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Restoration of expression of signal-transduction molecules in lymphocytes from patients with metastatic renal cell cancer after combination immunotherapy

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Abstract A decrease in lymphocyte signal-transduction molecules, described in cancer patients and patients with chronic infectious diseases, has been proposed as a possible mechanism leading to an impaired immune response in cancer patients. Here we report the effects of combination immunotherapy on the levels of T cell receptor ζ chain and $p56^{lck}$ tyrosine kinase in a retrospective study of cryopreserved lymphocytes from 26 metastatic renal cell carcinoma patients treated with high-dose interleukin-2 (IL-2), interferon α (IFNα) and ex vivo IL-2-activated lymphocytes. Of the 26 patients, 12 were responders (5 complete and 7 partial) and 14 were non-responders (6 stable and 8 with progressive disease). Prior to treatment, 21 of 26 patients (81%) and 13 of 21 patients (62%) respectively expressed ζ chain and $p56^{lck}$ at less than 50% of the levels observed in healthy controls. During therapy, this low ζ chain and $p56^{lck}$ expression increased to at least 50% of normal in 13 of the 21 patients (62%) and in 6 of the 13 patients (46%) respectively; in the remaining patients expression levels remained at 50% of normal or more, or declined. Although, in this limited study, pretreatment levels of ζ and $p56^{lck}$ did not show significant correlation with antitumor response, 4 of 5 patients that achieved a complete response (80%) corrected *both* ζ chain and $p56^{lck}$ levels to at least 50% of normal, while restoration of both signal-transduction molecules to such levels was only observed in 3 of 7 partial responders (43%), 1 of 5 patients with stable disease (20%) and 2 of 7 patients with progressive disease (29%). Thus, these results suggest that analysis of changes in signal-transduction molecules may a be useful tool for immunological monitoring of patients throughout immunotherapy, and could provide important information for designing new clinical trials that restore impaired signal transduction while activating T cell responses.

Key words Signal transduction · Lymphocytes · Immunotherapy · Renal cell carcinoma

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Introduction

The progression of cancer is accompanied by a general reduction of immune functions such as natural killer activity [25], the cytolytic function of T lymphocytes [26], lymphokine production [7] and delayed-type hypersensitivity [29]. In this context, the alterations in signal transduction of T lymphocytes from tumor-bearing mice, i.e., altered patterns of protein-tyrosine phosphorylation and the significant reductions of the protein tyrosine kinase $p56^{lck}$ and ζ chain [17], have raised much interest. As ζ chain and $p56^{lck}$ fulfill pivotal functions in signal transduction and subsequent activation of T lymphocytes, their impaired expression might contribute to the reduced immune cell functions associated with advanced cancer. This finding has been reported in patients with cancer of several histological types and in various stages of progression. For example, reductions in ζ chain and $p56^{lck}$ expression have been observed in tumor-infiltrating and peripheral blood lymphocytes (PBL) from patients with metastatic renal cell carcinoma (RCC), colon carcinoma, ovarian carcinoma, melanoma and prostate cancer [2, 4–6, 11, 13, 14, 21, 27, 31], as well as some hematological malignancies [22]. In some studies the magnitude of reductions in ζ chain and protein-tyrosine kinase expression appeared to be inversely correlated with the distance between the sampling site of the lymphocytes and the tumor lesions. These observations suggest that direct contact between

tumor cells and lymphocytes, or tumor-derived factors acting over a short range may be responsible for these reductions [4, 14]. In addition, the impairment of ζ chain expression becomes more severe with increasing tumor burden [14].

We have observed significant therapeutic efficacy in patients with metastatic RCC treated with combination immunotherapy [i.e., high-dose interleukin-2 (IL-2), interferon α (IFNα) and autologous IL-2-activated lymphocytes]: overall response rates ranging between 24% and 37% [10, 12]. The severe toxicity associated with this type of treatment calls for the development of a means to monitor the immune response and identify patients who are likely to show a clinical antitumor response. Our analysis of several basic immune parameters (i.e., leukocyte and lymphocyte subset counts, cytolytic activities and serum cytokine concentrations) during treatment has not yielded a parameter with predictive value in this respect [10]. We therefore decided to test the effect on the expression of ζ chain and $p56^{lck}$ in PBL of an immunotherapy combination known to induce a clinical response in RCC patients.

