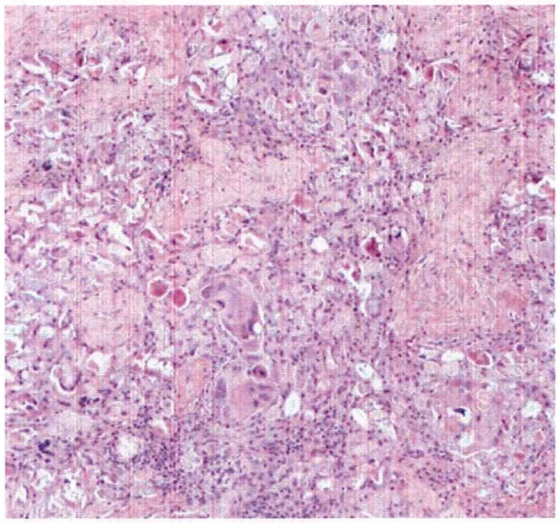
Sample ID	Histology	Ki-67		p53		CD56		Synapto		Chr-A		PAS	
		S	X	S	X	S	X	S	X	S	X	S	X
441921	Small	++	++	-	-	+	+	-	-	+	+		
465111	Small	+++	NE	+	+	+	+	-	-	Focal	-		
460011	Squamous	++	++	-	-								
477711	Squamous	+++	+++	+	+								
487711	Adenoca	+++	+++									Focal	Focal
499811	Adenoca	+	+	-	-								

S: Surgical sample, X; Xenografted tumor
All samples were negetive for k-ras or EGFR mutation.
Semiquantitative analysis of ki-67: + ~30%, ++ 31~60%, +++ 61%~

Figure8.Pathological evaluation of chemo-response



Control



After CDDP treatment

Ⅲ. 研究成果の一覧　雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kanzaki R, Higashiyama M, Okami J, Kodama K.	Surgical treatment for patients with solitary metastasis in the mediastinal lymph node from renal cell carcinoma.	Interact Cardiovasc Thorac Surg.	Apr;8(4):	485-487	2009
Kodama K, Higashiyama M, Takami K, Oda K, Okami J, Maeda J, Akazawa T, Matsumoto M, Seya T, Wada M, Toyoshima K.	Innate immune therapy with a Bacillus Calmette-Guérin cell wall skeleton after radical surgery for non-small cell lung cancer: a case-control study.	Surg Today.	39(3):	194-200	2009
Higashiyama M, Oda K, Okami J, Maeda J, Kodama K, Imamura F.	Malignant pleural mesothelioma with long-term tumor disappearance of a local relapse after surgery: a case report.	J Med Case Reports.	Mar 27;3:	6800	2009
Okami J, Higashiyama M, Asamura H, Goya T, Koshiishi Y, Sohara Y, Eguchi K, Mori M, Nakanishi Y, Tsuchiya R, Miyaoka E,Japanese Joint Committee of Lung Cancer Registry.	Pulmonary resection in patients aged 80 years or over with clinical stage I non-small cell lung cancer: prognostic factors for overall survival and risk factors for postoperative complications.	J Thorac Oncol.	Oct;4(10):	1247-1253	2009
Okami J, Tomita Y, Higashiyama M, Kodama K.	Solitary pulmonary metastasis of mucoepidermoid carcinoma of the palate 43 years after the initial treatment. Interact Cardiovasc.	Thorac Surg.	Oct;9(4):	728-729	2009

Takami K, Omiya H, Higashiyama M, Maeda J, Okami J, Oda K, Tsujinaka T, Kodama K.	A case report of large thymic hyperplasia associated with hyperthyroidism.	Ann Thorac Cardiovasc Surg.	Dec;15(6):	404-407	2009

Interactive CardioVascular and Thoracic Surgery

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Interactive CardioVascular and Thoracic Surgery 8 (2009) 485-487

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Case report - Thoracic oncologic

Surgical treatment for patients with solitary metastasis in the mediastinal lymph node from renal cell carcinoma

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Abstract

We performed surgical treatment on two patients, each with a solitary metastasis in a mediastinal lymph node from a renal cell carcinoma (RCC). The first case was a 58-year-old male with a chief complaint of chest discomfort due to pretracheal mediastinal lymph node (#3) swelling. He had undergone a right nephrectomy for RCC 13 years previously. Because of difficulty in establishing the diagnosis, a mini-thoracotomy was performed, and this lymphadenopathy was judged to be metastasis from the RCC. The pretracheal lymph nodes were completely resected, and he has experienced no recurrence for two years postoperatively. The second case was a 60-year-old female who had undergone a left nephrectomy for RCC two years previously. Because of the Botallo's lymph node (#5) swelling, a mini-thoracotomy was performed. This swollen lymph node was resected, and it was finally diagnosed to be metastasis from the RCC. Unfortunately, the tumor recurred in the mediastinal lymph nodes with multiple lung metastases five years later. A solitary metastasis in a mediastinal lymph node from a RCC is an unusual event, particularly in the absence of lung metastasis. The diagnostic and clinicopathological problems associated with this unique disease are herein discussed.

