comparable to those in previous reports. In the present investigation, due to the retrospective nature of the study design, the level of anastomosis was calculated from the tumor location and distal resection margin when data were not available. And in some patients, tumor location was measured only by digital examination and not by rectoscopy, these might introduce bias. Although the anastomotic level was not associated with leakage, this data should be evaluated with caution.

High ligation of IMA was the only leakage risk factor on univariate analysis in the present study. Lange et al. [24] systematically reviewed the literature concerning the level of ligation and concluded that preserving IMA and left colic artery was anatomically less invasive with respect to circulation and autonomous innervations of the proximal limb of anastomosis. Seike et al. [25] measured the colonic blood flow at the proximal site of the anastomosis by laser Doppler flowmetry to evaluate the influence of high ligation. They proved a significant reduction of colonic blood flow at the proximal site after clamping IMA. Our result also suggested the possibility that blood flow reduction on anastomotic sites leads to more leakage.

In the present study, we reported our low leakage rate in cases without DS (11.5%; 24 of 209). This rate is comparable to the leakage rate in cases with DS in a randomized controlled trial by Matthiessen et al. (10.3%; 12 of 116) [1]. This may have some association with our patient population that neoadjuvant radiotherapy or chemo-radiotherapy was not performed in this series. Neoadjuvant radiation therapy is considered to be a risk factor by some authors [13, 14]. Although randomized multicenter trials have shown that neoadjuvant radiation does not increase postoperative morbidity [26–28], Peeters et al. [18] retrospectively analyzed risk factors from the database of the Dutch Colorectal Cancer Group, and reported that a defunctioning stoma was constructed more often in patients who had received radiation, and that the absence of a DS was significantly associated with a higher leakage rate.

We also reported our low mortality. This reflects our low leakage rate in cases without DS and our appropriate decision of reoperation for peritonitis in cases without DS. We considered that our appropriate decision lead to low mortality rate and high reoperation rate (54.2%). In the present study, a DS constructed at the time of initial surgery obviously reduced the necessity of an urgent reoperation after overt leakage, proving the clinical benefits of DS in this regard. The important objective of DS was not to eliminate leakage but to decrease the risk of reoperation. However, DS construction did not guarantee the complete safety of LAR. In fact, we experienced one mortality in a patient with DS in this series, so complete elimination of leakage and severe septic complications was not feasible.

In conclusion, we clearly demonstrated the outstanding safety of LAR with very low mortality and acceptable leakage rate in our group. Although this retrospective study could not prove whether DS can prevent leakage itself, we found that it could mitigate the need for urgent abdominal reoperation for leakage. To define clear criteria for DS construction, a well-designed randomized control study is genuinely needed in the future.

Acknowledgments This study was supported by the "Studies on the standardization for diagnosis, treatment, and follow-up of colorectal cancer patients", sponsored by Grant-in-Aid 18-2 for Cancer Research from the Ministry of Health, Welfare and Labor of Japan. The authors thank the following surgeons for generously providing data: S. Yamamoto (National Cancer Center Hospital), S. Noura (Osaka Medical Center for Cancer and Cardiovascular Diseases), Y. Komori (Aichi Cancer Center Hospital), and T. Kobatake (National Hospital Organization Shikoku Cancer Center Hospital).

References

1. Matthiessen P, Hallbook O, Rutegard J, Simert G, Sjodahl R (2007) Defunctioning stoma reduces symptomatic anastomotic leakage after low anterior resection of the rectum for cancer: a randomized multicenter trial. Ann Surg 246:207–214
2. Chude GG, Rayate NV, Patris V, Koshariya M, Jagad R, Kawamoto J et al (2008) Defunctioning loop ileostomy with low anterior resection for distal rectal cancer: should we make an ileostomy as a routine procedure? A prospective randomized study. Hepatogastroenterology 55:1562–1567
3. Huser N, Michalski CW, Erkan M, Schuster T, Rosenberg R, Kleeff J et al (2008) Systematic review and meta-analysis of the role of defunctioning stoma in low rectal cancer surgery. Ann Surg 248:52–60
4. Tan WS, Tang CL, Shi L, Eu KW (2009) Meta-analysis of defunctioning stomas in low anterior resection for rectal cancer. Br J Surg 96:462–472
5. Japanese Classification of Colorectal Carcinoma 2nd English edition, Japanese Society for Cancer of the Colon and Rectum. Tokyo: Kanehara; 2009
6. JSCCR Guidelines 2009 for the Treatment of Colorectal Cancer, Japanese Society for Cancer of the Colon and Rectum. Tokyo: Kanehara; 2009
7. Zaheer S, Pemberton JH, Farouk R, Dozois RR, Wolff BG, Ilstrup D (1998) Surgical treatment of adenocarcinoma of the rectum. Ann Surg 227:800–811
8. Sato H, Maeda K, Hanai T, Matsumoto M, Aoyama H, Matsuoka H (2006) Modified double-stapling technique in low anterior resection for lower rectal carcinoma. Surg Today 36:30–36
9. Moriya Y, Hojo K, Sawada T, Koyama Y (1989) Significance of lateral node dissection for advanced rectal carcinoma at or below the peritoneal reflection. Dis Colon Rectum 32:307–315
10. Sobin LH (2002) TNM classification of malignant tumours, 6th edn. Wiley-Liss, New York
11. Fain SN, Patin CS, Morgenstern L (1975) Use of a mechanical suturing apparatus in low colorectal anastomosis. Arch Surg 110:1079–1082
12. Yeh CY, Changchien CR, Wang JY, Chen JS, Chen HH, Chiang JM et al (2005) Pelvic drainage and other risk factors for leakage after elective anterior resection in rectal cancer patients: a prospective study of 978 patients. Ann Surg 241:9–13

13. Eriksen MT, Wibe A, Norstein J, Haffner J, Wiig JN (2005) Anastomotic leakage following routine mesorectal excision for rectal cancer in a national cohort of patients. Colorectal Dis 7:51–57
14. Matthiessen P, Hallbook O, Andersson M, Rutegard J, Sjodahl R (2004) Risk factors for anastomotic leakage after anterior resection of the rectum. Colorectal Dis 6:462–469
15. Rullier E, Laurent C, Garrelon JL, Michel P, Saric J, Parneix M (1998) Risk factors for anastomotic leakage after resection of rectal cancer. Br J Surg 85:355–358
16. Law WI, Chu KW, Ho JW, Chan CW (2000) Risk factors for anastomotic leakage after low anterior resection with total mesorectal excision. Am J Surg 179:92–96
17. Pakkastie TE, Luukkonen PE, Jarvinen HJ (1994) Anastomotic leakage after anterior resection of the rectum. Eur J Surg 160:293–297, discussion 299–300
18. Peeters KC, Tollenaar RA, Marijnen CA, Klein Kranenbarg E, Steup WH, Wiggers T et al (2005) Risk factors for anastomotic failure after total mesorectal excision of rectal cancer. Br J Surg 92:211–216
19. Hallbook O, Pahlman L, Krog M, Wexner SD, Sjodahl R (1996) Randomized comparison of straight and colonic J pouch anastomosis after low anterior resection. Ann Surg 224:58–65
20. Marusch F, Koch A, Schmidt U, Geibetaler S, Dralle H, Saeger HD et al (2002) Value of a protective stoma in low anterior resections for rectal cancer. Dis Colon Rectum 45:1164–1171
21. Wong NY, Eu KW (2005) A defunctioning ileostomy does not prevent clinical anastomotic leak after a low anterior resection: a prospective, comparative study. Dis Colon Rectum 48:2076–2079
22. Pakkastie TE, Ovaska JT, Pekkala ES, Luukkonen PE, Jarvinen HJ (1997) A randomised study of colostomies in low colorectal anastomoses. Eur J Surg 163:929–933
23. Graffner H, Fredlund P, Olsson SA, Oscarson J, Petersson BG (1983) Protective colostomy in low anterior resection of the rectum using the EEA stapling instrument. A randomized study. Dis Colon Rectum 26:87–90
24. Lange MM, Buunen M, van de Velde CJ, Lange JF (2008) Level of arterial ligation in rectal cancer surgery: low tie preferred over high tie. A review. Dis Colon Rectum 51:1139–1145
25. Seike K, Koda K, Saito N, Oda K, Kosugi C, Shimizu K et al (2007) Laser Doppler assessment of the influence of division at the root of the inferior mesenteric artery on anastomotic blood flow in rectosigmoid cancer surgery. Int J Colorectal Dis 22:689–697
26. Kapiteijn E, Marijnen CA, Nagtegaal ID, Putter H, Steup WH, Wiggers T et al (2001) Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer. N Engl J Med 345:638–646
27. Swedish Rectal Cancer Trial (1997) Improved survival with preoperative radiotherapy in resectable rectal cancer. N Engl J Med 336:980–987
28. Bosset JF, Calais G, Daban A, Berger C, Radosevic-Jelic L, Maingon P et al (2004) Preoperative chemoradiotherapy versus preoperative radiotherapy in rectal cancer patients: assessment of acute toxicity and treatment compliance. Report of the 22921 randomised trial conducted by the EORTC Radiotherapy Group. Eur J Cancer 40:219–224

Original Paper

Digestive Surgery

Dig Surg 2011;28:404–409
DOI: 10.1159/000332007

Received: July 12, 2011
Accepted after revision: August 16, 2011
Published online: December 20, 2011

Short-Term Outcomes of Laparoscopic Intersphincteric Resection for Lower Rectal Cancer and Comparison with Open Approach

Seiichiro Yamamoto Shin Fujita Takayuki Akasu Ryo Inada Masashi Takawa Yoshihiro Moriya

Division of Colorectal Surgery, National Cancer Center Hospital, Tokyo, Japan

Key Words

Laparoscopic surgery, complications · Intersphincteric resection, laparoscopic, open · Rectal cancer, lower

Abstract

Background/Aims: To evaluate the short-term surgical outcomes of laparoscopic intersphincteric resection (ISR) for lower rectal cancer, and to compare them with a case-control series of open ISR. ***Methods:*** Between July 2002 and March 2011, 29 patients with lower rectal cancer underwent laparoscopic ISR, and 22 of 29 patients who underwent laparoscopic ISR were compared with the control open ISR group of patients matched for age, gender, operative procedure and pathological stage. ***Results:*** There was no perioperative mortality, 8 complications occurred in 7 patients, and the morbidity rate was 24.1% (7/29). Leakage occurred in 1 patient (3.4%) in the laparoscopic ISR group. Regarding the matched case-control study, the operative time was significantly longer ($p = 0.0007$), but blood loss was significantly lower ($p = 0.0003$) in the laparoscopic ISR group. The median postoperative hospital stay was 8 days in the laparoscopic ISR group, which was significantly shorter than in the open ISR group (14 days). Postoperative complication rates were similar. In the laparoscopic ISR group, the levels of C-reactive protein on postoperative days 1–3 were significantly lower than in the open ISR group. ***Conclusions:*** Laparoscopic ISR for lower rectal cancer provides benefits in the early postoperative period without increasing morbidity or mortality.

Introduction

Controversy still persists regarding the appropriateness of laparoscopic surgery (LS) for patients with rectal cancer because of concerns over the safety of the procedure and the uncertainty of the long-term outcome. The advantages of LS for rectal cancer have been reported; LS for rectal cancer is associated with a reduction in intraoperative blood loss and the number of transfused patients; however, laparoscopic rectal excision has procedural complexities and technical difficulties, and LS in patients with rectal cancer is technically demanding [1]. Due to the high complication rate, it is unclear whether LS for rectal cancer should be regarded as a minimally invasive surgery [2].

Abdominoperineal resection was originally the standard surgery for patients with rectal adenocarcinoma located within 5 cm from the anal verge [3]. Intersphincteric resection (ISR) was developed in the 1980s to avoid permanent colostomy for such patients, and this procedure by the open approach became well established in the 1990s [4–6]. ISR involves resection of part or all of the internal sphincter from a per anal approach and restoration of bowel continuity while obtaining sufficient margins for rectal cancers involving or close to the anal canal, and ISR is performed in combination with total mesorectal excision.

At our institution, open ISR was introduced in the 1990s, and laparoscopic ISR was started in 2002 following

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0253–4886/11/0286–0404$38.00/0

Accessible online at:
www.karger.com/dsu

Dr Seiichiro Yamamoto
Division of Colorectal Surgery
National Cancer Center Hospital
5-1-1, Tsukiji, Chuo-ku, Tokyo 104-0045 (Japan)
Tel. +81 3 3542 2511, E-Mail seyamamo@ncc.go.jp

advances in laparoscopic techniques. In previous reports, we have demonstrated that open ISR for rectal cancer is technically feasible and oncologically safe; however, laparoscopic ISR is still not an established technique, and there are only a few reports on the use of this procedure [7–12]. Moreover, due to the lack of comparative study, it is currently still controversial as to whether laparoscopic ISR can be regarded as a minimally invasive surgery. The aims of the present study are to evaluate the surgical outcomes of laparoscopic ISR for lower rectal cancer, and to compare these outcomes with a control series of cases treated by open ISR.

