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A Small GTP-binding Protein is Frequently Overexpressed in Peripheral Blood Mononuclear Cells From Patients With Solid Tumours

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***ras* oncoproteins and *ras*-related proteins constitute a large family of the small GTP-binding protein family. The *rab* branch of the *ras* superfamily is involved in the intracellular transport along the secretory and endocytic pathway in eukaryotic cells. We here demonstrate that a member of the *rab* branch, the *rab2* protein, is frequently overexpressed in peripheral blood mononuclear cells from patients with solid neoplasms. Moreover, this expression is shown to be greatly modified during the course of therapy. Our results provide strong evidence for the implication of a small GTP-binding protein in immunological events associated with neoplastic diseases. The precise cellular population involved as well as the potential prognostic value of this process remains to be determined.**

Key words: *rab* proteins, GTP-binding proteins, peripheral blood cells, solid tumours
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INTRODUCTION

RECENT DISCOVERIES indicate that the *ras* oncoproteins belong to a large family of low molecular weight (21–27 kD) monomeric proteins capable of binding and hydrolysing GTP [1, 2]. All members of the so-called *ras* superfamily contain highly conserved domains required for guanine nucleotide binding, GDP/GTP exchange and GTP hydrolysis [3]. These domains interact with regulatory proteins which stimulate guanine nucleotide dissociation, inhibit GDP exchange or promote GTP hydrolysis [4]. According to the sequence homologies of the proteins,

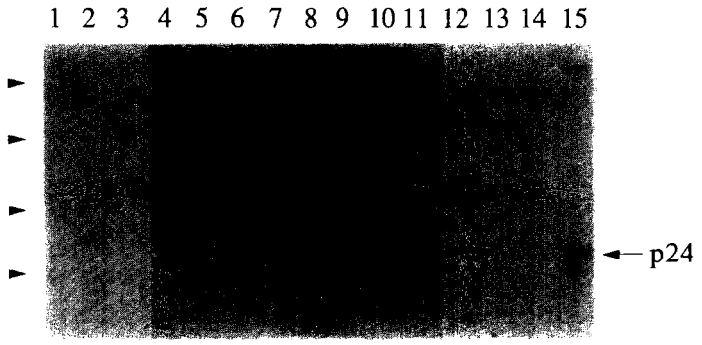

Figure 1. Expression of the *rab2* protein in PBM cells from 15 healthy donors. Each lane contains 30 μg of cytosolic proteins. Arrows on the left indicate molecular weight marker positions (respectively, 69 000, 46 000, 30 000 and 21 000). Non-specific high molecular weight signals are seen in the upper part of the gel.

three main branches are recognised which constitute the *ras* superfamily. The first one contains the three *ras* oncoproteins (H-*ras*, K-*ras* and N-*ras*) as well as the closely related R-*ras*, *ral* and *rap* proteins. *Rho* and *rac* proteins constitute the second arm, while at least 30 *rab* proteins are members of the third branch [5].

Members of the *rab* branch are associated with subcellular compartments in both the endocytic and exocytic pathways. These proteins are likely to be key regulatory components of protein complexes catalysing the fission and fusion of transport vesicles between distinct subcellular compartments [6]. We previously demonstrated the overexpression of the *rab2* protein in peripheral blood mononuclear (PBM) cells from 7 out of 12 patients exhibiting solid tumours [7]. In the present report, we assessed the incidence of this phenomenon in 104 patients, and prospectively studied the influence of therapy on the expression of the *rab2* protein levels in 24 patients who were treated with chemotherapy or immunotherapy.

MATERIALS AND METHODS

PBM cells

PBM cells were prepared by Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) density centrifugation of heparinised blood from healthy donors or patients with solid tumours, and stored in liquid nitrogen until analysis. Blood samples were harvested before each cycle of chemotherapy and at days 1, 8, 15, 29, 43, 50 and 64 during immunotherapy.

Protein extraction and analysis

Cells (10^7–10^8) were suspended in 50–500 μl ice-cold buffer [pH 7.5, 0.01 M Tris–HCl, 0.005 M $MgCl_2$, 0.001 M dithiothreitol (DTT), 0.001 M phenylmethysulfonyl fluoride (PMSF)] and homogenised using 10 strokes in a glass homogeniser as described previously [8]. The lysates were centrifuged at 600 g for 10 min to pellet nuclei. The supernatants were then centrifuged at 100 000 rpm for 12 min to generate cytosolic fractions and membrane crude extracts. The latter were washed, resuspended in 50–500 μl buffer containing 0.05 M Tris–HCl, 0.005 M $MgCl_2$, 0.05 M NaCl, 0.001 M DTT, 0.001 M PMSF, pH 7.5, treated with 1% Triton 100X on ice for 30 min, and centrifuged at 100 000 rpm for 12 min. The quantities of proteins were estimated with the Bio-Rad Protein Assay (München, Germany). The protein samples were resolved in 12.5% sodium dodecyl sulphate–polyacrylamide gel after boiling, electrophoretically transferred to nitrocellulose filters, and probed with an anti-*rab2* rabbit immune serum previously shown to be specific for the *rab2* protein [9] and revealed with [^{125}I]protein A.