Keywords: Renal cell carcinoma; Mediastinal lymph node; Thoracic surgery

1. Introduction

The most common site of the initial recurrence in patients who have undergone a radical nephrectomy for renal cell carcinoma (RCC) is the lung. However, solitary metastasis in a mediastino-hilar lymph node in the absence of lung metastasis is uncommon. We herein describe the surgical treatment of two patients with a solitary metastasis to a mediastinal lymph node from a RCC. We also discuss several diagnostic and clinicopathological problems associated with this unique disease.

2. Case report

2.1. Case 1

A 58-year-old male underwent a radical right nephrectomy for RCC (clear cell carcinoma G1, pT1b N0 M0) in October 1992. He had a medical history of hypertension and hyperlithuria and had a one pack-per-day smoking history. No postoperative adjuvant chemotherapy was performed. Thirteen years later, he consulted his primary physician regarding chest discomfort. A computed tomographic (CT) scan demonstrated an anterior mediastinal mass, considered to be pretracheal lymphadenopathy (#3), which measured 2 cm in diameter (Fig. 1). A whole body Fluorine-18-2-fluoro-D-glucose positron emission tomography/CT (FDG-PET/CT) study revealed intense focal FDG uptake [standard uptake value (SUV) max=2.6] in the anterior mediastinal mass and no other abnormal FDG uptake. No serum tumor marker, including carcinoembryonic antigen, was elevated. An exploratory mini-thoracotomy was performed on November 29, 2006, and the anterior mediastinal mass was removed. An intraoperative histological examination using a frozen specimen resulted in a diagnosis of metastatic RCC to the pretracheal lymph node (#3, Fig. 2). Immediately, the mediastinal lymph nodes (#1–#4) were completely re-resected, but metastasis was finally observed only in the swollen #3 node without extracapsular invasion. The patient had an uneventful postoperative recovery and was given no postoperative therapy. To date, at 21 months after the operation, he has experienced no recurrence of the RCC.

2.2. Case 2

A 60-year-old female underwent a radical left nephrectomy for RCC (granular cell carcinoma G2, pT2 N0 M0) in March 1999. Two years later, a follow-up chest CT-scan demonstrated a solitary swelling of the left Botallo's lymph node (#5) which measured 3 cm in diameter, but no metastatic nodules in the pulmonary parenchyma were observed. Thereafter, only the swollen lymph node was removed through a mini-thoracotomy, and it proved to be

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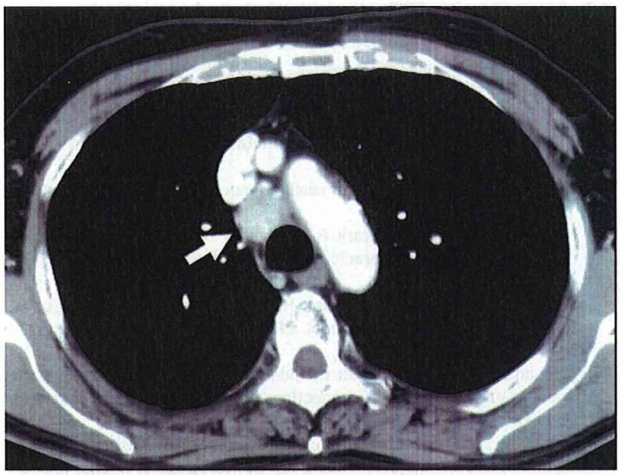

Fig. 1. A contrast-enhanced chest CT-scan showed an anterior mediastinal mass measuring 2 cm that is well enhanced (arrow).