Patients and Methods

Between July 2002 and January 2011, we performed 29 continuous laparoscopic ISR for selected patients with lower rectal cancer, and the study took the form of a single-center, prospective, observational, case-series analysis. Moreover, 22 of 29 patients who underwent laparoscopic ISR were compared with 22 of 159 control open ISR patients matched for age, gender, operative procedure and pathological stage. Seven patients who underwent laparoscopic ISR were excluded from the comparative study because we could not find a matched open ISR case.

Selection criteria for open ISR were as follows: (1) sufficient medical fitness; (2) normal sphincter function; (3) distance between the lower edge and the dentate line of <3 cm; (4) no involvement of the external sphincter, and (5) no signs of disseminated disease or clinical T4 disease. Because the safety of LS in cancer patients remains to be established, candidates for laparoscopic ISR were basically patients who were preoperatively diagnosed with T1 or T2 disease. Laparoscopic ISR was also performed in patients who were preoperatively diagnosed with T3/4 but wished to undergo LS. Six patients registered for the clinical trial, a phase II trial to evaluate laparoscopic surgery for stage 0/I rectal carcinoma [13], are included in the present study. We excluded the following groups of patients from laparoscopic resection: patients with tumors of >8 cm; patients with a prior history of extensive adhesions; patients with severe obesity (body mass index >30); patients with intestinal obstruction, and patients who did not consent to LS.

All patients were evaluated before surgery by clinical investigation, including barium enema or computed tomographic colonography, total colonoscopy, chest X-ray, abdominal ultrasonography, endorectal ultrasonography, thin-section helical CT, or high-resolution magnetic resonance.

LS was converted to open surgery when open techniques were used to cope with unexpected intraoperative difficulties, regardless of the size of the wound.

The techniques of open and laparoscopic ISR have been thoroughly described previously [7–9, 14, 15]. After mobilization of the left colon and splenic flexure, intracorporeal high ligation of the inferior mesenteric vessels was performed. Recently, the laparoscopic median-to-lateral approach has been indicated. In this approach, medial-to-lateral retroperitoneal dissection of the mesocolon and early division of the inferior mesenteric vessels were performed, which preserved the inferior mesenteric plexus and superior hypogastric plexus. After full mobilization of the rectum, the intersphincteric plane between the puborectalis and the internal sphincter was cautiously dissected as caudad as possible under laparoscopic vision. After retractors were applied to the anal canal, it was closed just below the tumor by purse-string sutures, and then irrigated with povidone iodine followed by saline. After irrigation, the anal canal mucosa and internal sphincter were circumferentially incised, and the intersphincteric plane was dissected cephalad. A resection margin of at least 1 cm was always attempted. After removal of the rectum through the anus, the pelvic cavity and anal canal were washed, and then a coloanal anastomosis was made using 4-0 absorbable vertical mattress sutures. A pelvic drain was placed, and a defunctioning ileostomy was made. In all cases, the retroperitoneum was not repaired.

Parameters analyzed included gender, age, body mass index, prior abdominal surgery, preceding local resection, ASA classification, pathological stage, size of the tumor, lymph nodes removed, operative time, operative blood loss, conversion, combined surgery, colonic pouch, days to resume diet, duration of postoperative hospital stay, and both intraoperative and postoperative complications within 30 days of surgery. Pathological staging was performed according to the TNM stage. White blood cell count and C-reactive protein (CRP) in serum were measured preoperatively and on postoperative day 1 routinely, and on postoperative days 2 and 3 if necessary. Data on combined surgical techniques were all included in the analyses of cancer surgeries.

Our institutional review board does not mandate obtaining its approval for the collection of patient clinical records prospectively and for publication as an institutional case-series study. All patients gave their informed consent for usage of their data for analysis in the future.

Statistical analysis was performed using SPSS ver. 11.0 software (SPSS, Chicago, Ill., USA), and Student's t test, the Mann-Whitney U test, and Fisher's exact test were used as appropriate. A p value of <0.05 was considered significant.

Results

Patient demographics of the case-series analysis are summarized in table 1. All the operations were completed laparoscopically in this series. Positive margin rate was 0 in the present series. With regard to simultaneous surgical techniques, 1 patient underwent laparoscopic cholecystectomy for a gallbladder stone. There was no perioperative mortality, 8 complications occurred in 7 patients, and the morbidity rate was 24.1% (7/29). Anastomotic leakage occurred in 1 patient (3.4%). The postoperative course of the patient with anastomotic leakage was uneventful except for urinary tract infection that was managed by per oral antibiotics, and the patient was discharged on the 8th postoperative day without symptoms. Two months after the initial operation, routine radiological examination before ileostomy closure demonstrated a minor anastomotic leakage. The patient was symptom

Table 1. Demographic data of patients who underwent laparoscopic ISR

	Lap. ISR group
Number of patients	29
Sex ratio (male:female)	19:10
Mean age, years	57 (34–70)
Mean body mass index	22.0 (16.8–26.7)
Prior abdominal surgery	8 (27.6)
Preceding local resection	10 (34.5)
ASA (I:II)	16:13
Pathological stage	
UICC stage I	20
UICC stage II	3
UICC stage III	6
Mean tumor size, mm[1]	25 (15–60)
Median Lymph nodes resected	13 (3–40)
Median operative time, min	335 (256–500)
Median blood loss, ml	109 (27–477)
Conversion	0
Preservation of left colic artery (yes:no)	25:4
Combined surgery (yes:no)	1:28
Colonic pouch (yes:no)	4:25
Median time to liquid intake, days	1 (1–2)
Median time to solid intake, days	2 (2–3)
Median length of hospital stay, days	8 (7–10)
Mortality	0
Morbidity	
Mucosal prolapse	2
Anastomotic leakage	1
Bowel obstruction	1
Wound sepsis	1
Perianastomotic abscess	1
Dehydration	1
Urinary tract infection	1
Total number of patients	7

Values in parentheses are ranges or percentages. [1] Preoperatively locally resected cases are not included.

free, and after conservative observation for 3 months, the leakage disappeared, and the patient underwent ileostomy closure 5 months after the initial operation. At the end of the study period, 2 patients developed recurrence of cancer (6.9%). One patient with pathological stage IIIC developed para-aortic and mediastinum lymph node metastasis 4 years after the initial operation, and another patient with pathological stage I developed pulmonary metastasis 2 years after the initial operation. All the patients are still alive. At 24 months or more after stoma closure, daytime and nocturnal leakage was observed in 6 (1/17) and 18% (3/17), respectively.

The demographic characteristics of the case-control study are shown in table 2. Cases and controls were well matched; however, the body mass index of the open ISR group was slightly higher ($p = 0.0804$).

Operative and postoperative results are shown in table 3. In the laparoscopic ISR group, the operative time was significantly longer ($p = 0.0007$), but blood loss was significantly lower ($p = 0.0003$) than in the open ISR group. The median postoperative hospital stay was 8 days in the laparoscopic ISR group, which was significantly shorter than in the open ISR group (14 days).

Postoperative complications are listed in table 4. There was no perioperative mortality. The morbidity rate was 32% (7/22) in the laparoscopic ISR group, and 59% (13/22) in the control group. No significant differences were observed in complication rates between the 2 groups.

White blood cell count and CRP levels after surgery are presented in figure 1. In laparoscopic ISR, the levels of CRP on postoperative days 1–3 were significantly lower than in the open ISR group.

In the laparoscopic ISR group, all patients underwent ileostomy closure.

Discussion

ISR was first introduced as an alternative option to avoid permanent colostomy for selected patients, and is now regarded as the standard surgical treatment for sphincter preservation and excision for extremely low rectal cancer. In the early stages, it was unclear whether there was an increased risk of local recurrence with ISR; however, recent studies have shown that short-term outcomes and oncological results after ISR are satisfactory in patients with low rectal cancer [4–9, 16, 17]. Unfortunately, most of these reports have involved open ISR, whereas there have been few reports on laparoscopic ISR.

Laparoscopic ISR was first described by Watanabe et al. [18] in 2000. Some case series on laparoscopic ISR have subsequently been published, but the technique requires a higher level of skill than laparoscopic low anterior resection (LAR) and has yet to be recognized as a common procedure [10–12]. In our institution, open ISR was introduced in the 1990s and laparoscopic ISR was started in 2002 following accumulation of experience with the open approach and advances in laparoscopic techniques [7–9, 14, 15]. In the early era in both open and laparoscopic ISR, the indications for newly developed techniques were patients at relatively early stages, because the safety of the technique remained to be established. After confirmation of technical safety, we gradually ex-

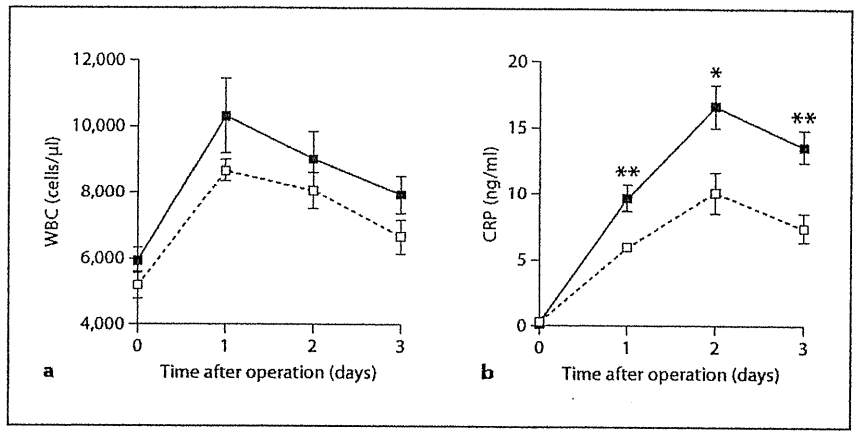

Fig. 1. Changes in white blood cell count (WBC; **a**) and CRP levels (**b**). □ = Laparoscopic ISR; ■ = open ISR. The difference between the 2 groups in CRP levels was significant: * $p < 0.05$; ** $p < 0.01$. Each bar represents the mean ± standard error.

Table 2. Patient characteristics in the case-control study

	Laparoscopic ISR group	Open ISR group	p value
Number of patients	22	22	
Sex ratio (male:female)	16:6	16:6	1
Mean age, years	55 (34–68)	58 (35–69)	0.4334
Mean body mass index	21.8 (16.8–26.7)	22.5 (19.3–28.9)	0.1804
Prior abdominal surgery	6 (27.2)	7 (31.8)	1
Preceding local resection	7 (31.8)	4 (18.2)	0.4876
ASA (I:II)	14:8	17:5	0.1464
Pathological stage (TNM stage)			
Stage I	17	18	
Stage II	1	1	
Stage III	4	3	
I:II+III	17:5	18:4	1
Mean tumor size, mm[1]	22 (15–38)	28 (11–55)	0.1549
Median lymph nodes resected	13 (3–27)	14 (529)	0.929

Values in parentheses are ranges or percentages. [1] Preoperatively locally resected cases not included.

Table 3. Intraoperative and postoperative results

	Laparoscopic ISR group	Open ISR group	p value
Operative time, min	385 (305–500)	299 (202–475)	0.0007
Blood loss, ml	139 (45–477)	434 (76–1108)	0.0003
Conversion	0	–	–
Preservation of left colic artery (yes:no)	4:18	4:18	1.0000
Combined surgery (yes:no)	2:20	1:21	1.0000
Colonic pouch (yes:no)	4:18	4:18	1.0000
Time to liquid intake, days	1 (1–2)	3 (2–11)	<0.001
Time to solid intake, days	2 (2–3)	5 (3–12)	<0.001
Length of hospital stay, days	8 (7–10)	14 (10–40)	<0.001

Values are numbers or medians (range).

Table 4. Morbidity and mortality

	Laparoscopic ISR group	Open ISR group	p value
Mortality	0	0	
Morbidity			
Anastomotic leakage	1	1	
Wound sepsis	1	4	
Bowel obstruction	1	2	
Perianastomotic abscess	1	1	
Urinary tract infection	1	1	
Dehydration	1	2	
Mucosal prolapse	2	1	
Cholecystitis	0	1	
Total number of patients	7 (32%)	13 (59%)	0.12922

panded the indication for new procedures. In the present study, a review was performed of laparoscopic ISR for lower rectal cancer, and our results demonstrated that it is a safe procedure and provides benefits in the early postoperative period without increasing morbidity or mortality. Moreover, this is the first report to conduct a comparative study between laparoscopic and open ISR, and the findings of the current study demonstrated the feasibility and safety of laparoscopic ISR compared to open ISR for selected patients with lower rectal cancer.