Materials and methods

Patients

We performed a retrospective analysis, using specimens of peripheral blood mononuclear cells (PBMC) that had been cryopreserved and stored in liquid N_2, in the setting of a longitudinal study of the immunological effects of combination immunotherapy of metastatic renal cell carcinoma [10, 12]. The selection of patients for this study was determined by the availability of cryopreserved PBMC specimens. Briefly, the treatment schedule consisted of two 5-week induction cycles of continuous i.v. high-dose IL-2 [Teceleukin (Hoffmann-la Roche, Basle, Switzerland), 18×10^6 IU m^{-2} $(24\ h)^{-1}$ for 120 h] and i.m. IFNα [Roferon-A (Hoffmann-la Roche), 5×10^6 IU m^{-2} day^{-1} for 5 days], followed by three apheresis sessions (days 6–8) to obtain lymphocytes for ex vivo activation using IL-2, and reinfusion of the activated lymphocytes on days 11–13 combined with i.v. IL-2 [18×10^6 IU $m^{-2}(24\ h)^{-1}$ for 108 h] and i.m. IFNα (5×10^6 IU m^{-2} day^{-1} for 4 days). On day 70 following the start of treatment, patients having a complete response (CR) or a partial response (PR), evaluated against WHO criteria, were followed with four 4-week maintenance cycles of i.v. IL-2 [18×10^6 IU m^{-2} $(24\ h)^{-1}$ for 96 h] and i.m. IFNα (5×10^6 IU m^{-2} day^{-1} for 4 days), while those with stable (SD) or progressive disease (PD) did not receive further immunotherapy. PBMC specimens were available from 26 patients; 4 of them were treated with Proleukin IL-2 (Eurocetus, Amsterdam, The Netherlands) and did not receive IFNα during days 1–5. We compared the changes in the expression of signal-transduction proteins T cell receptor ζ chain and $p56^{lck}$, with natural killer (NK) and lymphokine-activated killer (LAK) activity and clinical response in each patient.

Assessment of expression of signal-transduction molecules

Peripheral blood mononuclear cells were enriched from heparinized venous blood specimens by Ficoll density-gradient centrifugation and were cryopreserved [10]. After thawing, the PBMC were centrifuged and resuspended for 5 min at 4 °C in 10 µl lysis buffer/10^6 PBMC. The lysis buffer consisted of 50 mM HEPES, pH 7.2, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 1 mM sodium orthovanadate, 100 mg/ml soybean trypsin/chymotrypsin inhibitor, 100 mg/ml aprotonin, 100 mg/ml leupeptin, 100 mg/ml chymostatin and 2 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, Mo.). This combination of protease inhibitors has previously been shown to prevent the artifactual degradation of signal-transduction proteins during the extraction process [8]. The lysates were centrifuged at 15 000 rpm for 10 min, after which the supernatant (i.e., the cytoplasmic extract) was collected, extreme care being taken not to disturb the pellet of cellular debris. Aliquots were immediately frozen at −70 °C.

Determinations of ζ chain and $p56^{kic}$ tyrosine kinase were by Western blotting. Cytoplasmic extracts of 10^6 PBMC were electrophoresed (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) in 14% (ζ chain) and 8% ($p56^{lck}$) TRIS-glycine gels (Novex Experimental Technology, San Diego, Calif.). The membranes were immunoblotted with Onco-Zeta 1 serum (a kind gift from Biomira USA, Cranbury, N.J.) and anti-(human CD3-ϵ) serum (Dako, Glostrup, Denmark) at 1:1000 and 1:6000 dilutions, respectively, or with anti-$p56^{lck}$ serum (UBI, Lake Placid, N.Y.) at 1:1000 dilution for 1 h. The membranes were then washed and incubated with horseradish-peroxidase-conjugated anti-(rabbit immunoglobulin) (Amersham Pharmacia, Rainham, UK) for 20 min, and developed with the ECL enhanced chemiluminescence kit (Amersham Pharmacia). X-OMAT AR film (Eastman Kodak Co., Rochester, N.Y.) was used to obtain autoradiographs after 1.0-, 2.5- and 5.0-min exposure times. The resulting bands were quantified by an Alpha Imager 2000 densitometer (Alpha Innotech Co., San Leandro, Calif.) and the resulting values were compared between healthy controls and patients.