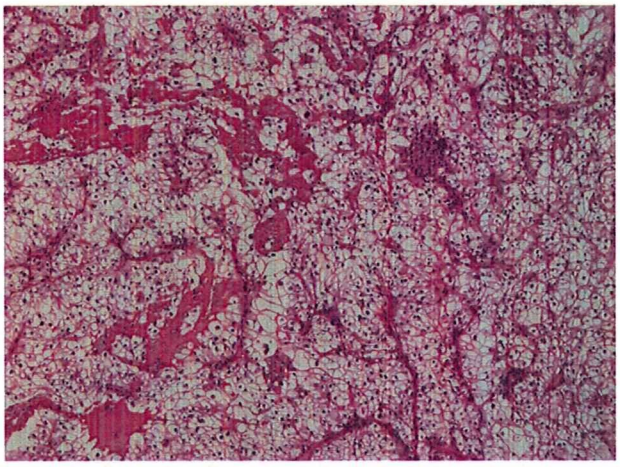

Fig. 2. A histological examination of the anterior mediastinal lymph node metastasis showing renal cell carcinoma.

involved with RCC metastasis. Intraoperatively, no metastatic lesion was found in the left thorax. In postoperative histological examinations, no extracapsular invasion was observed in the resected node. The patient had an uneventful postoperative recovery and was given no postoperative therapy. Unfortunately, five years after the thoracotomy, the tumor recurred in the lung and mediastinal lymph nodes. As a result, the patient was administered interferon-alpha. At present, at seven years after the operation, the patient is alive with recurrence in the lung.

3. Discussion

Metastases to the mediastinal lymph nodes from RCC are usually accompanied by lung metastasis. In 1965, Arkless [1] first reported patients showing such metastases associated with lung metastases. They reported that 11 of 152 patients with RCC had mediastinal lymph node involvement, and interestingly, all of them showed lung metastasis. In a necropsy series of 1451 patients with RCC [2], 75 (5%) had mediastinal lymphadenopathy, and surprisingly, more than 90% of these patients had lung metastases. Therefore, solitary metastasis in the mediastinal lymph nodes, in the absence of lung metastasis, is thus considered to be clinically uncommon.

The pathway by which RCC can metastasize directly to the mediastinal lymph nodes through the pulmonary parenchyma is interesting. Two possible pathways have so far been proposed. Firstly, the lymphogenous route via the thoracic duct or the inferior pulmonary ligament was presented. McLoud et al. [3] reported the lymphogenous route via the thoracic duct. They described that cancer cells pass through a retrograde flow to the peribronchial lymphatics from the thoracic duct because of its incompetent valves. In fact, reflux from the thoracic duct during lymphangiography occurs in 10–15% of all the tested patients, probably due to incompetent valves [4]. Interestingly, based on the recent study for renal lymphatic drainage, it was also shown that renal lymphatics always connect to the origin of the thoracic duct [5]. In addition, Wright [6] reported another lymphogenous route which passes through the retroperitoneal lymphatics into the inferior pulmonary ligament and finally reaches the mediastino-hilar lymph nodes. In contrast, as a second possibility, mediastinal lymph node metastasis has been reported to possibly occur as a second step from micrometastases in the lung. Specifically, pulmonary metastases, which usually spread through a hematogenous route from the RCC, are too small to be incidentally detected.

The metastatic lymph nodes in our cases were solitary and were limited to the upper part of the mediastinum without any involvement in the lower part, such as the subcarinal (#7) or inferior pulmonary ligament (#9) lymph nodes, at the time of the initial recurrence. In Case 2, considering the postoperative multiple recurrences in the lung and the mediastinal lymph nodes, it was likely that undetected micrometastasis in the tissues had remained behind at the time of the thoracotomy. In contrast, from our understanding of the clinical course of the Case 1 patient, the mechanism of a solitary metastasis remains to be elucidated. Considering the late recurrence at 13 years after the nephrectomy, it is difficult to explain the mechanism of recurrence in Case 1 with any degree of certainty. A postoperative follow-up may be required in the future, while careful attention must be paid to the lung and the regional lymph node status.

In these patients, we performed a mini-thoracotomy not only for diagnostic purposes but also to potentially effect a complete resection. Luckily, no extracapsular invasion was observed in the resected metastatic lymph nodes in postoperative histological examinations. Therefore, the metastatic lesions were judged to be completely removed in both patients. In cases of positive extracapsular nodal involvement, postoperative radiation therapy should therefore be considered in order to obtain complete local control. In Case 1, a diagnosis may have been possible by other methods, for example by a mediastinoscopy or an endobronchial ultrasonography (EBUS). However, since the pretracheal lesion was strongly contrasted according to the enhanced CT findings, such procedures were not chosen because of the risk of bleeding. In both cases, since we

had planned to perform a potentially radical resection, a mini-thoracotomy was intentionally selected.