ISR is a demanding technique that requires experienced colorectal surgeons, regardless of whether it is performed as open or LS, and the number of surgeons who can perform laparoscopic ISR is particularly limited. In a comparison of open and laparoscopic ISR in a relatively small number of cases, Fujimoto et al. [12] found that the complication rates of the two methods did not differ. The results of the present study are similar to their results, and moreover, we found that postoperative inflammatory reactions were significantly lower after laparoscopic ISR than open ISR, based on decreased CRP levels after laparoscopic ISR. These differences in inflammatory markers suggest that laparoscopic ISR may be less invasive than open ISR. In addition, the oncological outcomes after laparoscopic ISR were acceptable with a low recurrence rate, although we note that many of the patients who underwent laparoscopic ISR had early-stage disease. These results suggest that the indications for laparoscopic ISR can be expanded, provided that the operation is conducted by an experienced surgical team.

It is noteworthy that anastomotic leakage was relatively low after laparoscopic ISR in the present study. Anastomotic leakage after rectal cancer surgery performed by open or laparoscopic techniques with per anal hand-sewn anastomosis or the double-stapling technique (DST) can result in reoperation, morbidity, mortality, permanent stoma, prolonged hospitalization, anal stenosis and anal dysfunction, and may be associated with a higher local recurrence rate. Tension-free anastomosis with full mobilization and anastomosis at a site with good blood flow are important factors to avoid leakage. In addition, the high anastomotic leakage rate in ISR in previous reports suggests that a covering ileostomy is needed to stabilize the anastomotic region in a resting position [4–6, 9–12]. In our institution, the anastomotic leakage rate in open ISR has decreased with the accumulated experience of surgeons, and fortunately, the anastomotic leakage rate was relatively low in laparoscopic ISR. Thus, when performed by surgeons with sufficient LS skills, laparoscopic ISR can be regarded as a safe procedure.

In the previous study, Laurent et al. [19] reported that the risk of anastomotic leakage is increased in male patients with lower rectal cancer in laparoscopic LAR with DST reconstruction; therefore, they recommended open or coloanal hand-sewn anastomosis in male patients with rectal cancer. In our institution, the anastomotic leakage rate was 7.7% (3/39) in patients with low rectal cancer who underwent laparoscopic LAR with DST reconstruction. This rate was higher than that in patients who underwent laparoscopic ISR. Therefore, LS with DST reconstruction may not be the best choice in male patients or in patients in whom laparoscopic LAR with DST reconstruction is difficult, and coloanal anastomosis should be considered in these cases.

There are several limitations in the design of the study. First, the study was not randomized but was performed retrospectively, which may have caused bias. Thus, a prospective, multicenter, randomized clinical trial (RCT) is required to demonstrate that laparoscopic total mesorectal excision with ISR is a feasible procedure for very low rectal cancer; however, due to the lack of sufficient patients to perform an RCT, we chose to analyze the safety of laparoscopic ISR in a single-center study. Second, a longer follow-up is required to assess the incidences of local recurrence, cancer-free survival, and functional outcome. Third, patients who underwent preoperative adjuvant chemoradiotherapy or lateral lymph node dissection were not included because most of the patients who underwent laparoscopic ISR in the present study were in clinical stage I. Another concern for preoperative adjuvant chemoradiotherapy is that preoperative chemoradiotherapy was identified as the risk factor with the greatest negative impact on anal

function after ISR [20]. The outcomes of patients without preoperative adjuvant chemoradiotherapy in our hospital have been reported previously, and we conducted preoperative adjuvant chemoradiotherapy only in patients with clinical T4 cancer and/or involvement of lateral pelvic lymph nodes [21]. Open surgery is still our gold standard approach for patients with locally advanced rectal cancer, and the safety of the laparoscopic approach requires further examination in patients with advanced rectal cancer.

Laparoscopic ISR for lower rectal cancer provides benefits in the early postoperative period without increasing morbidity or mortality, and shows long-term benefits that are comparable to those after open ISR in selected patients with lower rectal cancer. In the absence of a large-scale RCT comparing open and laparoscopic ISR, and given the small number of institutions capable of conducting high-quality laparoscopic ISR, the safety of this procedure requires confirmation through prospective accumulation of more cases.

References

1 Ohtani H, Tamamori Y, Azuma T, Mori Y, Nishiguchi Y, Maeda K, Hirakawa K: A meta-analysis of the short- and long-term results of randomized controlled trials that compared laparoscopy-assisted and conventional open surgery for rectal cancer. J Gastrointest Surg 2011;15:1375–1385.

2 Guillou PJ, Quirke P, Thorpe H, Walker J, Jayne DG, Smith AM, Heath RM, Brown JM; MRC CLASICC trial group: Short-term endpoints of conventional versus laparoscopic-assisted surgery in patients with colorectal cancer (MRC CLASICC trial): multicentre, randomised controlled trial. Lancet 2005; 365:1718–1726.

3 Nicholls RJ, Hall C: Treatment of non-disseminated cancer of the lower rectum. Br J Surg 1996;83:15–18.

4 Basso N, Minervini S, Marcelli M: Modified abdominotransanal resection for cancer of the lower third of the rectum. Dis Colon Rectum 1987;30:641–643.

5 Kusunoki M, Shoji Y, Yanagi H, Fujita S, Hatada T, Sakanoue Y, Yamamura T, Utsunomiya J: Modified anoabdominal rectal resection and colonic J-pouch anal anastomosis for lower rectal carcinoma: preliminary report. Surgery 1992;112:876–883.

6 Schiessel R, Karner-Hanusch J, Herbst F, Teleky B, Wunderlich M: Intersphincteric resection for low rectal tumors. Br J Surg 1994;81:1376–1378.

7 Akasu T, Takawa M, Yamamoto S, Fujita S, Moriya Y: Incidence and patterns of recurrence after intersphincteric resection for very low rectal adenocarcinoma. J Am Coll Surg 2007;205:642–647.

8 Akasu T, Takawa M, Yamamoto S, Ishiguro S, Yamaguchi T, Fujita S, Moriya Y, Nakanishi Y: Intersphincteric resection for very low rectal adenocarcinoma: univariate and multivariate analyses of risk factors for recurrence. Ann Surg Oncol 2008;15:2668–2676.

9 Akasu T, Takawa M, Yamamoto S, Yamaguchi T, Fujita S, Moriya Y: Risk factors for anastomotic leakage following intersphincteric resection for very low rectal adenocarcinoma. J Gastrointest Surg 2010:14:104–111.

10 Rullier E, Sa Cunha A, Couderc P, Rullier A, Gontier R, Saric J: Laparoscopic intersphincteric resection with coloplasty and coloanal anastomosis for mid and low rectal cancer. Br J Surg 2003:90:445–451.

11 Bretagnol F, Rullier E, Couderc P, Rullier A, Saric J: Technical and oncological feasibility of laparoscopic total mesorectal excision with pouch coloanal anastomosis for rectal cancer. Colorectal Dis 2003:5:451–453.

12 Fujimoto Y, Akiyoshi T, Kuroyanagi H, Konishi T, Ueno M, Oya M, Yamaguchi T: Safety and feasibility of laparoscopic intersphincteric resection for very low rectal cancer. J Gastrointest Surg 2010;14:645–650.

13 Yamamoto S, Yoshimura K, Konishi F, Watanabe M: Phase II trial to evaluate laparoscopic surgery for stage 0/I rectal carcinoma. Jpn J Clin Oncol 2008;38:497–500.

14 Yamamoto S, Fujita S, Akasu T, Moriya Y: A comparison of the complication rates between laparoscopic colectomy and laparoscopic low anterior resection. Surg Endosc 2004;18:1447–1451.

15 Yamamoto S, Fujita S, Akasu T, Uehara K, Moriya Y: Reduction of prolonged postoperative hospital stay after laparoscopic surgery for colorectal carcinoma. Surgical Endoscopy 2006;20:1467–1472.

16 Chamlou R, Parc Y, Simon T, Bennis M, Dehni N, Parc R, Tiret E: Long-term results of intersphincteric resection for low rectal cancer. Ann Surg 2007;246:916–921.

17 Portier G, Ghouti L, Kirzin S, Guimbaud R, Rives M, Lazorthes F: Oncological outcome of ultra-low coloanal anastomosis with and without intersphincteric resection for low rectal adenocarcinoma. Br J Surg 2007;94: 341–345.

18 Watanabe M, Teramoto T, Hasegawa H, Kitajima M: Laparoscopic ultralow anterior resection combined with per anum intersphincteric rectal dissection for lower rectal cancer. Dis Colon Rectum 2000;43:S94–S97.

19 Laurent C, Leblanc F, Gineste C, Saric J, Rullier E: Laparoscopic approach in surgical treatment of rectal cancer. Br J Surg 2007;94: 1555–1561.

20 Ito M, Saito N, Sugito M, Kobayashi A, Nishizawa Y, Tsunoda Y: Analysis of clinical factors associated with anal function after intersphincteric resection for very low rectal cancer. Dis Colon Rectum 2009;52:64–70.

21 Fujita S, Yamamoto S, Akasu T, Moriya Y: Outcome of patients with clinical stage II or III rectal cancer treated without adjuvant radiotherapy. Int J Colorectal Dis 2008;23: 1073–1079.

CASE REPORT

Cecal schwannoma with laparoscopic wedge resection: Report of a case

T Matsumoto, S Yamamoto, S Fujita, T Akasu & Y Moriya*

National Cancer Center Hospital, Colorectal Surgery Division, Tokyo, Japan

Keywords
Laparoscopic wedge resection; schwannoma; submucosal tumor

Correspondence
Seiichiro Yamamoto, National Cancer Center Hospital, Colorectal Surgery Division, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan.
Tel: +81 3 3542 2511
Fax: +81 3 3542 3815
Email: seyamamo@ncc.go.jp

Received: 16 March 2011; revised 14 April 2011; accepted 9 May 2011.

DOI:10.1111/j.1758-5910.2011.00089.x

Abstract

Schwannomas of the large intestine are relatively rare. Here, we present a case of schwannoma of the cecum in a 59-year-old woman that was successfully resected by laparoscopic wedge resection. In a medical checkup, a colonoscopy revealed a 2 cm submucosal lesion, without mucosal changes, located in the cecum wall contralateral to Bauhin's valve. Abdominal contrast CT and contrast MRI indicated a smooth-surfaced, semi-round tumor of about 2 cm that gave an enhanced homogeneous signal in the cecum. Laparoscopic wedge resection was performed after the diagnosis of benign submucosal tumor. The lesion was 2.5 × 2.0 cm, was histologically composed of spindle neoplastic cells arranged in cords, was positive for S-100 and vimentin, and was diagnosed as schwannoma. The details of this case are reported herein and focus on the successful application of laparoscopic wedge resection for treatment of the colonic submucosal lesion.

Introduction

Laparoscopic surgery (LS) is a minimally invasive procedure that can improve quality of life. Randomized controlled trials have demonstrated that LS for colon cancer can improve quality of life through early recovery and that its long-term survival rates are not inferior to that of open surgery (1–3).

We sometimes encounter intestinal submucosal tumors (SMT) that require diagnostic resection. However, the surgical treatment for SMT requires careful planning based on multimodality images that identify the location of the tumor. Here, we report a rare case of cecal schwannoma treated successfully with laparoscopic wedge excision.

Case Presentation

A 59-year-old woman was referred to the Division of Colorectal Surgery at the National Cancer Center Hospital, Tokyo, Japan, for the treatment of cecal SMT, which was diagnosed by colonoscopy at a local hospital during a medical checkup. She only had a history of appendectomy. Laboratory workup showed no abnormality. The abdominal tumor was not palpable in physical findings. A colonoscopy performed at our hospital showed a SMT of about 2 cm without mucosal changes in the cecum wall contralateral to Bauhin's valve (Figure 1). A biopsy demonstrated no abnormality. Abdominal contrast CT indicated a clearly demarcated, semi-round tumor of about 2 cm that gave an enhanced and relatively homogeneous signal (Figure 2). There were no signs of malignancy such as enlarged lymph nodes and distant metastases. On abdominal MRI, the tumor gave homogeneous low-intensity signals on T_1- and T_2-weighted images. On opposed-phase T_1 images, there was no signal intensity loss compared to the in-phase images, which suggested that there was little fat inside. Therefore, we diagnosed cecal SMT (e.g. gastrointestinal stromal tumor [GIST] or neurogenic tumor). The tumor was considered to be benign based on image findings, and surgical resection or close follow-up was offered to the patient. The patient requested surgical resection, and we performed a laparoscopic cecal wedge resection.

