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International Journal of Cancer

Long-term follow-up of patients with resected pancreatic cancer following vaccination against mutant K-ras

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K-ras mutations are frequently found in adenocarcinomas of the pancreas and can elicit mutation-specific immune responses. Targeting the immune system against mutant Ras may thus influence the clinical course of the disease. Twenty-three patients who were vaccinated after surgical resection for pancreatic adenocarcinoma (22 pancreaticoduodenectomies, one distal resection), in two previous Phase I/II clinical trials, were followed for more than 10 years with respect to long-term immunological T-cell reactivity and survival. The vaccine was composed of long synthetic mutant ras peptides designed mainly to elicit T-helper responses. Seventeen of 20 evaluable patients (85%) responded immunologically to the vaccine. Median survival for all patients was 27.5 months and 28 months for immune responders. The 5-year survival was 22% and 29%, respectively. Strikingly, 10-year survival was 20% (four patients out of 20 evaluable) *versus* zero (0/87) in a cohort of nonvaccinated patient treated in the same period. Three patients mounted a memory response up to 9 years after vaccination. The present observation of long-term immune response together with 10-year survival following surgical resection indicates that K-ras vaccination may consolidate the effect of surgery and represent an adjuvant treatment option for the future.

Although pancreatic carcinoma ranks eighth among solid tumors worldwide, it ranks fourth as a cause of death from cancer. Most patients with pancreatic adenocarcinoma have locally advanced or disseminated disease at the time of diagnosis. The subgroup of patients with resectable disease has a median survival time of approximately 18 months and 5-year survival following adjuvant chemotherapy is approximately 18%.[1–3] Thus, new treatment modalities are urgently needed. Point mutations at specific positions of the *K-ras* gene have been identified in up to 90% of patients with pancreatic cancer. Such mutations occur early in carcinogenesis[4,5] and they are essential for the maintenance of the malignant phenotype of the cancer cells. In pancreatic cancer, mutations generally result in amino-acid substitutions at position 12 of the ras protein, giving rise to potential neo-antigens specific to the cancer cells. Such point mutations can be recognized both by helper T-cells (Th) and cytotoxic T-cells (CTL), as demonstrated in several studies by independent laboratories.[6–8] Mutant ras has therefore emerged as a bona fide tumor-specific antigen of particular interest in the treatment of pancreatic cancer. These observations led to the first peptide vaccine trial in humans, where patients with irresectable pancreatic cancer were treated with a personalized peptide vaccine corresponding to the *K-ras* mutations present in their tumor.[9] This and subsequent trials[10] confirmed that vaccination with mutant ras peptides is safe, with very few adverse events, and results in combined Th- and CTL-cell responses in the patients,[11] which may result in a more efficient attack on the tumor and in prolonged immunological memory. In our earlier trials, and in subsequent trials by others,[12,13] patients who responded immunologically against the mutant ras vaccine had a more favorable clinical course. These results indicate that some clinical benefit may be obtained by mutant ras vaccine as a monotherapy. However, faced with the rapid progression of nonresectable pancreatic cancer, the induced immune response may be too weak and come too late to have an effect for the majority of patients. Thus, for patients with advanced disease, combination therapy with several different treatment modalities might be required for more substantial clinical benefit. The high rate of early recurrence during the first 1−2 years after resection of patients with pancreatic adenocarcinoma reflects the limitations of surgery in this aggressive disease. Indeed, recent evidence indicates that pancreatic resections are left with positive resection

Key words: K-ras vaccine, immunotherapy, pancreatic cancer, long-term survival
The study has been presented in part at AGA meeting, 2004, and ASCO Meeting, May 2007.
Grant sponsor: Norwegian Research Council
DOI: 10.1002/ijc.25449
History: Received 4 Feb 2010; Accepted 28 Apr 2010; Online 12 May 2010
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Table 1. Demographic data and outcome for treatment for single patients