Solitary or isolated metastatic RCC may generally be surgically resected, if possible. The 5-year survival rates of such patients have been reported to range from 30% to 60%, when a complete resection is possible [7]. In fact, in cases with solitary metastasis in the lymph nodes similar to ours, a favorable prognosis has been obtained by several authors. Recently, Whiston et al. [8] reported nine surgical cases for asynchronous renal cell carcinoma metastases to the mediastinal lymph nodes out of the 386 patients in their series. According to this report, there were no surgical complications, and interestingly, their median survival after resection of metastases was significantly longer than that of the other patients with stage IV disease. Therefore, surgical treatment for mediastinal lymph node metastases from RCC is considered to be a safe and effective modality, which appears to prolong the survival while also potentially curing the disease. In contrast, it is now thought that the therapeutic effect of conventional chemotherapy or radiation therapy might be poor. Recently, immunotherapy, such as the administration of recombinant human interleukin-2 and interferon-alpha, has developed as new modalities for such diseases, but the overall response rate is only 15%–20% [9, 10]. Therefore, in cases with a solitary enlarged mediastino-hilar lymph node occurring after a nephrectomy for RCC, surgery may be aggressively selected for both establishing the diagnosis and potentially to achieve a complete therapy. Therefore, the surgical approach for an isolated metastatic RCC disease may be appropriate, not only as a means of local control, but also to improve the understanding of this disease, itself.

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Innate Immune Therapy with a Bacillus Calmette-Guérin Cell Wall Skeleton After Radical Surgery for Non-Small Cell Lung Cancer: A Case–Control Study

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Abstract

Purpose. We investigated whether adjuvant immunotherapy with Bacillus Calmette-Guérin (BCG) cell wall skeleton (CWS) and surgical resection was better than resection, with or without other adjuvant therapy, for patients with non-small cell lung cancer (NSCLC).

Methods. The case group comprised 71 patients who underwent radical surgery for NSCLC, followed by BCG-CWS immunotherapy, with follow-up data available. The case–control study was designed with one control selected for each case-group patient. Each control was matched by pathological stage and year of birth (±5 years). BCG-CWS 200 μg was inoculated intracutaneously in the upper arm four times per week (sensitization phase); then at 4-week intervals (therapeutic phase).

Results. The case-group patients received 45 ± 22.6 (average ± SD) cycles of BCG-CWS inoculation. Overall 5-year and 10-year survival rates were 71% and 61% for the case-group patients, and 63% and 43% for the control-group patients. The survival rate of the case group was better than that of the control group (not significant; $P = 0.114$). The same trend was seen in the patients with stage III or N+ NSCLC (not significant; $P = 0.114$, $P = 0.168$). There were no life-threatening adverse events.

Conclusions. BCG-CWS immunotherapy seemed to improve survival after resection of NSCLC, especially locally advanced NSCLC. Moreover, this immunotherapy did not compromise quality of life during treatment.

Key words Bacillus Calmette-Guérin cell wall skeleton · Immunotherapy · Non-small cell lung cancer · Surgical resection · Case–control study

Introduction

The human immune system consists of innate and acquired arms. Recent advances in the field of tumor immunology have revealed two novel findings in these two systems: first, most solid tumors express tumor-associated antigens (TAAs) which are rooted in the aberrance of tumor-related genes;[1] and second, activation of the innate immune system before the acquired system is indispensable for full activation of lymphocyte effectors, or cell-mediated immunity.[2] Considering the former issue, immunotherapy for cancer has been designed with TAA peptides and many cytokines, and this augments lymphocyte-based therapies.[3] Rosenberg et al. challenged clinical trials of a peptide vaccine therapy in which a variety of TAAs were administered to melanoma patients. However, the overall rate of remission (including incomplete remission) was only 2.6%.[4] They used peptide vaccines without adjuvant conjugated, or only with aluminum (non-Toll-like receptor (TLR)-directed adjuvant). These results suggest that innate immunity must be stimulated before the induction of acquired effectors to raise antitumor therapeutic potential.

Microbial components that activate the host innate immune system have been designated as adjuvants. Adjuvants are often used for immunization with pure antigens (Ag) for effective induction of antibody (Ab) production, cytotoxic T cells (CTL), and natural killer (NK) cell activation.[5] Many adjuvants have been identified as ligands for microbial pattern-recognition recep-

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tors such as TLRs.[6] Through sensing microbe patterns, dendritic cells mature to present extrinsic Ag and release lymphocytes from an anergic state.[2,6] The cell-mediated immune system is thereafter activated to target Ag-bearing cells. This concept was demonstrated only recently, and it is now apparent that TLR agonists were being used as popular adjuvants for therapeutic purposes without knowledge of their mechanistic function.