We used three trocars in surgery. Pneumoperitoneum was established by the open laparotomy technique through supraumbilical incision. Under laparoscopic guidance, one 5 mm port was inserted on the right epigastric midclavicular line and one 12 mm port in the mid-lower abdominal region. The operating table, tilted to the left, was placed in the head down position. Then 2 cm tumor

*All authors took part in the work and agree with the contents of this manuscript.

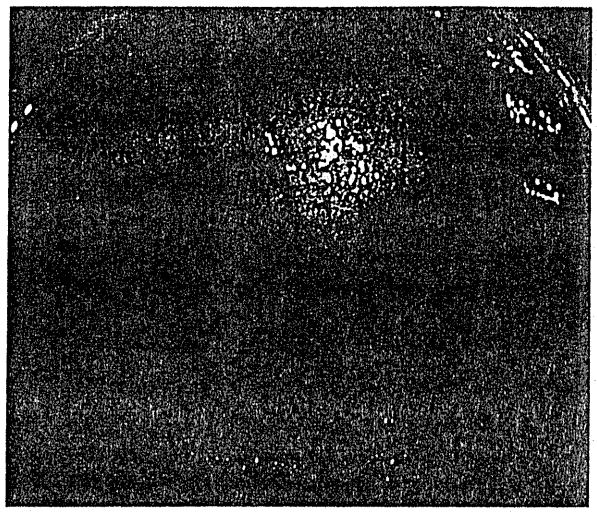

Figure 1 A colonoscopy shows a 2 cm-sized submucosal tumor, without mucosal changes, in the cecum wall contralateral to Bauhin's valve.

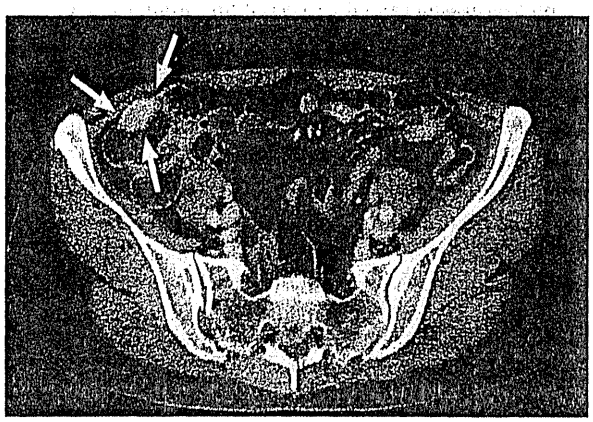

Figure 2 Abdominal contrast CT depicts a well-demarcated, semi-round tumor (white arrows) with relatively homogeneous enhancement.

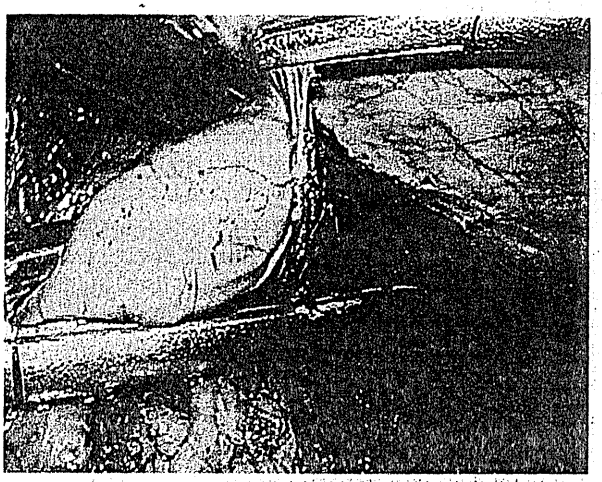

Figure 3 The tumor was fully elevated with a clamp and then extirpated while a sufficient margin was secured with endolinear stapler. Two stapler cartridges were needed to complete the resection.

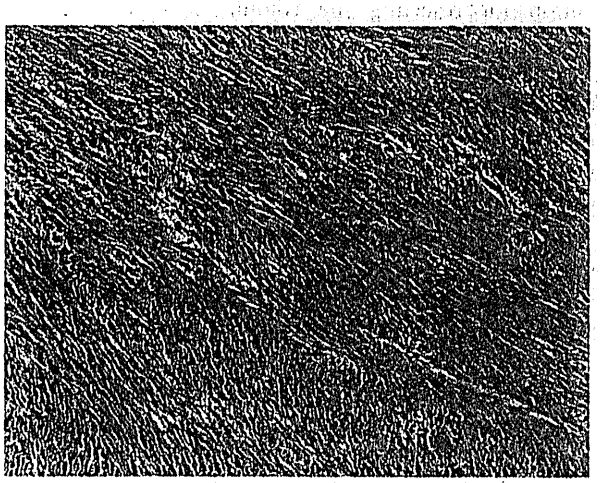

Figure 4 Most parts of the whole tumor were located in the muscularis propria. The tumor was histopathologically composed of spindle neoplastic cells arranged in cords (HE stain × 100).

in the ventrolateral part of the cecum was confirmed by laparoscopy. After mobilization of the ileocecum, the tumor was fully elevated with a clamp and then extirpated based on secured sufficient margin using an endolinear stapler (Figure 3). The tumor was removed from the umbilicus port using Endo Catch (Covidien, Mansfield, USA). Two stapler cartridges were needed to complete the resection. The total operation time was 115 minutes, and blood loss was 4 mL. An elastic hard SMT of about 2.5 × 2.0 cm was detected macroscopically, but no macroscopic invasion was found on the mucosal surface and serosa. A margin of 5 mm was secured. The tumor was solid and the cut surface was uniform yellowish-white. Histopathological examination demonstrated that most of the tumor was located in the muscularis propria and was composed of spindle neoplastic cells arranged in cords (Figure 4). Immunostaining of the tumor was positive for S-100 and vimentin, and negative for c-kit, CD34, αSMA, h-caldesmon, and desmin. The MIB-1 index was 3% or less. Finally, the diagnosis was benign schwannoma. Postoperative recovery was good, and the patient was discharged 4 days postoperative. At present, 2 years after surgery, she is free of symptoms and continues an uneventful course.

Discussion

Here, we have described a case with the preoperative diagnosis of benign cecal SMT, which resulted in a successful wedge resection by laparoscope. The ratio of benign to malignant tumors in large intestinal SMT is almost 6 to 4, and pathologically, large intestinal SMT

may be lipoma, lymphangioma, leiomyoma, malignant lymphoma, carcinoid tumor, or GIST (4). Hence, treatment strategy (e.g. endoscopic or open resection) should be decided after a multimodality image is obtained.

In our case, the lesion was an SMT without ulceration. The pathological diagnosis could not be confirmed by preoperative biopsy, but the tumor was considered benign based on image findings and minimally invasive surgery was indicated. This surgical technique potentially poses a threat of postoperative intestinal stenosis. Fortunately, because the tumor was located contralateral to Bauhin's valve, safe wedge resection could be performed laparoscopically using vertical stapling to the intestine axis, which decreased the potential for intestinal stenosis. Had extended surgery been required based on the intraoperative findings, a technical change to laparoscopic right hemicolectomy would have been straightforward. Combined laparoscopic-endoscopic resection was another option for resecting the colonic SMT that did not require lymph node removal (5). Thus, LS should be considered for suspected benign SMT in the cecum or large intestine. For SMT in the lower rectum, transanal resection, transsacral resection, transanal endoscopic microsurgery, or minimally invasive transanal surgery should be considered.

Schwannomas are benign tumors originating from the peripheral perineurium that have a predilection for the surface of flexor muscles of the extremities, neck, mediastinum, retroperitoneum, back of the spinal root, and cerebellopontine angle (6). Based on immunostaining findings, many cases with various causes and symptoms have been reported under the general name of schwannoma, but cases of gastrointestinal schwannomas are relatively rare (4). Furthermore, almost all these cases involved lesions in the stomach and small intestine, and the percentage of schwannomas in the large intestine is about 4.8% among all gastrointestinal schwannomas (7). In many cases, schwannomas were SMT or polyps with ulceration on the top and were sometimes accompanied by bleeding (8,9). Schwannomas in the cecum have been reported in Western patients (10), while many Japanese reports have shown lesions in the rectum (9). Differential diagnosis from GIST is essential and the judgment of myogenicity or neurogenicity cannot be made based on HE staining only. Immunostaining is needed for accurate diagnosis, especially as schwannomas are typically positive for S-100 and vimentin, and negative for desmin, keratin, c-kit, CD34 and αSMA (4,6). The index for malignancy is based on the dimension of the nucleus and mitosis, but the size of the nucleus in schwannomas often varies and there is no established index. Cases of malignant changes are rare (8).

In summary, we have reported a case of cecal schwannoma removed by laparoscopic wedge resection. Laparoscopic wedge resection may be a useful procedure for the treatment of small, colonic submucosal lesions.

Acknowledgement

We all did not have any financial support or relationships that may pose a conflict of interest.

References

1. The Clinical Outcomes of Surgical Therapy Study Group. A comparison of laparoscopically assisted and open colectomy for colon cancer. *N Engl J Med* 2004; **350**: 2050–2059.
2. Fleshman J, Sargent DJ, Green E *et al.* Laparoscopic colectomy for cancer is not inferior to open surgery based on 5-year data from the COST Study Group trial. *Ann Surg* 2007; **246**: 655–662, discussion.
3. Buunen M, Veldkamp R, Hop WC *et al.* Survival after laparoscopic surgery versus open surgery for colon cancer: Long-term outcome of a randomised clinical trial. *Lancet Oncol* 2009; **10**: 44–52.
4. Hamilton SR & Aaltonen LA. Pathology and genetics tumors of the digestive system. In: Kleihues P & Sobin LH, (eds). *World Health Organization classification of tumors*. Lyon: International Agency for Research on Cancer, 2000; 65.
5. Wilhelm D. Combined laparoscopic-endoscopic resections of colorectal polyps: 10-year experience and follow-up. *Surg Endosc* 2009; **23**: 688–693.
6. Rosai J. Soft tissues. In: Rosai J., (ed). *Surgical pathology*, 9th ed. London: Mosby, Elsevier's Health Sciences, 2004 pp 2264–2269.
7. Stout AP. The peritoneal manifestation of the specific nerve sheath tumor (neurilemoma). *Am J Cancer* 1935; **24**: 751–796.
8. Fotiadis CI, Kouerinis IA, Papandreou I *et al.* Sigmoid schwannoma: A rare case. *World J Gastroenterol* 2005; **11**: 5079–5081.
9. Tomozawa S, Masaki T, Matsuda K *et al.* A schwannoma of the cecum: Case report and review of Japanese schwannomas in the large intestine. *J Gastroenterol* 1998; **33**: 872–875.
10. Miettinen M, Shekitka KM, Sobin LH. Schwannomas in the colon and rectum: A clinicopathologic and immunohistochemical study of 20 cases. *Am J Surg Pathol* 2001; **25**: 846–855.

Radiofrequency ablation for hepatocellular carcinoma induces glypican-3 peptide-specific cytotoxic T lymphocytes

DAISUKE NOBUOKA[1,4*], YUTAKA MOTOMURA[1,5*], HIROFUMI SHIRAKAWA[1], TOSHIAKI YOSHIKAWA[1], TOSHIMITSU KURONUMA[1], MARI TAKAHASHI[1], KOHEI NAKACHI[2], HIROSHI ISHII[2], JUNJI FURUSE[2], NAOTO GOTOHDA[3], SHINICHIRO TAKAHASHI[3], TOSHIO NAKAGOHRI[3], MASARU KONISHI[3], TAIRA KINOSHITA[3], HIROYUKI KOMORI[5], HIDEO BABA[5], TOSHIYOSHI FUJIWARA[4] and TETSUYA NAKATSURA[1]

[1]Section for Cancer Immunotherapy, Investigative Treatment Division, Research Center for Innovative Oncology, [2]Division of Hepatobiliary and Pancreatic Oncology, [3]Division of Digestive Surgery, National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa, Chiba 277-8577; [4]Department of Gastroenterological Surgery, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama 700-8558; [5]Department of Gastroenterology and Hepatology, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan

Received July 9, 2011; Accepted August 25, 2011

DOI: 10.3892/ijo.2011.1202

Abstract. Glypican-3 (GPC3), a carcinoembryonic antigen, is an ideal target for anticancer immunotherapy against hepatocellular carcinoma (HCC). In this study, we attempted to compare the induction of the GPC3-specific T-cell-mediated immune response after locoregional therapies in HCC patients and tumor-bearing mice. Twenty-seven HCC patients treated with locoregional therapies, including radiofrequency ablation (RFA), surgical resection and transcatheter arterial chemoembolization (TACE), were prospectively enrolled in this study. Additionally, we performed RFA experiments using a mouse model. GPC3-specific T-cell response was investigated pre-treatment and post-treatment by an interferon-γ enzyme-linked immunospot assay using peripheral blood mononuclear cells from HCC patients and lymph node cells from tumor-bearing mice. Circulating GPC3-specific cytotoxic T lymphocytes (CTLs) were increased in 5 of 9 patients after RFA and in 4 of 9 patients after TACE, but in only 1 of 9 patients after surgical resection. All 7 patients with GPC3-expressing HCCs exhibited an increase in GPC3-specific CTLs after RFA or TACE, whereas none of the 7 patients did after surgical resection. The number of increased GPC3-specific CTLs after RFA was significantly larger than that after surgical resection (P=0.023). Similarly, the frequency of GPC3-specific CTLs after RFA was significantly greater than that after surgical resection in the mouse model (P=0.049). We validated for the first time the stronger effect on the immune system brought by RFA compared with surgical resection for HCC patients and tumor-bearing mice. Combined treatment of RFA and immunotherapy is a reasonable strategy against HCC.