Immunophenotyping and cytotoxicity assays

Absolute lymphocyte counts were obtained from EDTA-anticoagulated blood in a Technicon H1 counter (Bayer, Tarrytown, N.Y.). Flow-cytometric immunophenotyping was performed on freshly obtained heparinized venous blood specimens [10]. Cytotoxicity assays were performed on cryopreserved and thawed PBMC specimens [10].

Statistical analysis

For some analyses, patients with an overall antitumor CR or PR were classified as responders, and those with SD and PD as nonresponders. Western blot results of ζ chain and $p56^{lck}$ were expressed on a scale of 0–4 relative to those in PBMC of healthy controls, i.e. 4, at least 75% of normal; 3, 51%–74% of normal; 2, 26%–50% of normal; 1, 1%–25% of normal; and 0, undetectable signal. For some analyses, Western blot results were divided into those that were at least 50% (≥50%) of normal, i.e., level 3 or 4, and those below 50% (<50%) of normal, i.e., levels 0–2. The correlation between the levels of ζ chain or $p56^{lck}$ tyrosine kinase expression and antitumor response were analyzed by two-sided χ^2- and Fisher's exact tests, while the effects of pretreatment ζ chain or $p56^{lck}$ levels on survival and time to progression of disease were studied by the log-rank test. The interactions between ζ chain or $p56^{lck}$ levels and other immune parameters (absolute lymphocyte count, immunophenotype and cytolytic activities) were evaluated by Spearman's rank-correlation test.

Results

Expression of ζ chain and $p56^{lck}$ tyrosine kinase prior to and after immunotherapy

Most patients expressed lower levels of ζ chain prior to immunotherapy than did healthy controls: 21 of 26 patients (81%) showed levels between 0 and 2 (i.e., <50%

of normal) on a scale of 0–4 as measured by densitometry (Table 1). Similarly, the majority of patients showed significantly lower levels of $p56^{lck}$ prior to treatment than did healthy controls: 13 of 21 patients (62%) had levels between 0 and 2 on a scale of 0–4 (Table 2). These results confirm the reduced levels of signal-transduction molecules in RCC patients as reported by us [6] and others [5, 13]. During immunotherapy, 13 of 21 patients (62%) who initially had low ζ chain expression, upregulated this molecule to normal levels, i.e., they showed an increase from 0, 1 or 2 prior to treatment, to 4 (Table 1). Three other patients maintained ζ chain expression at ≥50% of normal levels (i.e., 3 or 4), while the remaining 2 patients showed a reduction of ζ chain expression during treatment (i.e., from 4 to 2). Furthermore, 6 of 13 patients (46%) with $p56^{lck}$ expression at <50% of normal levels showed re-expression to ≥50% of normal after immunotherapy. The remaining 7 patients maintained $p56^{lck}$ expression at ≥50% of normal levels.

Correlation between expression levels of ζ chain and $p56^{lck}$ and clinical antitumor response

We did not observe any statistically significant associations between pretreatment levels of ζ chain and $p56^{lck}$, and clinical antitumor response. Specifically, 2 of 12 responding patients expressed ζ chain at ≥50% of normal levels compared to 3 of 14 nonresponding patients (Table 1); 3 of 11 responding patients expressed $p56^{lck}$ at ≥50% of normal levels compared to 5 of 10 nonresponding patients (Table 2). However, the analysis of alterations in expression levels of ζ chain and $p56^{lck}$ upon treatment in relation to antitumor response yielded some interesting results.