For more than 6 years, clinical trials of many TLR-directed adjuvants have been conducted, aiming at adjuvant-augmented immunotherapy.[6,7] Most of these trials are still in progress with fruitful or anticipated results. Our earlier studies suggested that Bacillus Calmette-Guérin cell wall skeleton (BCG-CWS) has the potential to activate human antigen-presenting dendritic cells and induce interleukin 6 (IL-6), IL-12, tumor necrosis factor alpha (TNF-α),[8] and possibly, CTL.[9] Interferon-gamma (IFN-γ) levels increase in response to BCG-CWS.[10] A sole BCG-CWS without peptides was used in these studies since cancer patients are usually exposed to their own TAAs.[10] Studies on mouse tumor implant models suggest that BCG-CWS induces cross-priming facilitating class I presentation of exogenous antigens.[9] An efficient CTL response against Ag-bearing cells appears evident. These immune responses are attributed to TLR2 and TLR4 in antigen-presenting dendritic cells.[8,11]

The current clinical study was designed to investigate these basic findings on BCG-CWS adjuvant. We anticipated that BCG-CWS alone has the ability to evoke an antitumor immune response because patients with cancer postoperatively still possess TAAs.[10,12] To find out if patients who undergo radical surgery followed by adjuvant BCG-CWS immunotherapy for NSCLC are more likely to have a favorable outcome, we conducted a case–control study.

Materials and Methods

Preparation of BCG-CWS and Its Inoculation Schedule

BCG-CWS, donated by Dr. Azuma,[13] was used as an immunotherapeutic agent in the form of an oil-in-water emulsion, using either mineral oil (Drakeol 6VR) or a metabolizable oil such as squalene or squalane. After sterilizing by heating for 30 min at 60°C, the oil-attached BCG-CWS suspension was inoculated intracutaneously at a final concentration of 1 mg/ml in the upper arm according to the schedule described by Hayashi et al.[12,14] In the sensitization phase, 200 μg was inoculated four times weekly, whereas in the therapeutic phase, the amount inoculated, at 4-week intervals, ranged between 10 and 200 μg, depending on the patient's biological responses, including IFN-γ induction, local skin reaction at the inoculation site, various physical conditions (fever or general malaise), and indicators of laboratory tests showing liver function or inflammatory reactions.

Interferon-γ Induction Test

To evaluate the effect of immunotherapy on BCG-CWS, an IFN-γ induction test was performed at the time of the fourth inoculation in the sensitization phase, and at the time of the first and sixth inoculations in the therapeutic phase. The level of IFN-γ in the peripheral blood was measured before inoculation and 18 h after inoculation. Interferon-γ levels were detected with an enzyme-linked immunosorbent assay at the laboratory of Otsuka Assay (Tokushima, Japan), with the lower limit of sensitivity for detecting human serum IFN-γ being 7.8 pg/ml.

Case–Control Study

In May 1994, the protocol of a pilot study on BCG-CWS immunotherapy for patients with various malignant neoplasms was approved by the Ethical Review Board of Osaka Medical Center for Cancer and Cardiovascular Diseases. At the time of informed consent, we explained to patients about the expected effectiveness and side effects based on previous reports on immunotherapy,[15] chemotherapy,[16] and our survival data of surgery alone. In the 1990s, with the exception of one article published in 1995 from the NSCLC Collaborative Group,[16] there was no clear evidence of the survival benefit of adjuvant chemotherapy for NSCLC.

Written informed consent was obtained from all patients who chose to be treated with immunotherapy, which was started 4–6 weeks after the operation. Among the patients who received BCG-CWS, we recruited 83 NSCLC patients. Between 1994 and 2000, these patients received immunotherapy with BCG-CWS alone as adjuvant therapy after radical surgery for NSCLC. Since the clinical records of 12 patients were unavailable because their operations had been performed at other hospitals, they were excluded from the final analysis. Thus, 71 patients with both clinical records and follow-up data were enrolled in this study as the case group. They did not receive any other adjuvant therapy until recurrence was confirmed.

The case–control study was designed with one control selected for each patient. The control was matched to the patient by pathological stage and year of birth (±5 years). The matched control was recruited from among patients who underwent radical surgery for NSCLC, regardless of adjuvant chemo- and/or radiotherapy, with the shortest interval between the operation from our medical history data file.