	Patient no.	Date of surgery	Date of first vaccination	Age/Sex	Immune response DTH/T-cell	No. of vaccine tions, Stand. Boost	Overall survival months from surgery	+ still living	Long term T-cell memory response
*CTN-95002, single peptide vaccination	1 (103)	January 09, 1997	March 5, 1997	F/72.7	+/+	6, 8	144	+	Positive
	2 (104)	May 27, 1997	June 25, 1997	M/67.9	−/−	6, 4	26	−	
	3 (105)	September 29, 1997	December 1, 1997	M/64.2	−/−	6	37	−	
	4 (201)	January 27, 1997	March 10, 1997	M/34.8	+/+	6, 24	144	+	Negative
	5 (202)	March 10, 1997	June 10, 1997	M/60.6	NT/−	6	19,5	−	
	6 (203)	April 21, 1997	October 15, 1997	F/60.6	−/+	6	16	−	
	7 (204)**	June 16, 1997	December 15, 1997	M/58.9	+/−	6, 24	140	+	Negative
	8 (205)***	February 02, 1998	March 30, 1998	M/71.8	NT/−	3	28	−	
	9 (206)	January 19, 1998	April 27, 1998	F/58.0	+/+	6, 4	28	−	
	10 (207)	March 2, 1998	June 22, 1998	F/58.9	+/+	6	33	−	
	Total no immune responses				60%				
	Total no immune responses (evaluable)				67%				
CTN-98010, seven peptide vaccination	11 (702)	June 5, 1998	November 2, 1998	F/67.0	+	6	23.5	−	
	12 (704)	June 2, 1998	November 30, 2000	M/59.4	+	6, 4	27.5	−	
	13 (705)	December 1, 1998	January 4, 1999	M/60.3	+	6, 4	121	+	Positive
	14 (707)	August 10, 1998	February 1, 1999	F/73.7	+	6, 4	34	−	
	15 (710)	February 22, 1999	April 6, 1999	F/60.7	+	6, 4	90	−	Positive
	16 (711)	January 25, 1999	February 26, 1999	F/57.0	+	6	16	−	
	17 (713)****	January 12, 1999	May 7, 1999	F/67.8	+	6	14.5	nk	
	18 (715)***	August 30, 1999	September 27, 1999	F/59.3	−	2	10.5	−	
	19 (718)***	June 3, 1999	October 18, 1999	M/62.4	−	1	11.5	−	
	20 (721)	December 13, 1999	January 10, 2000	M/74.2	+	nk	28	−	
	21 (751)	July 9, 1997	October 25, 1998	M/73.8	+	5	19	−	
	22 (759)	November 2, 998	March 19, 1998	M/68.5	+	6	11.5	−	
	23 (760)	November 4, 1998	March 19, 1999	M/57.9	+	6, 4	15.5	−	
	Total no immune responses				85%				
	Total no immune responses (evaluable)				100%				

*immunological results published earlier by Gjertsen et al. **This patient had a distal resection and splenectomy, all others were Whipple operated. The histology specimen was revised from adenocarcinoma to endocrine carcinoma. Six years after primary surgery he developed a metastasis in liver segment 4, treated by left hemihepatectomy in June 2003. He is clinically and radiologically recurrence free at follow up January 2009. He has a ras mutation. All the others had histological proven adenocarcinoma. ***Not evaluable. Evaluable patients, i.e. patients having received at least 4 vaccinations ****Emigrated, last observed alive 30.03.2000nk: not known. NT: not tested

Table 2. TNM classifications

Patient no.	T	N	M	R	G
103	T1	N0	M0	R0	G1
104	T1	N0	M0	R0	G3
105	T3	N0	M0	R0	G2
201	T3	N0	M0	R0	G3
202	T2	N0	M1	R0	G3
203	T2	N1	M0	R0	G2
204	T3	N1	M0	nk	G2
205	T3	N1	M0	R1	G3
206	T3	N1	M0	R0	G2
207	T2	N0	M0	R	G2
702	T3	N1	M0	R0	G2
704	T3	N1	M0	R0	G2
705	T2	N1	M0	R1	G2
707	T3	N1	M0	R1	G2
710	T2	N0	M0	R0	nk
711	T3	N0	M0	R0	G2
713	T2	N1	M0	R0	G2
715	T4	N1	M0	R0	G2
718	T4	N1	M0	R0	nk
721	T3	N1	M0	R0	G3
751	T2	N0	M0	R0	G2
759	T3	N1	M0	R0	G3
760	T2	N1	M0	R1	G2

TNM classifications for all included patients, together with histological resectional margin (R0, free margin; R1, microcopic tumor-infiltration of resectional margin) and differentiation grade (G 1–4). Abbreviation: nk, not known, TNM: Tumor (T), lymph node (N), Metastases (M).