Whilst ζ chain expression at ≥50% of normal levels was observed at any time during or after immunotherapy in 8 of 12 responding (67%) and 8 of 14 nonresponding patients (57%), $p56^{lck}$ expression at ≥50% of normal levels was significantly more frequent in responding than in nonresponding patients. Out of 12 responding patients, 10 (83%) expressed $p56^{lck}$ at levels ≥50% of normal at any time during or after immunotherapy, whilst only 4 of 12 nonresponding patients (33%) did so ($P = 0.04$).

When levels of ζ chain and $p56^{lck}$ upon start of treatment were analyzed *in combination*, the following pattern became evident. Four of 5 (80%) patients (patients 1, 3, 4 and 5) achieving a complete response re-expressed both ζ chain and $p56^{lck}$ to (sub)normal levels (i.e, level 3 or 4). The fifth patient (patient 2) showed a decrease of ζ chain and $p56^{lck}$ from level 4 prior to therapy to levels 3 and 2 respectively after treatment. Three of 7 (43%) patients (patients 8, 11 and 12) achieving PR recovered both ζ chain and $p56^{lck}$ expression to levels 3 or 4. Two other patients (patients 7 and 10) only (re-)expressed their $p56^{lck}$ levels to level 3;

Table 1 Immunotherapy-associated changes in ζ chain expression (scored 1–4) by peripheral blood lymphocytes. *CR* complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease, – no data

Patient	Clinical antitumor response	Change in ζ chain expression relative to start of immunotherapy					
		Days −30–0	Days 18–20	Day 35	Days 51–55	Days 60–102	Days 110–165
1	CR	2	–	–	–	4	4
2	CR	4	–	–	–	–	2
3	CR	2	–	–	–	–	4
4	CR	2	–	–	4	–	–
5	CR	2	–	–	–	–	4
6	PR	2	–	–	2	–	–
7	PR	1	–	–	–	2	1
8	PR	1	–	–	–	–	4
9	PR	1	–	–	4	–	–
10	PR	4	–	–	–	2	–
11	PR	0	–	–	–	4	–
12	PR	1	–	4	–	–	4
13	SD	1	–	–	–	1	2
14	SD	1	–	0	–	–	–
15	SD	0	–	–	1	–	–
16	SD	1	–	–	–	–	2
17	SD	4	–	–	–	4	–
18	SD	2	–	–	4	–	4
19	PD	4	–	4	–	–	–
20	PD	3	–	–	3	–	–
21	PD	1	4	–	–	–	–
22	PD	1	4	–	–	–	–
23	PD	1	–	–	–	4	–
24	PD	2	2	–	–	–	–
25	PD	2	–	–	–	4	–
26	PD	2	2	–	–	–	–

Table 2 Immunotherapy-associated changes (scored 1–4) in $p56^{lck}$ expression by peripheral blood lymphocytes. – No data

Patient	Clinical antitumor response	Change in $p56^{lck}$ expression relative to start of immunotherapy					
		Days −30–0	Days 18–20	Day 35	Days 51–55	Days 60–102	Days 110–165
1	CR	2	–	–	–	4	4
2	CR	4	–	–	–	–	3
3	CR	1	–	–	–	–	3
4	CR	3	–	–	4	–	–
5	CR	2	–	–	–	–	4
6	PR	1	–	–	2	–	–
7	PR	2	–	–	–	2	3
8	PR	2	–	–	–	–	4
9	PR	2	–	–	2	–	–
10	PR	4	–	–	–	3	–
11	PR	–	–	–	–	4	–
12	PR	2	–	–	–	–	3
13	SD	–	–	–	–	–	2
14	SD	–	–	–	–	–	–
15	SD	–	–	–	2	–	–
16	SD	4	–	–	–	–	4
17	SD	–	–	–	–	2	–
18	SD	3	–	–	4	–	4
19	PD	4	–	3	–	–	–
20	PD	2	–	–	2	–	–
21	PD	2	2	–	–	–	–
22	PD	4	–	–	–	–	–
23	PD	2	–	–	–	2	–
24	PD	2	2	–	–	–	–
25	PD	4	–	–	–	4	–
26	PD	2	2	–	–	–	–