Adverse effects of immunotherapy were graded from 0 (none) to 5 (fatal) according to the Common Terminology Criteria for Adverse Events; version 3 (CTCAE) of the National Cancer Institute.

Statistical Analysis

Continuous variables were analyzed by the *t*-test, and categorical variables were evaluated using χ^2 analysis. Overall survival rates and survival rates at each stage were compared between the patients and controls. We performed survival analysis with StatView version 5 (Abacus Computer, Berkeley, CA, USA), and survival curves were calculated with the Kaplan–Meier method.[17] Differences in survival were evaluated with the log-rank test. A P value of less than 0.05 was considered significant.

Results

The case-group patients were inoculated with 45 ± 22.6 (average ± SD) cycles of BCG-CWS over a range of 6–94 cycles (Fig. 1). Table 1 compares the patient characteristics of the case group with the control group. There were no significant differences between the groups in matched criteria, pathological stage (P = 1.000), or histology (P = 0.913). The mean age of the case-group patients was slightly less than that of the control patients (P = 0.087). The male to female ratio of the case-group patients was slightly lower than that of the control patients (P = 0.217). The types of lung resection and the pathological T and N factors were similar in the two groups (P = 0.967, 0.986, and 0.980, respectively). Among 40 control patients with stage II or III disease, 5 had received adjuvant chemo- and/or radiation therapy. The median follow-up was longer than 5 years and was similar in the two groups.

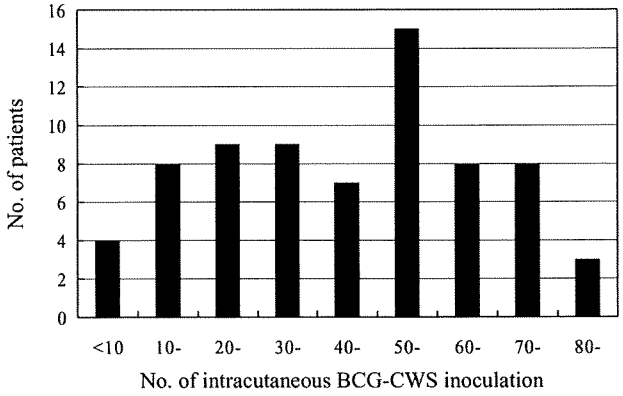

Fig. 1. Postoperative adjuvant immunotherapy with Bacillus Calmette-Guérin cell wall skeleton (*BCG-CWS*) for non-small cell lung cancer. Patient distribution according to the number of treatment courses

Table 1. Case–control study: patient backgrounds

Characteristic	Case patients	Control patients	*P* value
No. of patients	71	71	
Age, years (mean, range)	59 (37–78)	62 (38–78)	0.087
Sex			0.217
Male	43	50	
Female	28	21	
Surgery			0.967
Lesser resection	8	9	
Lobectomy	58	57	
Pneumonectomy	5	5	
Pathological T factor			0.986
T1	36	35	
T2	22	24	
T3	12	11	
T4	1	1	
Pathological N factor			0.980
N0	38	37	
N1	18	19	
N2	15	15	
Pathological stage			1.000
I	31	31	
II	21	21	
III	19	19	
Histology			0.913
Adenocarcinoma	51	49	
Squamous cell carcinoma	16	17	
Large cell carcinoma	4	5	
Median follow-up period (months)	68	66	