margins more often than previously reported, due to varying histological routines.[14–16] Furthermore, recent data have demonstrated that the presence of K-ras mutations in putative tumor-free resection margins indicate more aggressive tumor biology.[17] The knowledge that this is correlated with a higher rate of perineural and lymphovascular invasion and overall tumor aggressiveness may explain why surgery alone fails to cure the majority of patients. It also provides a rationale for adjuvant treatment with K-ras vaccination in patients with resected pancreatic cancer. An important goal for immunotherapy in this setting is therefore to consolidate surgery by eliminating those distant cells harboring a K-ras mutation. First, surgical debulking removes the main tumor mass and hence also changes the state of immune suppression induced by the tumor in favor of a less immunosuppressed environment. Second, prolonged survival (18–20 months) allows vaccinations to be repeated over an extended time period. In concert, this should potentially tip the balance in favor of the immune system. With this in mind, we initiated two clinical trials, with K-ras vaccination in surgical patients with resected pancreatic cancer. In the first trial (CTN-95002), 10 patients were treated with a single peptide vaccine, corresponding to the K-ras mutation in their cancer. In the second trial (CTN-98010), 13 patients were given a mixture of seven mutated ras peptides, corresponding to the most common mutations in pancreatic cancer. In both trials GM-CSF was used as an adjuvant. We have previously reported results of our first study, initiated in 1996.[18] The two studies, including follow-up protocols, had two objectives: First to investigate the safety and tolerability of ras peptide vaccination, either given as single peptides or as mixture of peptides; and second to register immune responses against the vaccine and the individual peptides. In this article, we present data on long-term T-cell responses and survival over a 10-year period of 23 patients from both trials.

Material and Methods

Patient selection

The treatment protocols were approved by the Regional Ethics Committee Health Regions I and II, and Norwegian Medicinal Agency and the studies were performed according to the principles of the Helsinki Declaration. Twenty-three patients with resectable pancreatic adenocarcinoma were enrolled into two clinical studies. Inclusion in the first protocol (CTN-95002) started in January 1997 ($n = 10$). Inclusion in the second protocol (CTN-98010) followed, ending in November 1998 ($n = 13$). Eight weeks before inclusion, abdominal CT-scans were taken of all patients. One week before vaccination, the following baseline studies were performed: physical examination (including performance status and medical history); hematological and biochemical testing. Inclusion required histologically proven adenocarcinoma of the pancreas, Karnofsky performance status >70% and adequate bone marrow, liver, heart and renal function. The age range was 18−75 years. For details see Table 1. HLA typing was not one of the inclusion criteria, thus the patients probably represent highly divergent HLA types. Exclusion criteria involved patients with active infection with hepatitis virus, HIV or patients treated with chemotherapy or radiation therapy within 4 weeks before vaccination. All patients gave informed consent before being enrolled. Demographic and therapeutic variables and response data are summarized in Tables 1 and 2. Twenty patients were evaluable (*i.e.*, completed visit four and received at least four vaccinations).

Vaccine and control peptides

Synthetic ras peptides encompassing residues 5–21 of p21 ras were synthesized and purified as clinical grade reagents under good manufacturing practice conditions (Norsk Hydro, Porsgrunn, Norway). Single peptides or peptide mix were supplied as a freeze-dried, sterile white powder soluble in water. Different preparations were employed for the two trials.

CTN-95002. The product was a kit consisting of four different separately freeze-dried peptides. Each peptide consisted of

17 amino acids *K-ras* 5–21: KLVVVGAXGVGKSALTI, where X corresponded to the amino acid substitution resulting from the mutation identified in the patient.

CTN-98010. The product kit consisted of a mixture of seven peptides (12ACDRSV13D/HCL) all peptides consisted of 17 amino acids (*K-ras* 5–21). Identification of *K-ras* mutations in the tumor was not a part of this protocol.

Treatment protocols

The trials were open, uncontrolled, Phase II studies of ras oncogene peptide vaccines, with two participating centers (Ulleval University hospital, Oslo, and Rikshospitalet University Hospital, Oslo), both tertiary referral centers. Pancreatic surgery was performed as classical Whipple procedures in 22 patients and as a distal resection including splenectomy in one patient. None of the patients underwent resection/reconstruction of major mesenteric vessels. Only patients with histologically proven ductal adenocarcinoma in the surgical specimen were included in the vaccination protocols. Median time from surgery to first vaccination was 2–4 months in both trials. Clinical, biochemical and radiological follow-up, similar to baseline, were performed at 3, 6, 9 and 12 months postoperatively. Thereafter, the patients were controlled every 6 months, during the next 2 years, followed by yearly controls.

In CTN-95002, eligible patients received six vaccinations of 100 μg peptide/injection i.d. in the para-umbilical area over a period of 12 weeks, at week 1, 2, 3, 4, 6 and 10. GM-CSF (40 μg Leucomax, Schering-Plough, Cork, Ireland) was used as an adjuvant. A follow-up clinical protocol (booster protocol) was designed for patients in a stable clinical situation, following the completion of the primary protocol (after week 14). The follow-up protocol started 3 to 6 months after the first protocol, and the interval between additional booster vaccinations (100 μg peptide/injection) was 3 to 4 months for up to 2 years.