the sixth patient (patient 9) only showed ζ chain up-regulation to level 4, and the levels of both signal transduction molecules remained low in patient 6. Re-expression of both ζ chain and $p56^{lck}$ to level 4 was observed in only 1 of 5 (20%) patients with SD (patient 18). Two other patients (16 and 17) expressed only one of these molecules at level 4 and the remaining 2 patients (13 and 15) expressed low levels of both ζ chain and $p56^{lck}$ upon treatment. Similarly, only 2 of 7 (29%) patients with PD (patients 19 and 25) recovered or maintained both ζ and $p56^{lck}$ expression at ≥50% of normal levels upon treatment. Three further patients with PD (20, 21 and 23) recovered or maintained ζ expression at ≥50% of normal levels but expressed $p56^{lck}$ at <50% of normal levels. The remaining 2 patients with SD (24 and 26) expressed both ζ chain and $p56^{lck}$ at <50% of normal levels upon treatment. Thus, re-expression of *both* ζ chain and $p56^{lck}$ to ≥50% of normal levels upon immunotherapy appeared to be more frequent in patients achieving CR, followed by those patients showing a PR and less frequent in patients with SD or even PD.

Correlation between expression of ζ and $p56^{lck}$ and other immune parameters

We also correlated the expressions of ζ chain and $p56^{lck}$ with the absolute numbers of lymphocyte subsets and cytolytic activities of PBMC assessed on the same specimens [10]. Most patients had normal numbers of NK ($CD56^+$,3^-) and T ($CD3^+$) lymphocytes prior to the start of immunotherapy. During immunotherapy, most patients had increased numbers of NK lymphocytes, a well-known effect of IL-2 treatment. T lymphocytes increased only above the normal range during the episodes of rebound lymphocytosis [10]. The level of ζ chain expression was positively correlated with absolute NK ($r = 0.63$) and, to a lesser extent, with T lymphocyte counts ($r = 0.41$) in the 57 samples available for this analysis (Fig. 1, upper panels). Similarly, NK ($r = 0.52$; Fig. 1, lower right panel) and lymphokine-activated killer (not shown) cytolytic functions also showed positive correlations with ζ chain expression. Although the levels of $p56^{lck}$ expression in the 57 PBMC samples were positively correlated with those of ζ ($r = 0.40$), they did not correlate significantly with cytotoxic function (not shown). This finding is not unexpected since both NK and T lymphocytes express ζ chain [1, 30]. However, it is unknown whether the re-expression of ζ chain was due to a preferential increase in NK or T cells, since these subsets were not analyzed separately.

Discussion

We have studied the expression of the signal-transducing molecules T cell receptor ζ chain and $p56^{lck}$ in PBL of patients with metastatic RCC prior to and during or