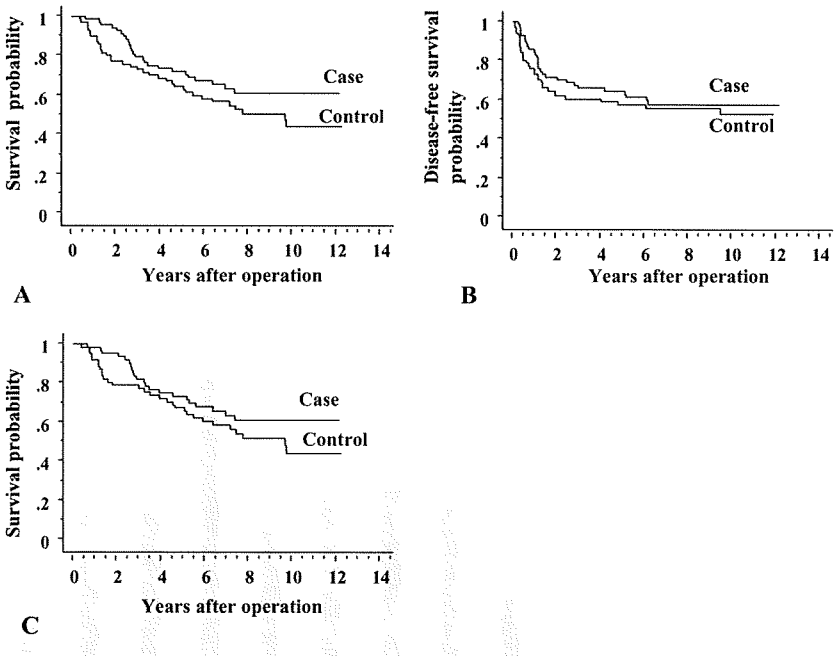

Fig. 2A–C. Kaplan–Meier overall survival estimates. **A** Survival curves. Case-group patients ($n = 71$) versus controls ($n = 71$). $P = 0.114$. **B** Disease-free survival curves. Case-group patients ($n = 71$) versus controls ($n = 71$). $P = 0.473$. **C** Survival curves of 62 gender-matched pairs. Case-group patients ($n = 62$) versus controls ($n = 62$). $P = 0.190$

When patients who had undergone radical surgery for NSCLC hoped to receive adjuvant immunotherapy in spite of a weak IFN-γ induction, the immunotherapy was continued if they had a local skin reaction at the injection site of BCG-CWS, such as an area of erythema greater than 20 mm in diameter, or an induration with an ulcer.[18] As a result, none of the patients given immunotherapy as postoperative adjuvant therapy stopped receiving it. The 5-year and 10-year survival rates were 74% and 62%, respectively, for 20 case-group patients given IFN-γ induction of 35 pg/ml or more, and 70% and 60%, respectively, for 39 given IFN-γ induction of less than 35 pg/ml ($P = 0.700$). The IFN-γ assay was not done for 12 patients.

The overall 5-year and 10-year survival rates were 71% and 61%, respectively, for the case-group patients, and 63% and 43%, respectively, for the control patients group (Fig. 2A). Although the difference was not significant, the survival rate of the case-group patients was better than that of the controls over the observation period ($P = 0.114$). The same trend was observed in disease-free survival between these two groups (Fig. 2B).

To exclude the influence of gender heterogeneity (Table 1) on survival, we selected 62 gender-matched pairs and compared their survival curves. The 5-year and 10-year survival rates were 73% and 61%, respectively, for the 62 case-group patients, and 67% and 44%, respectively, for the 62 control patients ($P = 0.190$; Fig. 2C).

According to the pathological stages, there were no significant differences in survival between the case and control group patients with stage I or II disease (Fig. 3A and 3B). The survival rate of the case-group patients with stage III disease was better than that of the control group patients with stage III disease, although the difference did not reach significance ($P = 0.114$; Fig. 3C). Ten multi-station N2 patients were included in the case group and 8 in the control group. When the survival of pathologically N+ patients was analyzed, both groups showed the same tendency ($P = 0.168$; Fig. 3D).

Table 2. Adverse effects of Bacillus Calmette-Guérin cell wall skeleton treatment

No. of patients	71 (100%)
Nonadverse effect	52 (73)
Adverse effect	19 (27)
Nonmalignant axillary and/or cervical lymph node swelling	9 (13)
ALT and AST elevation (≤grade 2)	6 (8)
Nonmalignant pleural effusion (grade 1)	2 (3)
Infection	1 (1.5)
Neuropathy (grade 2)	1 (1.5)

ALT, alanine aminotransferase; AST, aspartate aminotransferase

Adverse effects were seen in 19 (27%) of the 71 case-group patients (Table 2). These included mild or moderate elevation of alanine aminotransferase and aspartate aminotransferase in six patients, mild nonmalignant pleural effusion in two, and moderate focal infection at the BCG-CWS inoculation site and neuropathy in one patient each. Although there is no grade that refers to the severity of CTCAE, nonmalignant axillary and/or cervical lymph node swelling was observed in nine