In CTN-98010, the immunization regimen included two immunization period; first six administrations over a period of 12 weeks, at week 1, 2, 3, 4, 6 and 10 followed by one booster period of four weekly administrations. The interval between the first and second immunization period was 3 months. In each case, the dose of the individual peptides given was 100 μg/injection (totally 700 μg) i.d. in the para-umbilical area. GM-CSF (30 μg Leucomax) was used as an adjuvant. A final follow-up evaluation was performed at 1 year. Survival time was recorded from date of surgery to date of death, registered by the Norwegian National Population registry.

Delayed-type hypersensitivity

Delayed-type hypersensitivity (DTH) skin tests were performed with the vaccine injected i.d. (without GM-CSF) into the left para-umbilical area at a site distant from the vaccination site.

CTN-95002. DTH, skin test was performed at baseline and at week 12 (end of initiation period) and at each visit in the booster period (performed with 100 μg of the corresponding peptide vaccine).

CTN-98010. DTH skin test was performed in the same way as above at baseline and at each visit in the booster period, 700 μg peptide mix was used as DTH test at each treatment. A positive DTH test was defined as a $\geq$5 mm diameter erythematic/induration 48 hr after administration. The patients were instructed to measure and record the erythema/induration and report the result to the clinician.

In vitro T-cell responses

Patients included in CTN-95002 were analyzed for T-cell responses against the individual vaccine peptide given to each patient and control peptides using a standard proliferation assay. Prevaccine samples were analyzed in simultaneous assays with postvaccine samples.

A positive T-cell response is defined as a stimulatory index (SI) of $>$2 following vaccination. Patients with a positive T-cell response in baseline blood samples were only recorded as positive if vaccination resulted in a consistently increased SI. These results have been reported earlier[18] and are only summarized here (Table 1). In the *in vitro* T-cell assay used for documentation of memory responses, specific T cells were expanded from freshly isolated Periferal Blood Mononuclear Cells (PBMC) by one cycle of antigen-driven stimulation before assaying. Previously frozen ampoules harvested before and after vaccination were processed in parallel. No responses were observed in PBMC from normal blood donors using this procedure. Thawed PBMCs were seeded at 2×10^6 per well in 24-well plates (Costar, Cambridge, MA) in 1 ml of CellGro serum free medium (CellGenix, Germany) and antibiotics supplemented with mutant ras peptides or control peptides at 15 μM concentration. After 3 days of culture, the medium was supplemented with 10 U/ml of recombinant human interleukin-2 (Proleukine, Chirion Corporation, Emeryville, California, USA). Cultured cells were tested on day 10 for specific proliferating capacity against mutant ras and control peptides at 15 μM concentration, by using 5×10^4 T cells and autologous, irradiated (30 Gy) PBMCs (5×10^4 cells/well) as antigen-presenting cells (APCs). After 2 days, wells were pulsed with 3.7×10^4 Bq of ^{3}H-thymidine over night and counted. Values are given as mean counts per minute (cpm) from triplicate wells. SD of triplicates is usually $<$10% in this assay.

Statistical analysis

Survival time was recorded from date of surgery to date of death, registered by the Norwegian National Population Registry, updated last February 2009. Median survival time, 5- and 10-year survival rates were calculated by the Kaplan-Meier method.

Long-term T-cell memory response

Between February and April 2006, blood samples from the five 5-year survivors were collected and analyzed for long-

Table 3. Description of long term survivors (more than 5 years)

Patient number	Resection date	Date of first vaccination	Tumor localization	Tumor size mm	Histology	T status	N status	M status	R status	Ras mutation
705	December 1, 1998	January 4, 1999	Pancreatic head, also infiltrating papilla Vateri, duodenum	nk	Adenocarcinoma. Verified when reassessed	T2	N1	M0	R1	Not verified
710	February 22, 1999	April 6, 1999	Pancreatic head	25	Adenocarcinoma. Verified when reassessed	T2	N0	M0	R0	Not verified
103	January 9, 1997	March 5, 1997	Pancreatic head	10	Adenocarcinoma. Revised to ampullary origin	T1	N0	M0	R0	Val 12
201	January 27, 1997	March 10, 1997	Pancreatic head, also infiltrating papilla Vateri and the duodenal wall	20	Adenocarcinoma. Verified when reassessed	T3	N0	M0	R0	Cys 12
204	June 16, 1997	December 15, 1997	Pancreatic tail	42	Primarily: adeno-carcinoma, revised to endocrine carcinoma	T3	N1	M0	R1	Arg 12

Abbreviation: nk, not known.

term immunological response. The blood samples were analyzed using the T-cell assay described above. Data are expressed as cpm in the proliferation assay, following one round of *in vitro* stimulation.

Results

A summary of each patient's characteristics and results is given in Table 1. The Tumor (T), lymph node (N), Metastases (M) (TNM) data for all patients are given in Table 2, and a detailed description of the 5-year survivors are given in Table 3.