Correspondence to: Dr Tetsuya Nakatsura, Section for Cancer Immunotherapy, Investigative Treatment Division, Research Center for Innovative Oncology, National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa, Chiba 277-8577, Japan
E-mail: tnakatsu@east.ncc.go.jp

*Contributed equally

Abbreviations: GPC3, glypican-3; HCC, hepatocellular carcinoma; RFA, radiofrequency ablation; TACE, transcatheter arterial chemoembolization; CTL, cytotoxic T lymphocyte; CT, computed tomography; TNM, tumor-node-metastasis; UICC, the Union for International Cancer Control; PBMC, peripheral blood mononuclear cell; IFN, interferon; ELISPOT, enzyme-linked immunospot; HSP105, heat shock protein 105; CMV, cytomegalovirus; AFP, α-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonist II; hTERT, human telomerase reverse transcriptase; MRP3, multidrug resistance-associated protein 3

Key words: hepatocellular carcinoma, radiofrequency ablation, glypican-3, cytotoxic T lymphocyte, immunotherapy

Introduction

Hepatocellular carcinoma (HCC) is one of the most common and most serious cancers worldwide (1). Locoregional therapies, including radiofrequency ablation (RFA), surgical resection, and transcatheter arterial chemoembolization (TACE), are recognized as the gold-standard therapies for HCC patients whose cancer lesions are limited to the liver (2). However, the recurrence rate remains quite high despite potentially curative treatment (3,4). The reasons for this are as follows: first, a multicentric new tumor frequently occurs from underlying active hepatitis or cirrhosis and, second, a small tumor undetectable by imaging modalities frequently exists before treatment and would be left untreated (5). Therefore, the establishment of effective adjuvant therapy to prevent recurrence is urgently required, and

clinical trials are ongoing throughout the world (6). However, at the present time, there is no universal consensus (2,7,8).

Previous studies have reported that local tumor ablation treatments, such as RFA and cryoablation, not only destroy tumor tissue but also induce a marked inflammatory response both locally and systemically (9,10). Unlike surgical resection, tumor ablation treatment generates tumor cell necrosis (11), followed by the release of tumor-associated antigens (12). These antigens can be uptaken, processed, and presented by dendritic cells (10,13), and then an antigen-specific T-cell-mediated immune response can be induced (9). If this induction is sufficiently steady and reliable, it may provide the basis for adjuvant immunotherapy, which is an attractive strategy.

Glypican-3 (GPC3) belongs to the glypican family of heparan sulfate proteoglycans that are linked to the outer surface of the cell membrane through a glycosylphosphatidylinositol anchor (14). GPC3 is one of the carcinoembryonic antigens overexpressed in HCC (15-17). We have shown that GPC3 is an ideal target for anticancer immunotherapy because its expression is specifically detected in ~80% of HCCs even in the early stages and is correlated with a poor prognosis (18-21). Moreover, GPC3-specific cytotoxic T lymphocytes (CTLs) have a high level of killing activity against HCC tumor cells (22). We have finished the phase I clinical trial of a GPC3-derived peptide vaccine for patients with advanced HCC (unpublished data), and just started the phase II clinical trial for adjuvant therapy after curative resection or RFA.

In this study, our aim was to determine if the GPC3-specific T-cell-mediated immune response is strengthened after locoregional therapies in HCC patients and tumor-bearing mice. Moreover, we evaluated the hypothesis that the post-treatment immune response may provide the basis for adjuvant immunotherapy.

Materials and methods

Patient population and treatment of HCC. Twenty-seven patients with primary HCC were prospectively enrolled in this study from January to November 2007 at the National Cancer Center Hospital East, in Japan. The eligibility criteria included primary HCC, which would undergo locoregional therapies with curative intent. Three treatment groups of nine patients each would undergo RFA, surgical resection, or TACE, respectively. Treatment selection in each patient was in accordance with the Japanese HCC treatment guidelines (2). Other inclusion criteria included HLA-A24 or HLA-A2 gene-positive status, as determined by commercially-available genomic DNA typing tests (Mitsubishi Chemical Medience, Tokyo, Japan), and no other active malignancy. HCC was diagnosed using dynamic computed tomography (CT). Tumor stage was assigned according to the tumor-node-metastasis (TNM) classification of the Union for International Cancer Control (UICC) (23). All RFA procedures were performed percutaneously under ultrasound guidance. Curative treatment was defined as complete necrosis of the tumor lesion confirmed by dynamic CT after RFA, a negative surgical margin confirmed histopathologically after resection, and complete lipiodol deposition after TACE.

All patients gave written informed consent before entering the study and this study was approved by the Ethics Committee of the National Cancer Center, conforming to the ethical guidelines of the 1975 Declaration of Helsinki.

Collection of blood samples and preparation of peripheral blood mononuclear cells. Venous blood (20-30 ml) from each patient was collected both before treatment and one month after treatment. Peripheral blood mononuclear cells (PBMCs) were separated from whole blood using LeucoSep® tubes (Greiner Bio-One, Frickenhausen, Germany) by means of density gradient centrifugation.

Identification of GPC3-specific CTLs in HCC patients. In order to identify GPC3-specific CTLs, the proportion of cells producing interferon (IFN)-γ upon stimulation with GPC3 peptide was assessed by an *ex vivo* IFN-γ enzyme-linked immunospot (ELISPOT) assay using pooled PBMCs from HCC patients. Defrosted PBMCs ($1x10^6$ cells/well) were cultured in duplicate using 96-well flat-bottomed plates (BD Biosciences, San Jose, CA) with HLA-A24-restricted $GPC3_{298-306}$ peptide (EYILSLEEL) or HLA-A2-restricted $GPC3_{144-152}$ peptide (FVGEFFTDV) (10 μmol/l) with 100 U/ml recombinant human interleukin-2 (IL-2) for 20 h. The negative control consisted of medium alone or HLA-A24- or HLA-A2-restricted heat shock protein 105 (HSP105) peptide, and the positive control included the HLA-A24- or HLA-A2-restricted cytomegalovirus (CMV) peptide. The number of spots, which indicated the presence of IFN-γ secreting cells, was automatically counted using the Eliphoto system (Minerva Tech, Tokyo, Japan). For an exact comparison of the frequency of GPC3-specific CTLs existing at pre- and post-treatment, the obtained mean values of the number of spots with non-peptide-pulsed samples ($1x10^6$ PBMCs) at pre- and post-treatment were equalized and set to zero, and then the actual number of GPC3-, CMV-, or HSP105-specific spots was calculated. The Δspot was defined as the difference in the number of spots with each antigen between pre- and post-treatment.

Mice. Female BALB/c mice ($H\text{-}2^d$), 6-8 weeks of age, were obtained from Charles River Laboratories Japan (Yokohama, Japan). The mice were maintained under specific-pathogen-free conditions. All animal procedures were performed in compliance with the guidelines by the Animal Research Committee of the National Cancer Center, Japan.

Tumor cell lines. A subline of the BALB/c-derived GPC3-negative colorectal adenocarcinoma cell line, Colon 26 (24), was provided by Dr Kyoichi Shimomura (Astellas Pharma, Tokyo, Japan). Colon 26/GPC3 is an established stable GPC3-expressing cell line (18). The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in humidified 5% CO_2 at 37°C.

RFA experiment using a mouse model. The mice were shaved at the tumor area and the contralateral flank. After attachment of the electricity-conducting pad (ground pad) onto the contralateral side, an RFA needle with 5-mm active tip (Cool-tip™, Valleylab, Boulder, CO) was inserted into the middle of the tumor. Impedance could be evaluated on the RFA lesion generator system (RFG-3B model, Radionics, Burlington, MA).

Treatment was started by delivering RFA energy. During two treatment cycles of 10 sec, the temperature could be monitored using the thermistor and thermocouple in the tip of the probe. Treatment was considered successful if a tip temperature of 60-70˚C was reached.

Identification of GPC3-specific CTLs in mice. BALB/c mice were immunized beforehand by peptide vaccination with K^d-restricted $GPC3_{298-306}$ peptide (50 μg/mouse) emulsified with incomplete Freund's adjuvant twice at a 7-day interval as described previously (20). The day after the second vaccination, the mice were challenged subcutaneously with Colon 26/GPC3 tumor cells ($1x10^5$ cells/100 μl) on their shaved back and, 5 days later, the mice underwent therapeutic RFA or surgical resection for the established tumor. After the next 5 days, the mice were sacrificed and bilateral inguinal lymph nodes were obtained. $CD8^+$ T cells were isolated from lymph node cells using anti-mouse CD8α (Ly-2) microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and an IFN-γ ELISPOT assay was performed without prior *in vitro* stimulation. For the IFN-γ ELISPOT assay, $CD8^+$ lymph node cells ($3x10^5$ cells/well) were used as effector cells, and Colon 26 and Colon 26/GPC3 cells ($3x10^4$ cells/well) as target cells. These cells were cultured in duplicate using 96-well flat-bottomed plates (BD Biosciences) with 100 U/ml recombinant murine IL-2 for 20 h. The number of spots after RFA or surgical resection was compared with that without treatment.

Immunohistochemical analysis. To investigate GPC3 expression in HCC tissues, we performed immunohistochemical staining of GPC3 in biopsy specimens or resected specimens from HCC patients. The paraffin-embedded blocks were analyzed using monoclonal anti-GPC3 antibody (dilution 1:300, BioMosaics, Burlington, VT) as described previously (17,21). The results were classified into two groups according to the area of GPC3-positive staining cells as follows: -, negative (<10%) and +, positive (≥10%).

To investigate tumor-infiltrating lymphocytes, we performed immunohistochemical staining of CD4 and CD8 in resected specimens from an HCC patient using monoclonal anti-CD4 or CD8 antibody (dilution 1:20, Novocastra, Newcastle upon Tyne, UK).

Statistical analysis. Statistical analyses were performed using χ^2 test, Mann-Whitney U test, or Kruskal-Wallis rank test. Differences were considered significant at $P<0.05$. Data were analyzed with the StatView 5.0 software package (Abacus Concepts, Calabasas, CA).

Results

Demographics and clinical characteristics. The characteristics of all 27 patients are represented in Table I. The three groups of 9 patients received RFA (RFA1-9), surgical resection (RES1-9), or TACE (TAE1-9), respectively. Among them, 21 patients had the HLA-A24 gene and 7 had the HLA-A2 gene. One patient had both HLA-A24 and -A2, and the HLA-A2-restricted $GPC3_{144-152}$ peptide was used for the IFN-γ ELISPOT assay in this patient. Among the three treatment groups, tumor size in the RFA group (mean: 16.4 mm) was significantly smaller than that in the resection group (mean: 43.2 mm) ($P=0.001$) and the TACE group (mean: 44.1 mm) ($P=0.001$). Similarly, tumor stage in the RFA group was less advanced than that in the resection group ($P=0.018$) and TACE group ($P=0.005$). There was no statistically significant difference in Child-Pugh classification grade among the three groups ($P=0.128$). In this study, all treatments were considered to be curative according to the definitions described in Materials and methods. Moreover, all groups reduced the levels of α-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist II (PIVKA-II) in most of HCC patients after treatment (data not shown). The diagnosis of HCC was histopathologically confirmed by biopsy specimens or resected specimens from 21 patients. GPC3 expression was detected by immunohistochemical staining in 14 of 21 patients.

Analysis of GPC3-specific CTLs in HCC patients. As shown in Table I, GPC3-specific CTLs were detected in 11 and 15 of 27 patients at pre- and post-treatment, respectively. In total, 19 patients had GPC3-specific CTLs at either pre- or post-treatment. There was no statistically significant correlation between the presence of GPC3-specific CTLs and clinical features, including HLA-A type ($P=0.126$), age ($P=0.750$), gender ($P=0.764$), HCV infection ($P=0.674$), HBV infection ($P=0.764$), Child-Pugh classification grade ($P=0.404$), tumor multiplicity ($P=0.674$), tumor size ($P=0.650$), HCC staging ($P=0.155$), serum AFP level ($P=0.288$), and serum PIVKA-II level ($P=0.094$). Among the 21 patients who had the information about GPC3 expression in their HCC tissue, patients with GPC3-expressing HCCs had GPC3-specific CTLs more frequently than those with GPC3-negative HCCs, but the difference was not statistically significant ($P=0.053$).