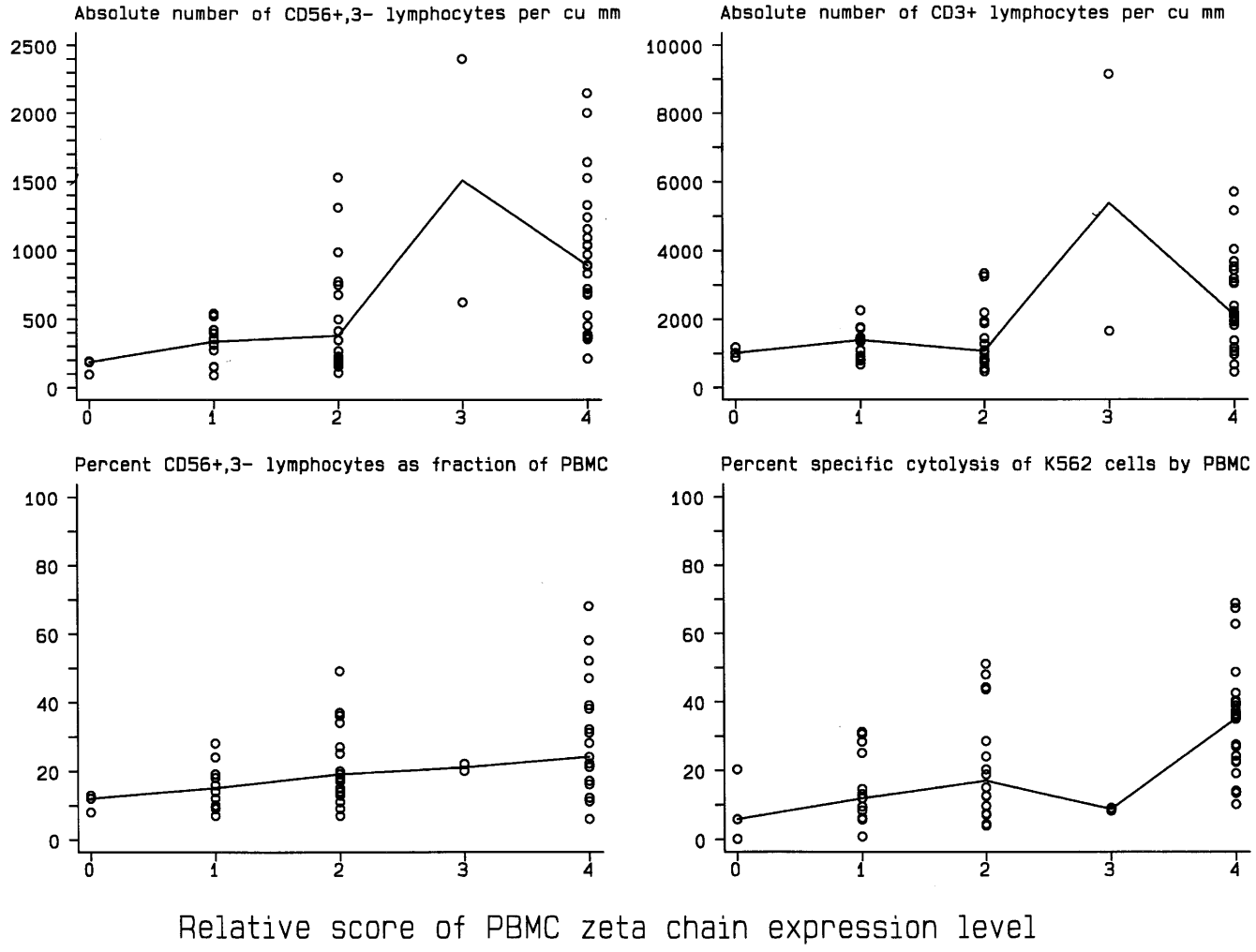

Fig. 1 Correlations between peripheral blood mononuclear cell (*PBMC*) ζ chain expression level and absolute number of $CD56^+,3^-$ natural killer (NK) lymphocytes (*upper left panel*), absolute number of $CD3^+$ T lymphocytes (*upper right panel*), the percentage of $CD56^+,3^-$ NK lymphocytes as fraction of PBMC (*lower left panel*) and NK activity of PBMC against K562 target cells (*lower right panel*). The lines connect the median values of each group defined by ζ expression level (i.e., 0–4)

after combination immunotherapy with high-dose-IL-2, IFNα and autologous IL-2-activated lymphocytes. In line with the observations of Rabinowich et al. [21], who observed that 3 patients with advanced melanoma achieving CR restored the levels of T cell ζ chain expression, we hypothesized that the levels of these molecules might predict the clinical antitumor response. Although this limited retrospective study did not show a predictive ability of these two assays, several interesting results were obtained.

The PBL of most of the RCC patients expressed decreased levels of ζ chain and $p56^{lck}$ prior to immunotherapy, which confirms the results of Farace et al. [5], Finke et al. [6] and Bukowski et al. [2] in patients with metastatic disease. Cardi et al. [3] failed to observe such signal-transduction changes in RCC patients. However, these apparently different observations may have been caused by the fact that Farace, Finke and Bukowski studied patients with advanced disease, while Cardi [3] studied patients at the time of resection of their primary tumor. In addition, the latter group used directly conjugated anti-ζ antibody, which yields a high fluorescence background and, therefore, possibly false positive results. The extent of ζ down-regulation has been found to be proportional to cancer dissemination [14].