Safety and toxicity

Data on all patients who received K-ras vaccination were included in the safety analysis. The patients were followed closely for signs of adverse events during and after each vaccination. Adverse events were recorded using the WHO toxicity criteria. Peptide vaccination was administered on an outpatient basis and was well tolerated in all 23 patients. In CTN-95002, we have previously reported adverse events[18] such as mild fever or erythema around the vaccination site, lasting 1 to 2 days. Similar vaccine-related side effects were observed in CTN-98010. Other possible drug-related side effects were fever, chills, pain, fatigue, nausea and vomiting. We observed no clinical signs of autoimmune disease or abnormal biochemical and hematological parameters related to the vaccinations. Importantly, no sign of toxicity and no clinically serious adverse events following peptide vaccination were observed in any of the patients including long-term survivors who received up to 30 injections each. We accordingly conclude that mutant ras peptides in combination with GM-CSF can be injected repeatedly over a substantial period of time without any serious side effects.

Immune responses against mutant ras vaccine

All patients who completed visit four (week 4) and complied with the inclusion criteria were considered evaluable and included in the analysis of immune responses.

Three patients were considered nonevaluable, one in CTN-95002 and two in CTN-98010. Altogether there were 20 evaluable patients. Patients with a positive DTH test or the presence of *K-ras*-specific T-cells in peripheral blood after vaccination were considered immune responders. In CTN-95002, six of nine patients (67%) mounted an immune response, measured as positive DTH and/or a positive T-cell test *in vitro*. This percentage is somewhat higher than observed when patients with nonresectable pancreatic cancer were vaccinated with the same peptides.[18] In CTN-98010, all 11 patients (100%) were immune responders, registered as DTH response. The observation that all patients responded to the vaccine clearly demonstrates that this group of patients is quite immunocompetent, being able to respond to this mixture of neo-epitopes. Because of the nature of the design of the study, we do not know which of the peptides in the mixture was immunogenic in the individual patients. Altogether, 17 of 20 patients (85%) were immune responders, testifying to the immunogenicity of the mutant ras peptide in combination with Granlocyte Macrophage-Colony Stimulating Factor (GM-CSF) as adjuvant and the immunocompetence of Whipple operated patients.

Long-term T-cell memory response and long-term survival

All patients surviving 5 years (103, 201, 204, 705 and 710; see Tables 1 and 2) were responders to the vaccine in the initial protocol. From these patients, blood samples were collected and analyzed 7–9 years after treatment. The samples were tested in parallel with thawed frozen samples where