Changes in GPC3-specific CTLs between before and after treatment. In order to analyze the effect of anticancer treatment on GPC3-specific T-cell response, we compared the frequency of GPC3-specific CTLs in PBMCs before treatment with that after treatment. As shown in Table I and Fig. 1, an increase in GPC3-specific CTLs was found in 5 of 9 patients after RFA and in 4 of 9 after TACE, but in only 1 of 9 patients after resection. Of note, all of the 7 patients with GPC3-expressing HCCs exhibited an increase in GPC3-specific CTLs after RFA or TACE, whereas none of the 7 patients with GPC3-expressing HCCs did after surgical resection. The Δspot of GPC3 in the RFA group (mean: 24.4 spots) was larger than that in the resection group (mean: -7.2 spots) ($P=0.023$). The Δspot of GPC3 in the TACE group (mean, 36.9 spots) was also larger than that in the resection group, but the difference was not statistically significant ($P=0.096$). In contrast, the Δspot of CMV showed no difference among the three groups ($P=0.498$). Neither the existence of GPC3-specific CTLs before or after treatment, nor the changes between before and after treatment had statistically significant correlation with patient survival according to the log-rank test in each treatment group (neither disease-free nor overall), with the 27-month mean follow-up period (data not shown).

The representative data on changes in CT images and serum levels of tumor markers between before and after treatment is shown in Fig. 2. All three patients (RFA3, RES6, and TAE5) had GPC3-expressing HCCs. Both the CT images and

Table I. Patient characteristics and glypican-3-specific cytotoxic T lymphocytes.

Patient	HLA	Age (yrs.)	Gender	Etiology	Child-Pugh	No. of tumor	Tumor size (mm)	T[1]	N[1]	M[1]	AFP (<9.5 ng/ml)	PIVKA-II (<40 mAU/ml)	GPC3 expression[2]	GPC3-specific CTLs[3] Pre	Post	Change	Δspot[4]
RFA1	A24	73	F	HBV	A	2	26	2	0	0	4.0	228	-	4	0	-	-4
RFA2	A24	68	M	HCV	B	1	20	1	0	0	5.0	300	+	10	24	+	+14
RFA3	A2	50	M	HCV	A	1	15	1	0	0	63.3	25	+	0	88	+	+88
RFA4	A24	79	F	HCV	A	1	10	1	0	0	484.2	30	+	0	10	+	+10
RFA5	A24	69	M	HCV	A	1	15	1	0	0	2.3	57	-	0	0	+/-	0
RFA6	A24	60	M	HCV	A	1	17	1	0	0	15.1	23	-	0	0	+/-	0
RFA7	A2	73	M	HCV	A	1	20	1	0	0	97.3	51	+	3	88	+	+85
RFA8	A2/A24	64	M	HBV/HCV	B	1	15	1	0	0	39.9	17	+	0	31	+	+31
RFA9	A2	60	M	HCV	B	1	10	1	0	0	92.0	19	-	19	15	-	-4
RES1	A24	48	M	HBV	A	1	20	1	0	0	19.7	38	+	32	15	-	-17
RES2	A24	66	F	HCV	A	1	26	2	0	0	63.4	77	+	20	3	-	-17
RES3	A24	64	M	HCV	A	2	30	2	0	0	10.1	276	+	12	0	-	-12
RES4	A2	72	M	-	A	1	60	2	0	0	9.2	1500	+	3	1	-	-2
RES5	A24	70	M	HCV	A	1	20	1	0	0	4.2	25	+	0	0	+/-	0
RES6	A24	42	M	HBV/HCV	A	2	98	3	0	0	15115.0	22477	+	50	30	-	-20
RES7	A2	75	M	-	A	1	75	2	0	0	22.8	10341	-	0	3	+	+3
RES8	A24	52	M	HCV	A	1	30	1	0	0	16.0	234	+	0	0	+/-	0
RES9	A24	60	M	HBV	A	1	30	1	0	0	15.6	23	-	0	0	+/-	0
TAE1	A2	64	M	-	A	3	30	2	0	0	10.7	98	+	0	330	+	+330
TAE2	A24	78	F	HCV	B	1	60	1	0	0	2483.0	3932	ND	34	0	-	-34
TAE3	A24	77	F	-	A	>5	35	3	0	0	180.2	11538	ND	0	3	+	+3
TAE4	A24	77	M	HCV	A	2	80	4	0	0	20014.0	241	ND	0	0	+/-	0
TAE5	A24	55	M	HBV	A	2	30	2	0	0	3.7	24	+	0	23	+	+23
TAE6	A24	77	M	-	A	>5	42	2	0	0	1407.0	1661	ND	0	20	+	+20
TAE7	A24	63	F	HCV	A	>5	32	2	0	0	640.3	270	ND	0	0	+/-	0
TAE8	A24	74	M	-	A	1	18	1	0	0	3.8	12	-	0	0	+/-	0
TAE9	A24	62	M	HCV	A	3	70	3	0	0	46.8	1907	ND	10	0	-	-10

[1]Tumor stage was assigned according to the tumor-node-metastasis (TNM) classification of the Union for International Cancer Control (UICC). [2]GPC3 expression was evaluated by immunohistochemical staining; +, positive; -, negative. [3]Peripheral blood was taken from each patient before and after treatment, and GPC3-specific CTLs were measured by *ex vivo* interferon-γ enzyme-linked immunospot assay; +, increase; -, decrease; +/-, no change. [4]The Δspot was defined as the difference in the number of spots with each antigen between pre- and post-treatment. F, female; M, male; HBV, hepatitis B virus; HCV, hepatitis C virus; AFP, α-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonist II; GPC3, glypican-3; ND, not determined.

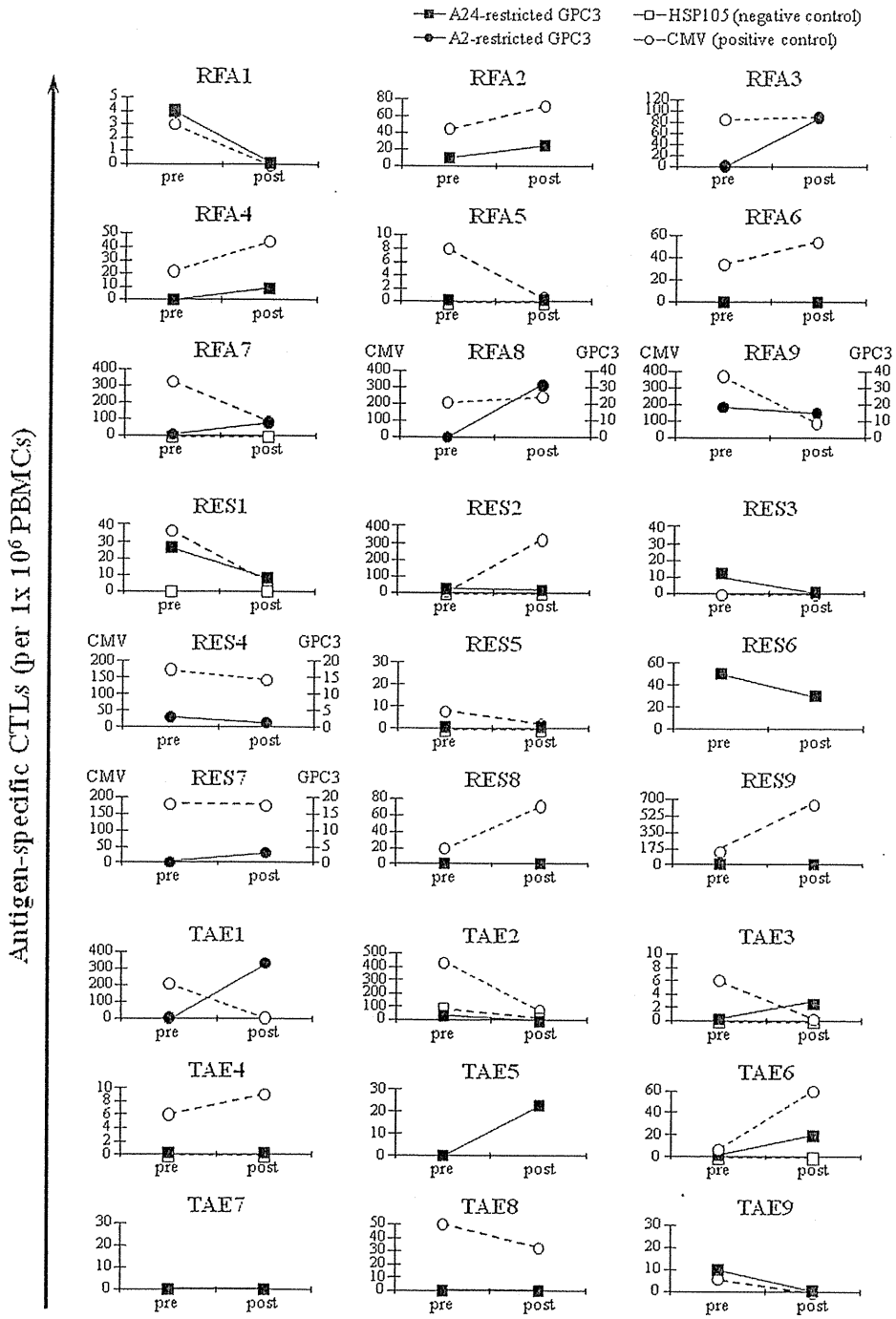

Figure 1. Kinetics of glypican-3 (GPC3)-specific CTLs between before and after treatment in each patient. A direct *ex vivo* interferon-γ enzyme-linked immunospot assay of PBMCs was performed before treatment and one month after treatment. The data are expressed as the number of interferon-γ producing cells, which indicate the CTLs specific with HLA-A24-restricted $GPC3_{298-306}$ peptide (EYILSLEEL) (▪) or HLA-A2-restricted $GPC3_{144-152}$ peptide (FVGEFFTDV) (•). Heat shock protein 105 (HSP105) peptide (□) and cytomegalovirus (CMV) peptide (○) were used as the negative and positive control, respectively.

kinetics of tumor markers indicated that their treatment was effective. The frequency of GPC3-specific CTLs increased after RFA (RFA3) and TACE (TAE5), whereas it decreased after surgical resection (RES6).

RFA has the potential to strongly induce T-cell-mediated immune response: A case report. A 70-year-old woman was admitted because of recurrent HCCs. Thirteen months earlier, the patient had undergone RFA for primary HCC located in the S5/8 region of the liver. CT detected two recurrent HCCs: one was contiguous to the previously ablated S5/8 region and the other was a distant tumor located in the S6 region. We performed surgical resection for these recurrent HCCs. Immunohistochemical examination of CD8 in the resected tumors revealed that a marked number of $CD8^+$ T cells had infiltrated not only into the surrounding recurrent tumor but also into the distant recurrent tumor after RFA (Fig. 3). On the other hand, few $CD4^+$ T cells were observed in these tumors (data not shown). Immunohistochemical analyses showed the expression of GPC3 and HLA class I in these tumors (data not

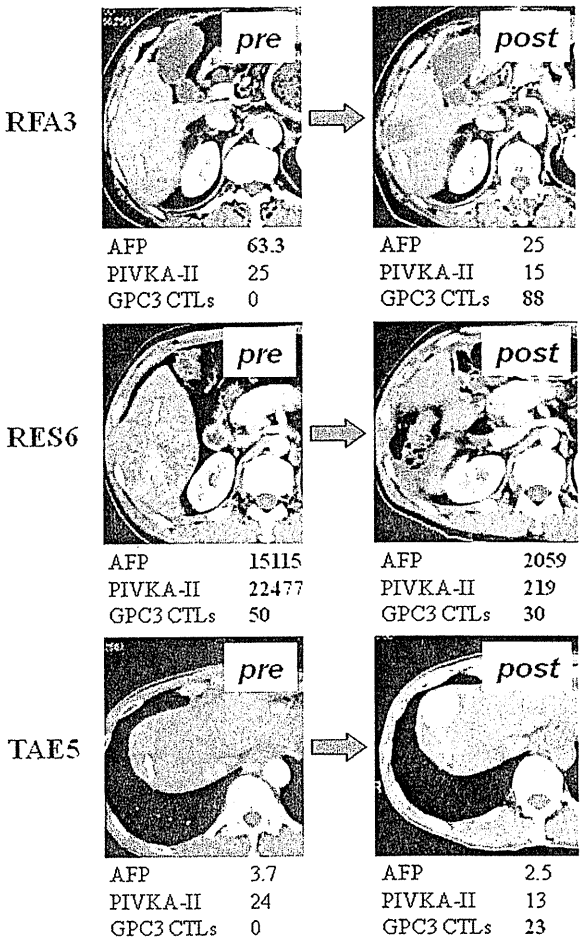

Figure 2. Changes in computed tomography images, serum levels of tumor markers, including α-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist II (PIVKA-II), and glypican-3 (GPC3)-specific CTLs in PBMCs between before and after treatment in patients RFA3, RES6, and TAE5. White arrows indicate nodules of hepatocellular carcinoma at pre- and post-treatment. The bold letters show the abnormal levels of tumor markers or the positive response of GPC3 specific CTLs.