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RESULTS

Frequency of the rab2 *protein overexpression among patients with solid tumours*

In PBM cells from healthy donors, the rabbit anti-*rab2* immune serum detected a specific signal with a 24 000 molecular weight in agreement with previously reported data [9–11]. The expression levels were weak in the 25 controls studied, as illustrated in Figure 1. After phytohemagglutinin stimulation of PBM cells, we observed no increase in the *rab2* expression levels (data not shown).

The expression of the *rab2* protein was studied in the PBM cells from 104 patients with solid tumours of various origins. Samples were harvested at different stages of the diseases, before or in the process of treatment with chemotherapy or immunotherapy. The main characteristics of the analysed patient population are listed in Table 1. An overexpression of the *rab2* protein was seen in 59 (57%) patients. Scanning of autoradiographs demonstrated a 2- to 5-fold overexpression in 30 (29%) patients, and a 6- to 12-fold overexpression in 29 (28%) patients. The *rab2*-specific signals, shown in Figure 2, were observed in the PBM cells from 24 consecutive patients. They illustrate the large variations in the *rab2* protein expression levels we saw in the studied population. Conversely, a uniform expression pattern was obtained in the same patients with a specific rabbit anti-*rab1* immune serum (Figure 3). The subcellular fractionation experiments evidenced a major cytosolic localisation of the *rab2* protein in the PBM cells from healthy donor as well as from patients with solid tumours. However, beyond a 5-fold overexpression, specific *rab2* signals were detected in the membrane fractions (data not shown). No correlation could be established between the *rab2* expression levels and the tumour origins, the histological subtypes or the status of the disease (Table 1). As a first approach to the characterisation of the peripheral blood cell population involved in the *rab2* overexpression, we carefully recorded the haemograms of the 104 patients. No obvious correlation was identified

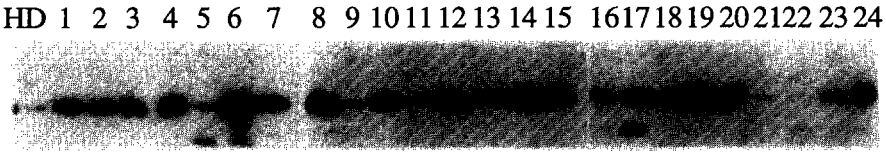

Figure 2. *Rab2* expression levels observed in PBM cells from a healthy donor (HD) and from 24 consecutive patients with various solid tumours. Each lane contains 30 μg of cytosolic proteins.

Table 1. Characteristics of the 104 solid tumour patients studied for rab2 expression

	Number of patients	Number of samples with *rab2* overexpression
Primary tumours		
Breast	23	14
Bladder	15	9
Ovary	14	9
Kidney	13	6
Testis	12	4
Lung	8	5
Colon-rectum	7	3
Other	12	9
Previous treatment		
None	16	11
Surgery	28	15
Chemotherapy	14	6
Surgery + chemotherapy	46	27
Status of the disease		
Local	11	9
Metastatic	87	46
Complete remission	6	4
Current treatment		
None	26	14
Chemotherapy	67	39
Immunotherapy	11	6

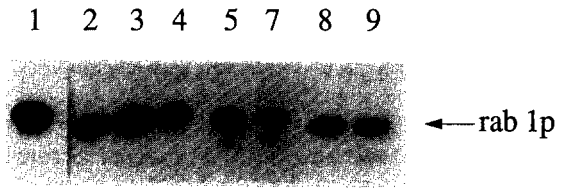

Figure 3. Expression of the *rab*1 protein in PBM cells from a healthy donor (lane 1) and from 7 patients with solid tumours (lanes 2 to 9). Each lane contains 30 μg of cytosolic proteins. Signals observed in the lower part of the gel are likely to represent degradation products.

between the *rab2* expression levels and the absolute monocyte or lymphocyte counts.