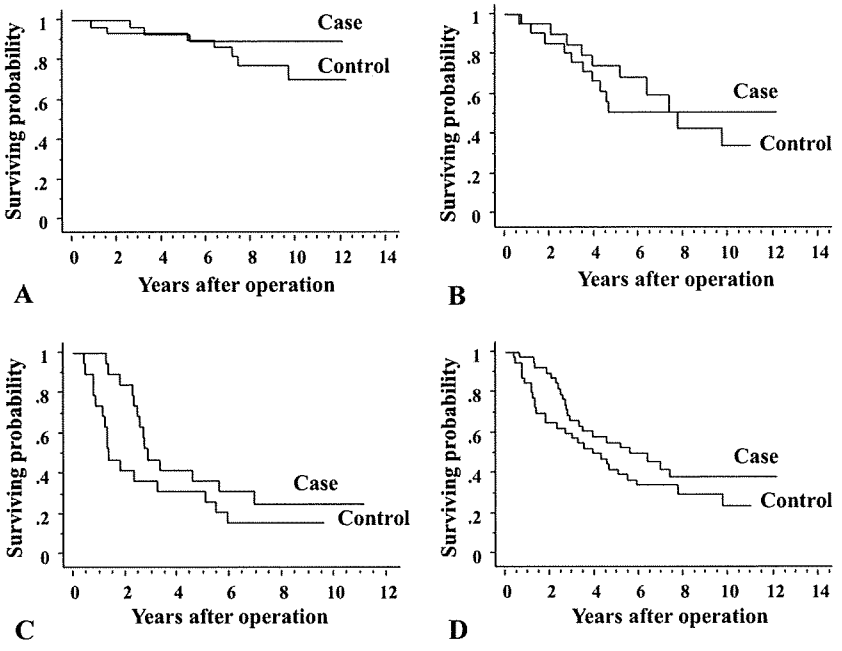

Fig. 3A–D. Kaplan–Meier survival estimates. **A** Stage I. Case-group patients (n = 31) versus controls (n = 31). P = 0.207. **B** Stage II. Case-group patients (n = 21) versus controls (n = 21). P = 0.420. **C** Stage III. Case-group patients (n = 19) versus controls (n = 19). P = 0.114. **D** N(+). Case-group patients (n = 33) versus controls (n = 34). P = 0.168

patients. There was no life-threatening or fatal event. Thirteen of the 19 patients were managed with a temporary dose reduction or discontinuation of BCG-CWS.

Discussion

Immune control of tumor growth can be mediated by the antigen-specific activity of CTLs or by the innate immune response of NK cells. Cytotoxic T cells recognize malignant cells in the context of antigen presentation via major histocompatibility complex (MHC) molecules.[19] Tumor cell recognition by NK cells is antigen-independent and MHC-unrestricted.[20]

Cancer cells share the same MHC as host cells and barely express pathogen-associated molecular patterns (PAMPs). We hypothesized that the inability of tumors to evoke the host immune response is due to the lack of PAMPs in patients with cancer. Supplementation with PAMPs as adjuvants may increase the efficacy of immune responses against tumor antigens (tumor vaccine). Adjuvants are materials added to a vaccine preparation to enhance its immunogenicity. One of the most powerful adjuvants is complete Freund's adjuvant; a suspension of killed mycobacteria in mineral oil. We used BCG-CWS as an adjuvant for this purpose.

Tsuji et al.[8] demonstrated that BCG-CWS can activate immature human dendritic cells (iDC). Antigen presentation and T-cell stimulation are enhanced by BCG-CWS, which also induces up-regulation of the DC maturation marker CD83, and the secretion of inflammatory cytokines such as IL-6, IL-12, and TNF-α. These responses and the increase in antigen-presenting ability indicate that the activation and maturation of DC is induced by CWS-containing mycobacterial peptidoglycan. This suggests that BCG-CWS induces TNF-α secretion from myeloid DC via Toll-like receptor (TLR) 2 and TLR4 and that the secreted TNF-α induces the maturation of DC.

Using a murine subcutaneous and lung metastatic sarcoma treatment model, Mason et al.[21] showed that a local injection of synthetic oligodeoxynucleotides (ODN) containing an unmethylated CpG motif (characteristic of bacterial DNA) could be given with conventional radiation therapy to augment therapeutic efficacy via an apparently immune-mediated mechanism. Combination cancer vaccines with TLR9 agonists such as ODN may induce tumor-specific CD4+ and CD8+ T cells, whose migration and killing activity would be enhanced by radiation therapy. Toll-like receptor expression differences exist between mice and humans; mouse plasmatoid and myeloid DC express TLR9, whereas only human plasmatoid DC does.[22] Mason et al.[21] hypothesized that when radiotherapy is given after TLR agonist injection, the tumor antigens released by dying tumor cells are taken up by activated DC, inducing a tumor-specific T-cell response.

The injection of BCG-CWS sometimes causes lymphadenopathy of the draining lymph nodes (Table 2). We demonstrated the uptake of fluorodeoxyglucose (FDG) into the enlarged lymph nodes not only in the axillary lymph nodes, but also in the cervical and mediastinal lymph nodes by positron emission tomography (PET)