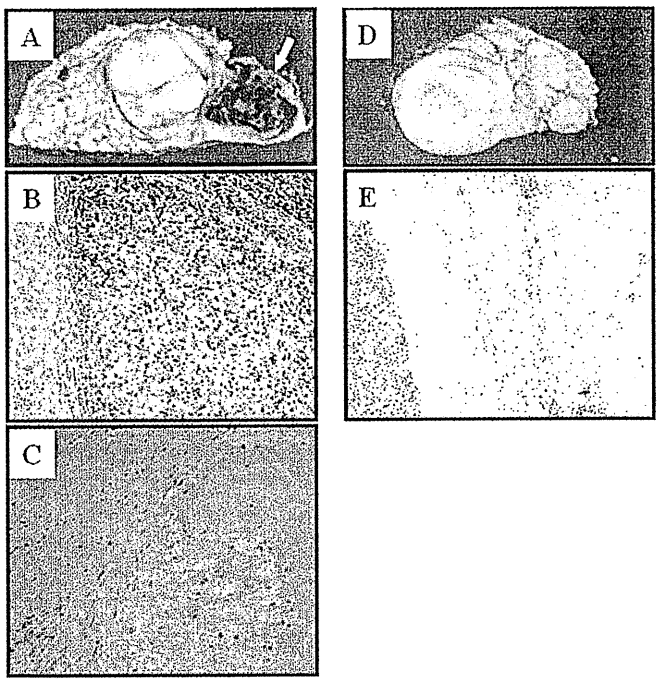

Figure 3. Macroscopic features and immunohistochemical examination of $CD8^+$ T cells in the resected tumors that had recurred after radiofrequency ablation. (A and D) show the cut surface of the resected specimens. (A) The white arrow indicates the post-ablated lesion to which a recurrent tumor was contiguous. The other recurrent tumor was distant from the post-ablated lesion (D). A marked number of $CD8^+$ T cells had infiltrated into the contiguous recurrent tumor (B) and the distant recurrent tumor (E), whereas few $CD8^+$ T cells had infiltrated into the post-ablated necrotic lesion (C). Magnification x100 (B and C) and x40 (E).

shown). These findings suggest that RFA not only activates the immune response systemically but also induces local infiltration of CTLs into the tumors.

Analysis of immune response induced by RFA in a mouse model. The experimental schedule is shown in Fig. 4A. The IFN-γ ELISPOT assay with $CD8^+$ T cells from the lymph nodes of mice demonstrated that the number of spots against both Colon 26 (P=0.049) and Colon 26/GPC3 (P=0.049) was larger after RFA compared to without treatment. On the other hand, the number of spots did not increase after surgical resection. These results suggest that RFA induced a significantly larger number of both Colon 26- and Colon 26/GPC3-reactive CTLs compared to no treatment or surgical resection (Fig. 4B).

The difference in number of spots between Colon 26 and Colon 26/GPC3 in each mouse, which represents GPC3-specific CTLs, is shown in Fig. 4C. As an effect of prior peptide vaccination, GPC3-specific CTLs were detected in the no treatment group. The frequency of GPC3-specific CTLs increased after RFA and decreased after surgical resection. As a result, the frequency of GPC3-specific CTLs after RFA was significantly greater than that after surgical resection (P=0.049). These results suggest that RFA induced a significantly larger number of GPC3-specific CTLs compared to surgical resection (Fig. 4C).

Discussion

We previously reported that 39% of HCC patients had detectable GPC3-specific CTLs by a direct *ex vivo* IFN-γ ELISPOT assay (25). In this study, GPC3-specific CTLs were detectable before treatment in 11 of 27 patients (41%). Additionally, when we analyzed the patients with a prior treatment for HCCs using the same methods, 11 of 21 (52%) patients had detectable GPC3-specific CTLs (data not shown). These results are favorable for anticancer immunotherapy because the antigen-specific T-cell-mediated immune response could be detected without *in vitro* stimulation. As for frequency, GPC3-specific CTLs were detectable in ~40% of HCC patients, whereas AFP-, human telomerase reverse transcriptase (hTERT)-, and multidrug resistance-associated protein 3 (MRP3)-specific CTLs have been detected in 5-20, 6-12, and 14-21% of HCC patients with a single epitope peptide, respectively (26-28). As for tumor stages, a GPC3-specific immune response is frequently detected even in the early stages (24), whereas AFP-specific CTLs are more frequently detected in patients with advanced HCC (26). These results suggest that GPC3 has strong immunogenicity and GPC3-specific T-cell-mediated immunotherapy is suitable for adjuvant therapy against HCC because the induction of tumor-specific immune response in

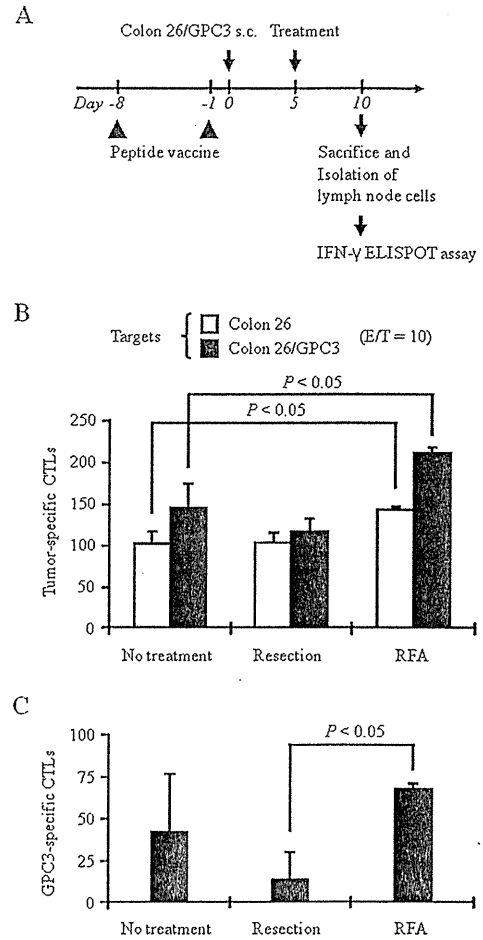

Figure 4. Investigation of the glypican-3 (GPC3)-specific immune response in a mouse model. (A) Experiment schedule. (B) An *ex vivo* interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assay of $CD8^+$ lymph node cells (effector, $3x10^5$ cells/well) against Colon 26 and Colon 26/GPC3 (target, $3x10^4$ cells/well). No treatment column indicates the group of mice that received only the peptide vaccination and no therapy for the established tumor. The data are expressed as the mean + SD. Three mice were used for each group. Effector/target ratio=10. (C) The frequency of GPC3-specific CTLs, which is calculated from the difference in the number of spots between Colon 26 and Colon 26/GPC3 in each mouse.

the early stages would be more effective for suppression of tumor growth.

The association between the induction of an antigen-specific immune response and the antigen expression in tumor tissue remains unclear. In this study, we obtained the result that the presence of GPC3-specific CTLs in PBMCs potentially had a positive correlation with GPC3 expression in tumor tissue, but the correlation was not statistically significant. On the other hand, Mizukoshi *et al* showed a negative correlation between the frequency of MRP3-specific CTLs and MRP3 expression level (28). Moreover, Benavides *et al* showed that even antigen-naïve patients had pre-existing immunity (29). First, this may be because of tumor heterogeneity of cancer tissue. In most cases, the whole tumor cannot be evaluated and, in the case of truly antigen-naïve patients, antigen-specific CTLs cannot exist in theory. Second, antigen expression may be negative if antigen-specific CTLs have killed all of the antigen-expressing tumor cells as described by Jäger *et al* (30). As for the changes in an antigen-specific immune response between before and after treatment, in this study, we showed impressive data that all patients with GPC3-expressing HCCs exhibited an increase in GPC3-specific CTLs after RFA or TACE, whereas no patient with GPC3-expressing HCCs did after surgical resection.

This is the first study to compare locoregional therapies, including RFA, surgical resection, and TACE, in terms of antigen-specific T-cell response in HCC patients and tumor-bearing mice. Half the patients after RFA or TACE showed an increase in GPC3-specific CTLs, which might have been induced by the treatment, whereas only 1 of 9 patients after resection showed an increase and more than half the patients after resection showed a decrease. Similarly, the frequency of GPC3-specific CTLs increased after RFA and decreased after resection in a mouse model. These results suggest that RFA induced a stronger GPC3-specific immune response compared to surgical resection. RFA destroys tumor tissue and causes local necrosis followed by the release of tumor-associated antigens (12), whereas all of the tumor-associated antigens must be completely removed after resection. With regard to TACE, whereas the results of an IFN-γ ELISPOT assay after TACE were as encouraging as that after RFA, we have no other favorable data on the immune response after TACE. Although further investigation is required, TACE, which is also a necrosis-inducing treatment, might induce an antigen-specific immune response.

A limitation of this study is the patient selection in the three kinds of locoregional therapy. Current treatment guidelines for HCC including the Japanese ones, which we followed in this study, recommend RFA to earlier HCCs and TACE to more advanced HCCs than those which receive surgical resection (2,31-33). Therefore, selection bias is unavoidable under the circumstances. To overcome this problem, we added a murine study. The advantage of RFA over surgical resection in the induction of GPC3-specific CTLs was demonstrated also in a mouse model.

The correlation between antitumor immune response and clinical response is controversial. In this study, a significant contribution of GPC3-specific CTLs toward an optimal prognosis was not demonstrated. Mizukoshi *et al* reported that enhancement of T-cell response did not last for long and did not contribute to the prevention of HCC recurrence (34). In view of the highly complex nature of the human immune system, patient prognoses might not be determined only by the CTL response. Previous studies have demonstrated that the release of tumor-derived antigens by necrosis-inducing treatment causes sufficient signaling to activate not only antigen-specific CTL response but also antigen-specific helper T-cell response (35,36), antigen-specific antibody response (36), and non-antigen-specific natural killer cell response (37). However, the mechanisms for cancer escape from immunosurveillance would suppress the efficiency of these immune responses (38). In the literature, tumor-infiltrating lymphocytes in HCC are associated with better prognosis (39), but, in our case, tumor-infiltrating CTLs were actually insufficient for suppression of cancer recurrence despite the massive infiltration. For successful anticancer immunotherapy, the development of an innovative strategy to link antitumor immune response with clinical response and to provide a survival benefit for cancer patients is necessary, and so we have just started the clinical trial of a GPC3-derived peptide vaccine for adjuvant therapy after RFA.

In conclusion, our results demonstrate that RFA has a stronger effect on the immune system compared with surgical resection. Although further investigation is necessary, the data on immune response support the rationale for combined immunotherapy for HCC patients.

Acknowledgements

D.N. would like to thank the Foundation for Promotion of Cancer Research (Japan) for the Third-Term Comprehensive Control Research for Cancer for awarding them a research resident fellowship. This study was supported in part by Health and Labor Science Research Grants for Research on Hepatitis from the Ministry of Health, Labor, and Welfare, Japan. J.F. received honoraria and consulting fee from Bayer, Chugai, Eisai, Eli Lilly, Taiho and Phizer; J.F.'s institute received grants from Pfizer and Bayer.