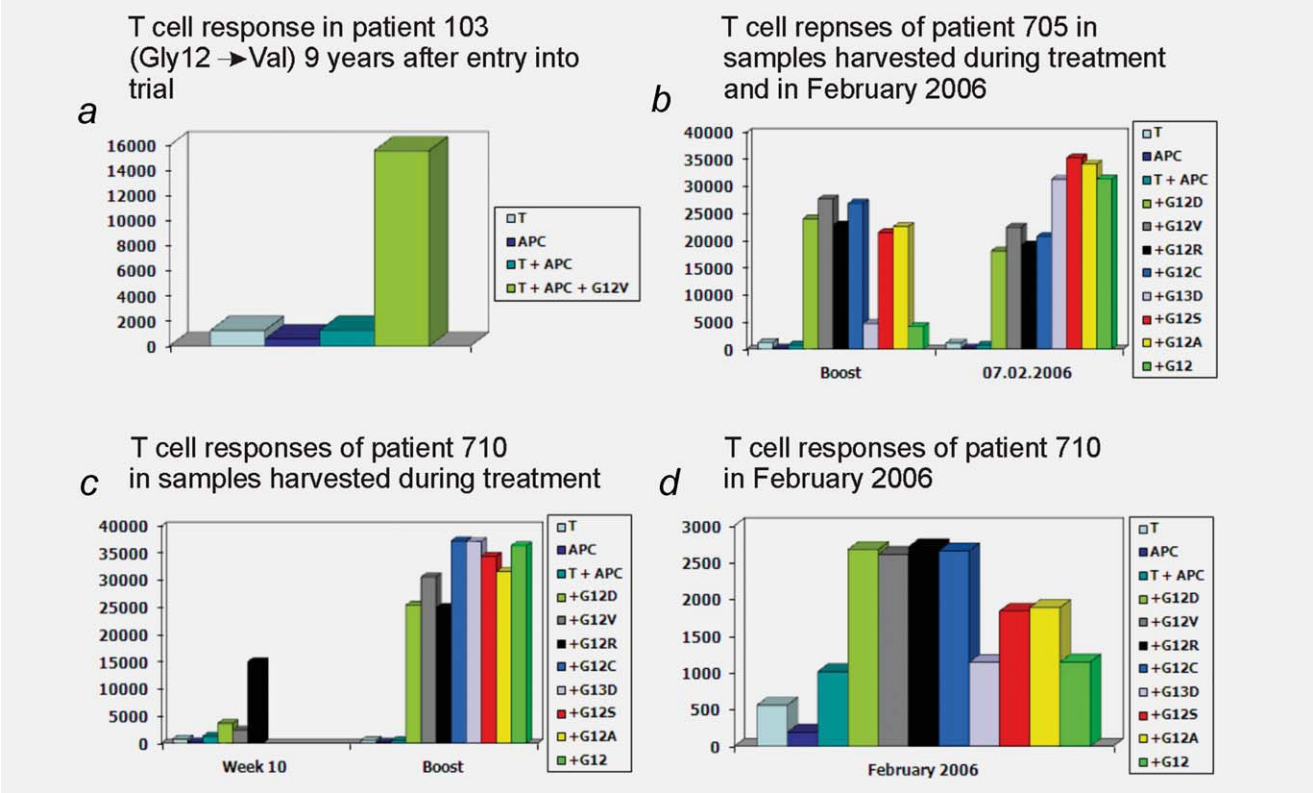

Figure 1. (*a*) Immune response of T cells from patient 103 from Protocol CTN-95002, harvested 9 years after treatment. The patient had received a single peptide vaccine representing the 12Gly → Val mutation. Results are recorded as mean cpm from triplicate wells containing 50,000 T-cells alone, 50,000 autologous, irradiated PBMC as antigen-presenting cells (APC) alone, 50,000 T cells + 50,000 APC, with or without the indicated peptide. (*b*) Immune response of T cells from patient 705 from Protocol CTN-98010, harvested in connection with the last booster injection and 7 years later. Results are expressed as in Figure 1*a*. The patient received a vaccine consisting of a mixture of peptides. Responses against the individual peptides and a peptide representing the normal K-ras sequence (G12) are given as in Figure 1*a*. (*c*) Immune response of T cells from patient 710 from Protocol CTN-98010, harvested during the vaccination regimen at week 10 and in connection with the last booster injection. The patient received a vaccine consisting of a mixture of peptides. Responses against the individual peptides and a peptide representing the normal K-RAS sequence (G12) are given as in Figure 1*a*. (*d*) T cell response of patient 710 harvested 7 years after treatment. Responses against the individual peptides and a peptide representing the normal K-ras sequence (G12) are given as in Figure 1*a*.

these were available, using a sensitive proliferation assay based on one cycle of *in vitro* stimulation with the vaccine peptides. Prevaccination samples from patients with pancreas cancer and samples from normal blood donors tested in the same assay did not respond to the ras vaccine peptides used. Two of the patients (201 and 204) had lost their immune response against the vaccine (data not shown). Interestingly, three patients (103, 705 and 710) mounted a long-term T-cell memory response against the vaccines. In patient 103 (Fig. 1*a*), the responding T cells were specific for the single vaccine peptide used (Gly12Val mutation), corresponding to what was observed earlier.[11] T-cell responses in the two other patients (705 and 710) had not been tested *in vitro* before as the responses were only measured as DTH. From patient 705, a blood sample harvested after boosting was available for comparison (Fig. 1*b*). It contained T-cells recognizing all of the peptides present in the vaccine. These data thus confirm and extend the DTH data from the trial, listing this patient as an immune responder. Interestingly, a weak reaction against the nonmutated 12G peptide was also observed, indicating the generation of T-cells with cross-reactivity for the wild-type peptide by the vaccination. Strikingly, the memory response observed in the blood sample harvested approximately 7 years later was of the same magnitude and showed the same broad reactivity against all the peptide constituents of the vaccine. For one of the vaccine peptides (Gly13Asp mutation) the T-cell response had increased in magnitude, and the same was observed for the cross-reactivity against the wild-type peptide indicating an enhancement of the immune response in the patient during the interval since boosting. For patient 710, two previous samples were available for *in vitro* T-cell analysis (Figs. 1*c* and 1*d*). A comparison of the samples harvested in week 10 and after boosting revealed some interesting features (Fig. 1*c*). Clearly,

boosting strongly increased the immune response against the three peptides recognized in week 10. In addition, a strong T-cell response against four other peptides was generated, as well as T-cells cross reacting against the wild-type peptide, which was not present in the vaccine. The same pattern of reactivity was seen in the blood sample harvested 7 years later, although the T-cell response was markedly reduced (Fig. 1*a*). The vaccine used consists of long peptides, mainly designed to generate potent T-helper responses. In accordance with this, the T-cell responses observed in these patients are T-helper responses. Few clinical trials have recorded survival data beyond 5 years for patients with pancreatic adenocarcinoma. To ensure correct interpretation of survival data the surgical specimens from the five long-term survivors were histopathologically reassessed by independent pathologists and the diagnosis was revised to an endocrine carcinoma in one patient (204) (for details see Table 3). However, also this patient had an identified K-ras mutation and was an immune responder.[18] This patient developed a liver metastasis in segment 7, 6 years after primary surgery. He was treated by left hemihepatectomy in June 2003 and was clinically and radiologically recurrence free at follow-up January 2009.