The mechanism by which growing cancer would down-regulate T and NK lymphocyte signal-transduction molecules is still unclear. It is important to note that ζ and $p56^{lck}$ have been down-regulated to a larger extent in tumor-infiltrating lymphocytes than in PBL [4, 6, 14]. Short-range effects of tumor-derived immunosuppressive factors [16] and/or hydrogen peroxide secreted by tumor-associated macrophages [19] on lymphocytes have been proposed to explain this down-regulation. However, the recent observations that patients with chronic infections such as HIV [28] and leprosy [32], and even those with auto-immune disease such as rheumatoid arthritis [15], also show decreased expression of ζ chain and $p56^{lck}$, suggest that tumor-specific factors are unlikely to be the only cause of these alterations. An

alternative explanation to chronic antigenic stimulation and oxidative stress is that down-regulation of signal-transduction molecules might be related to the predominance of T-helper type 2 responses reported in patients with advanced cancer [20]. In this context it is of interest that the type 2 cytokine IL-4 induces loss of lymphocyte ζ chain expression and antitumor activity in a murine fibrosarcoma model [9].

The immunotherapy combination used in this trial (i.e., high-dose IL-2, IFNα and autologous IL-2-activated lymphocytes) restored the expression of ζ chain and $p56^{lck}$ in 62% and 46% of our patients respectively. Increments of ζ chain expression by PBL have also been observed in melanoma patients treated with high-dose IL-2 [21] and in RCC patients treated with low-dose IL-2 and IFNα [2], but not in patients with advanced cancer treated with low-dose IL-2 [5, 21]. In contrast, neither low- or high-dose IL-2 up-regulated $p56^{lck}$ in PBL from patients with advanced melanoma [21]. Although IL-2 by itself is able to up-regulate ζ chain [21, 27, 28] and $p56^{lck}$ [21] in vitro, combination immunotherapy such as (high-dose) IL-2, IFNα and possibly IL-2-activated lymphocytes may be needed to achieve restoration of both ζ chain and $p56^{lck}$ in vivo.

Most patients with decreased ζ chain expression also showed a $p56^{lck}$ deficiency. One interesting observation was that patients achieving CR or PR more frequently restored their $p56^{lck}$ expression levels than did those with SD or PD. Furthermore, our data suggest that recovery of *both* signal-transduction molecules is more frequently observed in patients achieving CR than in those with SD or even PD. Our data also indicate that additional stimulatory signals may be needed to induce $p56^{lck}$ in these patients. The rapid turnover of the ζ chain in lymphocytes independent of the T cell receptor complex [18] may explain why changes in ζ precede those of the CD4- and CD8-associated protein-tyrosine kinase $p56^{lck}$ [24]. Therefore, it appears that the currently used combination immunotherapy is able to restore the expression of ζ chain in the majority of patients [i.e., 13 of 21 (62%) patients], but that re-expression of $p56^{lck}$ [observed in 6 of 13 (46%) patients] has additional requirements. These requirements could be a different timing of treatment because of the different kinetics of the expression of $p56^{lck}$ [18, 24], or the need for additional stimulatory signals that induce this particular tyrosine kinase. Alternatively, the combined signals of IL-2 and an antigen, such as those presented in cancer vaccines, might result in restoration of both ζ chain and $p56^{lck}$ levels. Both areas remain to be studied, but certainly could constitute an important area of research in the development of new cancer vaccines or immunotherapeutic approaches. In this context it is of interest that results of a recent melanoma vaccine trial suggested the increased therapeutic effect of the combination of antigen and IL-2 [23].

In summary, our results and those of others [13, 21] suggest that testing for signal-transduction alterations in cancer can be used as a means of monitoring the effect of immunotherapy in patients with cancer. Therefore, additional clinical trials that include the combined evaluation of signal-transduction molecules, such as ζ chain, $p56^{lck}$, ZAP-70 [21] and NFκB [13], will be needed to evaluate fully their use as a means of adequately monitoring changes in the immune response, leading to the development of a clinical response. Our preliminary data suggest that successful immunotherapy approaches should consider the inclusion of antigens, cytokines or even pharmaceuticals that induce the re-expression of signal-transduction molecules in lymphocytes.

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