References

1. Parkin DM: Global cancer statistics in the year 2000. Lancet Oncol 2: 533-543, 2001.
2. Makuuchi M, Kokudo N, Arii S, *et al*: Development of evidence-based clinical guidelines for the diagnosis and treatment of hepatocellular carcinoma in Japan. Hepatol Res 38: 37-51, 2008.
3. Imamura H, Matsuyama Y, Tanaka E, *et al*: Risk factors contributing to early and late phase intrahepatic recurrence of hepatocellular carcinoma after hepatectomy. J Hepatol 38: 200-207, 2003.
4. Tateishi R, Shiina S, Yoshida H, *et al*: Prediction of recurrence of hepatocellular carcinoma after curative ablation using three tumor markers. Hepatology 44: 1518-1527, 2006.
5. Nobuoka D, Kato Y, Gotohda N, *et al*: Postoperative serum alpha-fetoprotein level is a useful predictor of recurrence after hepatectomy for hepatocellular carcinoma. Oncol Rep 24: 521-528, 2010.
6. Printz C: Clinical trials of note. Sorafenib as adjuvant treatment in the prevention of disease recurrence in patients with hepatocellular carcinoma (HCC) (STORM). Cancer 115: 4646, 2009.
7. Schwartz JD, Schwartz M, Mandeli J and Sung M: Neoadjuvant and adjuvant therapy for resectable hepatocellular carcinoma: review of the randomised clinical trials. Lancet Oncol 3: 593-603, 2002.
8. Ishii H, Yamamoto J and Ikari T: Adjuvant treatments for resectable hepatocellular carcinoma. J Hepatobiliary Pancreat Surg 15: 459-462, 2008.
9. Wissniowski TT, Hänsler J, Neureiter D, *et al*: Activation of tumor-specific T lymphocytes by radio-frequency ablation of the VX2 hepatoma in rabbits. Cancer Res 63: 6496-6500, 2003.
10. den Brok MH, Sutmuller RP, Nierkens S, *et al*: Efficient loading of dendritic cells following cryo and radiofrequency ablation in combination with immune modulation induces anti-tumour immunity. Br J Cancer 95: 896-905, 2006.
11. McGahan JP, Brock JM, Tesluk H, Gu WZ, Schneider P and Browning PD: Hepatic ablation with use of radio-frequency electrocautery in the animal model. J Vasc Interv Radiol 3: 291-297, 1992.
12. Yang WL, Nair DG, Makizumi R, *et al*: Heat shock protein 70 is induced in mouse human colon tumor xenografts after sublethal radiofrequency ablation. Ann Surg Oncol 11: 399-406, 2004.
13. Ali MY, Grimm CF, Ritter M, *et al*: Activation of dendritic cells by local ablation of hepatocellular carcinoma. J Hepatol 43: 817-822, 2005.
14. Filmus J: The contribution of in vivo manipulation of gene expression to the understanding of the function of glypicans. Glycoconj J 19: 319-323, 2002.
15. Nakatsura T, Yoshitake Y, Senju S, *et al*: Glypican-3, over-expressed specifically in human hepatocellular carcinoma, is a novel tumor marker. Biochem Biophys Res Commun 306: 16-25, 2003.
16. Nakatsura T and Nishimura Y: Usefulness of the novel oncofetal antigen glypican-3 for diagnosis of hepatocellular carcinoma and melanoma. BioDrugs 19: 71-77, 2005.
17. Shirakawa H, Kuronuma T, Nishimura Y, *et al*: Glypican-3 is a useful diagnostic marker for a component of hepatocellular carcinoma in human liver cancer. Int J Oncol 34: 649-656, 2009.
18. Nakatsura T, Komori H, Kubo T, *et al*: Mouse homologue of a novel human oncofetal antigen, glypican-3, evokes T-cell-mediated tumor rejection without autoimmune reactions in mice. Clin Cancer Res 10: 8630-8640, 2004.
19. Komori H, Nakatsura T, Senju S, *et al*: Identification of HLA-A2- or HLA-A24-restricted CTL epitopes possibly useful for glypican-3-specific immunotherapy of hepatocellular carcinoma. Clin Cancer Res 12: 2689-2697, 2006.
20. Motomura Y, Ikuta Y, Kuronuma T, *et al*: HLA-A2 and -A24-restricted glypican-3-derived peptide vaccine induces specific CTLs: preclinical study using mice. Int J Oncol 32: 985-990, 2008.
21. Shirakawa H, Suzuki H, Shimomura M, *et al*: Glypican-3 expression is correlated with poor prognosis in hepatocellular carcinoma. Cancer Sci 100: 1403-1407, 2009.
22. Yoshikawa T, Nakatsugawa M, Suzuki S, *et al*: HLA-A2-restricted glypican-3 peptide-specific CTL clones induced by peptide vaccine show high avidity and antigen-specific killing activity against tumor cells. Cancer Sci 102: 918-925, 2011.
23. Sobin LH and Wittekind C: UICC: TNM Classification of Malignant Tumors. 6th edition, Wiley-Liss, New York, pp81-83, 2002.
24. Tanaka Y, Eda H, Tanaka T, *et al*: Experimental cancer cachexia induced by transplantable colon 26 adenocarcinoma in mice. Cancer Res 50: 2290-2295, 1990.
25. Hayashi E, Motomura Y, Shirakawa H, *et al*: Detection of glypican-3-specific CTLs in chronic hepatitis and liver cirrhosis. Oncol Rep 22: 149-154, 2009.
26. Mizukoshi E, Nakamoto Y, Tsuji H, Yamashita T and Kaneko S: Identification of alpha-fetoprotein-derived peptides recognized by cytotoxic T lymphocytes in HLA-A24$^+$ patients with hepatocellular carcinoma. Int J Cancer 118: 1194-1204, 2006.
27. Mizukoshi E, Nakamoto Y, Marukawa Y, *et al*: Cytotoxic T cell responses to human telomerase reverse transcriptase in patients with hepatocellular carcinoma. Hepatology 43: 1284-1294, 2006.
28. Mizukoshi E, Honda M, Arai K, Yamashita T, Nakamoto Y and Kaneko S: Expression of multidrug resistance-associated protein 3 and cytotoxic T cell responses in patients with hepatocellular carcinoma. J Hepatol 49: 946-954, 2008.
29. Benavides LC, Gates JD, Carmichael MG, *et al*: The impact of HER2/neu expression level on response to the E75 vaccine: from U.S. Military Cancer Institute Clinical Trials Group Study I-01 and I-02. Clin Cancer Res 15: 2895-2904, 2009.
30. Jäger E, Ringhoffer M, Karbach J, Arand M, Oesch F and Knuth A: Inverse relationship of melanocyte differentiation antigen expression in melanoma tissues and CD8$^+$ cytotoxic-T-cell responses: evidence for immunoselection of antigen-loss variants in vivo. Int J Cancer 66: 470-476, 1996.
31. Llovet JM, Burroughs A and Bruix J: Hepatocellular carcinoma. Lancet 362: 1907-1917, 2003.
32. Bruix J and Sherman M: Practice Guidelines Committee, American Association for the Study of Liver Diseases: Management of hepatocellular carcinoma. Hepatology 42: 1208-1236, 2005.
33. Omata M, Lesmana LA, Tateishi R, *et al*: Asian Pacific Association for the Study of the Liver consensus recommendations on hepatocellular carcinoma. Hepatol Int 4: 439-474, 2010.
34. Mizukoshi E, Nakamoto Y, Arai K, *et al*: Enhancement of tumor-specific T-cell responses by transcatheter arterial embolization with dendritic cell infusion for hepatocellular carcinoma. Int J Cancer 126: 2164-2174, 2010.
35. Ayaru L, Pereira SP, Alisa A, *et al*: Unmasking of alpha-fetoprotein-specific CD4(+) T cell responses in hepatocellular carcinoma patients undergoing embolization. J Immunol 178: 1914-1922, 2007.
36. Widenmeyer M, Shebzukhov Y, Haen SP, *et al*: Analysis of tumor antigen-specific T cells and antibodies in cancer patients treated with radiofrequency ablation. Int J Cancer 128: 2653-2662, 2011.
37. Zerbini A, Pilli M, Laccabue D, *et al*: Radiofrequency thermal ablation for hepatocellular carcinoma stimulates autologous NK-cell response. Gastroenterology 138: 1931-1942, 2010.
38. Zerbini A, Pilli M, Penna A, *et al*: Radiofrequency thermal ablation of hepatocellular carcinoma liver nodules can activate and enhance tumor-specific T-cell responses. Cancer Res 66: 1139-1146, 2006.
39. Wada Y, Nakashima O, Kutami R, Yamamoto O and Kojiro M: Clinicopathological study on hepatocellular carcinoma with lymphocytic infiltration. Hepatology 27: 407-414, 1998.

Identification of an HLA-A*0201-restricted cytotoxic T lymphocyte epitope from the lung carcinoma antigen, Lengsin

MUNEHIDE NAKATSUGAWA[1], KAZUTAKA HORIE[1], TOSHIAKI YOSHIKAWA[1], MANAMI SHIMOMURA[1], YAMATO KIKUCHI[1], NORIKO SAKEMURA[1], SHIRO SUZUKI[1], DAISUKE NOBUOKA[1], YOSHIHIKO HIROHASHI[2], TOSHIHIKO TORIGOE[2], KENJI HARADA[3], HIDEO TAKASU[3], NORIYUKI SATO[2] and TETSUYA NAKATSURA[1]

[1]Section for Cancer Immunotherapy, Investigative Treatment Division, Research Center for Innovative Oncology, National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa, Chiba 277-8577; [2]Department of Pathology, Sapporo Medical University School of Medicine, South-1, West-17, Chuo-ku, Sapporo 060-8556; [3]Dainippon Sumitomo Pharma Co., Ltd., 6-8 Doshomachi 2-chome, Chuo-ku, Osaka 541-0045, Japan

Received April 27, 2011; Accepted June 2, 2011

DOI: 10.3892/ijo.2011.1089

Abstract. Lengsin is an eye lens protein with a glutamine synthetase domain. We previously identified this protein as a lung carcinoma antigen through cDNA microarray analysis. Lengsin protein is overexpressed irrespective of the histological type of lung carcinoma, but not in normal tissues other than the lens. Therefore, to significantly extend the use of Lengsin-based T-cell immunotherapies for the treatment of patients with lung carcinoma, we searched for HLA-A*0201-restricted epitopes from this protein by screening predicted Lengsin-derived candidate peptides for the induction of tumor-reactive CTLs. Four Lengsin-derived peptides were selected by computerized algorithm based on a permissive HLA-A*0201 binding motif, and were used to immunize HLA-A*0201 transgenic (HHD) mice. Two of the immunizing peptides, Lengsin(206-215)(FIYDFCIFGV) and Lengsin(270-279)(FLPEFGISSA), induced peptide-specific cytotoxic T lymphocytes (CTLs) in HHD mice, and thus were used to stimulate human peripheral blood lymphocytes *in vitro*. Lengsin(206-215) and Lengsin (270-279) also induced human peptide-specific CTLs, and we were able to generate Lengsin(206-215)- and Lengsin(270-279)-specific CTL clones. The Lengsin(270-279)-specific CTL clone specifically recognized peptide-pulsed T2 cells, COS-7 cells expressing HLA-A*0201 and Lengsin, and HLA-A*0201$^+$/Lengsin$^+$ lung carcinoma cells in an HLA-A*0201-restricted manner. On the other hand, the Lengsin(206-215)-specific CTL clone failed to recognize HLA-A*0201$^+$/Lengsin$^+$ target cells in the absence of cognate peptide. These results suggest that Lengsin(270-279) is naturally processed and presented by HLA-A*0201 molecules on the surface of lung carcinoma cells and may be a new target for antigen-specific T-cell immunotherapy against lung cancer.

Correspondence to: Dr Tetsuya Nakatsura, Section Head, Section for Cancer Immunotherapy, Investigative Treatment Division, Research Center for Innovative Oncology, National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa, Chiba 277-8577, Japan
E-mail: tnakatsu@east.ncc.go.jp

Key words: Lengsin, lung carcinoma antigen, cytotoxic T lymphocyte epitope, HLA-A*0201-restricted

Introduction

Lung cancer is the leading cause of cancer death in the world (1). Current chemotherapy and radiotherapy regimens provide a limited survival benefit and are often toxic as well as ineffective. Tumor antigen-specific T-cell immunotherapy is a promising new approach to cancer treatment that is more effective and less toxic. Recent studies have shown that the adoptive transfer of normal peripheral lymphocytes genetically modified by the insertion of tumor-reactive T-cell receptors (TCRs) can mediate *in vivo* complete regression in patients with metastatic melanoma (2,3), and synovial cell sarcoma (4). Identification of naturally presented peptides derived from tumor-associated antigens on the surface of tumor cells, which can induce peptide-specific and tumor-reactive cytotoxic CD8$^+$ T cells (CTLs), is required for antigen-specific T-cell immunotherapy including TCR gene transfer therapy as the therapeutic target. To date, many immunogenic CTL epitopes in the context of HLA-A*0201, which is the predominant subtype in most ethnic groups (5), have been identified by reverse immunology approach (6-8). In addition, H-2D$^{b-/-}$, $\beta_2m^{-/-}$, HLA-A*0201 monochain transgenic (HHD) mouse is a useful animal model for assessing the ability of individual peptides to induce HLA-A*0201-restricted CTL response (9-15). Previously, we used genome-wide cDNA microarray analysis to identify a novel lung cancer antigen, Lengsin, as a potential target for immunotherapy (16). Lengsin protein is overexpressed irrespective of the histological type of lung carcinoma, but not in normal tissues other than the lens. Because the eye lens is an immune-privileged site (17,18), similar to the case of the testis, Lengsin can be an attractive target for tumor antigen-specific immunotherapy, as with cancer-testis antigens.