In the whole patient cohort, who had their tumors resected, followed by vaccination against mutant K-ras, the median survival time for all patients was 27.5 months. For all vaccinated patients ($n = 23$), the 5- and 10-year survival was 22% and 17%, respectively. The corresponding data for evaluable patients ($n = 20$) was 25% and 20%, respectively, and for immune responders ($n = 17$), 29% and 24%, respectively. When excluding the patient with the endocrine carcinoma in the pancreatic tail, 5-year survival in evaluable patients ($n = 19$) was 21% (4/19), and for immune responders ($n = 16$) 25% (4/16). The corresponding 10-year survival rates were 16% (3/19) and 19% (3/16), respectively. Patient 710, one of the long-time survivors died in 2006, having lived for 7 and a half years after diagnosis.

Discussion

We present here the data from two clinical protocols testing the safety and immunogenicity of mutant ras vaccines after pancreatic resections for adenocarcinoma, together with results of a 10-year follow-up of T-cell memory responses and overall survival. The most important finding reported here is the persistence of T-cells recognizing vaccine peptides many years after the last vaccination. Second, serious adverse events attributable to the vaccine treatment were not observed in any of the patients. Third, although the number of patients is relatively low, one might interpret the results as a correlation between initial immune response, long-term T-cell memory response and survival.

It should be noted that patients who generated robust immune responses to the vaccine and were given up to 24 booster injections over a prolonged period of time showed no sign of harmful side effects. The unique tumor specificity of the mutated K-ras antigen may explain this fact. Interestingly, two patients developed a T-cell response cross-reacting with the wild-type sequence in ras. This cross-reactivity, which is autoimmune in nature, did not however result in adverse effects, and one may speculate that the cross reactivity may have perpetuated the reactivity against the mutant peptides over time. In both studies, a high proportion of the patients were able to mount an immune response to the vaccine. This observation fits well with the promiscuous nature of the HLA-Class II binding of mutant ras peptides, which was described before the start of our vaccination studies.[19] In the first study CTN-95002, 67% (6/9) evaluable patients mounted an immune response to the vaccine. Importantly, these responses must be regarded as relevant for the patients, as they were directed against the specific mutation present in each of the patient's cancer. In the second study CTN-98010, all of the 11 evaluable patients demonstrated a positive immune response measured by the skin reaction against the vaccine. One hundred percent reactivity against a vaccine is rarely obtained in healthy individuals vaccinated with complex microbial vaccines and is quite remarkable in a cohort of cancer patients vaccinated with a self-antigen modified by a single amino acid substitution as a result of a point mutation. Obviously, these mutations can be highly immunogenic and giving a mixture of seven different mutant ras peptides to each patient may have greatly increased the chance that some T-cells in the patients' repertoire would recognize at least one of the injected peptides. Indeed, one of the conclusions of this study is that probably all patients have the capacity to recognize at least one and possibly more than one of the seven mutant ras peptides included in the vaccine. Thus, a broader immune response might have developed due to synergy between responses against single peptides, and response against a more immunogenic peptide might help T-cells reacting against a second peptide through paracrine cytokine secretion. Unfortunately, we did not have the opportunity to analyze the immune response of these patients in detail, as blood samples were not systematically collected for T-cell analysis in this protocol.

The persistence of T-cells immunity in vaccinated patients is most strikingly illustrated in patient 103. Here, vaccination with a peptide only differing by a single amino acid from the naturally occurring, nonmutated sequence, resulted in a >9 year persistence of T-cells specifically recognizing the mutation. Arguably, the immune response in this case might have been maintained by the remaining tumor cells containing the mutation. However, the fact that the patient is still alive and without evidence of cancer argues against this explanation. Pancreatic adenocarcinoma usually contains only a single ras mutation. The observation of long-term memory against all the seven peptides present in the peptide mixture used as a vaccine in CTN-98010 in patient 705 also argues strongly against this explanation, since it is very unlikely that several mutations in the patient's tumor would occur.

Long-term memory against peptide epitopes has rarely been investigated in longitudinal studies. It is therefore interesting that persistent T-cell responses against a survivin epitope, 7 years after complete remission following Il-2 immunotherapy of

malignant melanoma, has recently been described.[20] This observation clearly establishes a connection between persistent T-cell memory against a cancer antigen and clinical development. The persistence of T-cell memory reported here extends this observation and, more specifically, links the memory response to vaccination and long-term survival. Interestingly, the median survival both in evaluable patients and, in particular, in immune responders, is higher in the CTN-95002 than in the CTN-98006 trial. This result may be related both to the differences in the composition of the vaccines and the vaccination regimen. In CTN-95002, all included patients had a verified K-ras mutation; accordingly they were all given the relevant vaccine. In the CTN-98010 trial, no mutation analysis was performed. Some patients without a ras mutation at all may therefore have been included in this trial. Clearly they would not benefit from having responded to the vaccine. As discussed above, some of the patients recorded as immunological responders in the CTN-98010 trial might not have mounted an immune response against the peptide representing their own mutation, but against other peptides in the vaccine. Strictly speaking, these patients are nonresponders against the relevant vaccine component. Unfortunately, the limited number of patients included in the two trials is too small to allow any firm conclusion regarding the relative clinical efficacy of the two vaccination regimens.

Interestingly, two of the long-term survivors (204 and 705) in our study had metastasis to lymph nodes and had positive resection margins. Patient 204, who was reassessed to have an endocrine carcinoma, did, however, have a K-ras mutation. This observation is puzzling, as the presence of a K-ras mutation is highly frequent in pancreatic adenocarcinomas, but virtually absent in neuroendocrine tumors of the pancreas when these are benign.[21] Both the presence of K-ras mutations, and the fact that this patient developed a liver metastasis 5 years after the pancreatic resection, clearly shows that this endocrine carcinoma had a malignant potential.

A highly important factor for evaluation of survival in pancreatic cancer patients is a correct diagnosis and origin of the adenocarcinoma. In a nation-wide study of long-term survivors from the Finnish Cancer Registry, a re-evaluation of the histologically proven adenocarcinoma of the pancreas demonstrated that in only 10 of 26 long-term survivors was the diagnosis of adenocarcinoma confirmed. Hence the alteration of histological diagnosis in pancreatic tumors after critical reinvestigation also has an obvious relevance for survival analysis.[22] Also, even after systematic histopathological evaluation of pancreaticoduodenectomy specimen, the precise anatomical origin (pancreatic, ampullary, distal bile duct and duodenum) of the adenocarcinoma may be impossible to determine, with potential effects on survival evaluation.[23] This was the case for one of the patients (103) in this study, who was reclassified as an adenocarcinoma originating in the ampulla.

The role of adjuvant chemotherapy in pancreatic cancer patients has been extensively tested and significant survival benefit has been documented.[24–26] In the time period up to 2004, coinciding with the treatment period for the two trials studied, the national guidelines for chemotherapy for pancreas cancer in Norway did not recommend pre- or postoperative chemoradiation therapy. Thus, none of the patients, from any of the two study centers received adjuvant therapy, neither pre- nor postoperatively. The median survival of all other patients treated at Ulleval during this period ($n = 87$) was 16 months, their 5-year survival was 15.8% and 10-year survival was zero. The surgical specimens of these patients have not been histologically reinvestigated. There could thus probably be some endocrine carcinoma included in the 87 pancreatic resections. If these patients had been identified and excluded, survival in the group would be reduced. When we compare these data with the corresponding data from the vaccinated patients, the different 10-year survival is striking with 4/20 patients in the vaccinated group *vs.* 0/87 patients in the nonvaccinated group. The TNM status of the vaccinated group (Table 2) illustrates that numerous patients (52%) had lymph node metastases and hence advanced disease, illustrating that patient selection has been comparable with other published series of pancreatic tumors resection. Although randomized trials have shown significant effects of adjuvant 5-fluorouracil[24] and gemcitabine,[26] the effects are still modest, and a large majority of the patients suffers recurrence of the disease.[27] New adjuvant strategies are therefore needed. Immunotherapy in the form of vaccination against mutant K-ras as an adjunct to surgical resection appears as a promising principle of adjuvant therapy. Although the data presented here represents a nonrandomized study of a small number of patients with no control group, the findings support the hypothesis that immunotherapy has been clinically beneficial. Taking into account that ras vaccination is virtually free of side effects, the results should encourage a much larger controlled study on the same group of patients.

Acknowledgements

We thank study nurses Ms. Anne Mari Svardal and Ms. Wenche Risvik and bioengineer Ms. K. Lislerud for excellent technical assistance and Mericon AS for monitoring the study.

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