Influence of therapy on the rab2 *protein expression levels*

We prospectively analysed the expression of the *rab2* protein in the PBM cells from 24 patients with metastatic urological tumours before treatment and during the following courses of chemotherapy or immunotherapy they received. 14 patients were treated by four to six cycles of various chemotherapy regimens. Seven of them displayed a *rab2* protein overexpression before starting therapy. A large variability in the *rab2* expression levels was observed in the harvested successive samples, as illustrated in 4 patients (Figure 4a–d). In most of the patients

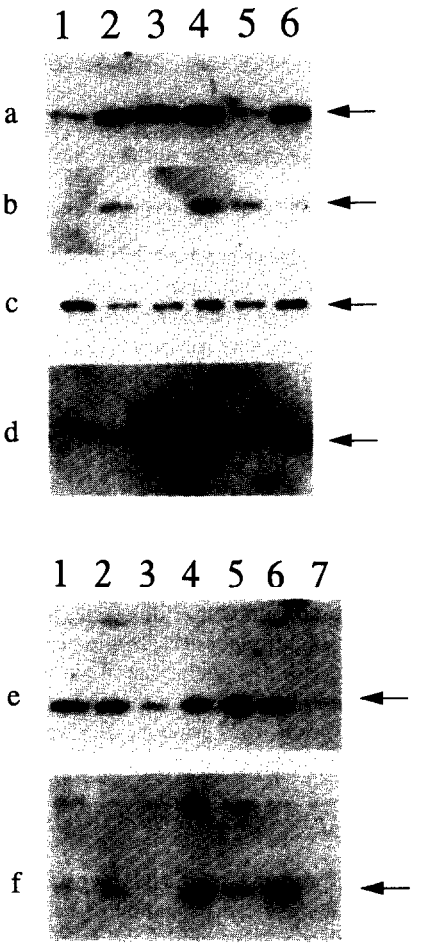

Figure 4. *rab2* expression levels observed in PBM cells during therapy in six urological cancer patients. Treatment applied was chemotherapy for patients a,b,c,d and interleukin-2 for patients e,f. Each lane contains 30μg of cytosolic proteins. Lanes 1–7, successive samples.

with initial high levels, low specific signals, similar to those obtained in healthy donors, were observed in at least one sample. Conversely, the majority of patients with initial low levels displayed a transient or sustained *rab2* overexpression. 10 patients with metastatic renal cancer were treated by immunotherapy and followed during treatment with interleukin 2 (6 patients) or interferon-α (4 patients). Similar results were obtained, with high variation rates in the *rab2* expression levels at the onset and during therapy, as shown in Figure 4 (patients e, f). Our observations are precisely reported in Table 2. Whatever the type of treatment, no obvious correlation could be established between the *rab2* protein expression levels and the absolute monocyte or lymphocyte counts. Likewise, the status of the disease at the end of therapy was not related to the expression levels before or after treatment.

Table 2. Clinical characteristics and rab2 *expression levels in PBM cells from patients with solid urological tumours during treatment with chemotherapy (C) or immunotherapy*

Patient number	Primary tumour	Treatment	Initial *tab2* expression level	Number of samples during therapy with: low expression levels	high expression levels	Final *rab2* expression level	Status at the end of therapy
1	Testis	C	++	1	3	+	CR
2	Testis	C	+	2	2	+	CR
3	Testis	C	+	3	1	+	PR
4	Testis	C	++	3	1	+-	CR
5	Testis	C	+	1	3	+-	PR
6	Bladder	C	++	2	2	+	PD
7	Bladder	C	+	4	0	+	PD
8	Bladder	C	++	3	2	++	PR
9	Bladder	C	++	1	4	+	PD
10	Bladder	C	+++	2	2	+	PR
11	Bladder	C	+	1	4	++	PR
12	Penis	C	+	2	3	+	PD
13	Penis	C	+	4	0	+	CR
14	Kidney	C	+	4	0	+	PR
15	Kidney	IL2	++	2	3	++	SD
16	Kidney	IL2	++	1	4	+	PR
17	Kidney	IL2	+	4	1	+	PD
18	Kidney	IL2	+	4	1	++	PD
19	Kidney	IL2	++	3	2	++	SD
20	Kidney	IL2	+	1	4	+	PD
21	Kidney	INF	+	2	3	+	PD
22	Kidney	INF	+	1	4	++	PD
23	Kidney	INF	++	4	1	++	PD
24	Kidney	INF	+	3	2	+	PD

IL2, interleukin 2; IFN, interferon-α; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease. + ≤2-fold overexpression as compared with healthy donor; ++, ≤5-fold overexpression; +++, >5-fold overexpression.

DISCUSSION

The present study clearly demonstrates that the overexpression of the *ras*-related *rab2* protein is frequently observed in the PBM cells from patients with solid tumours. This phenomenon was demonstrated in 59/104 (57%) patients at time of treatment onset or during chemotherapy or immunotherapy. These results led us to suggest that such an overexpression might be observed in each cancer patient at least once in the course of their disease. The knowledge of the subset of circulating cells involved in the *rab2* protein overexpression is currently not solved. We did not identify any obvious correlation between the *rab2* protein levels and the absolute counts of lymphocytes or monocytes. Previous studies in patients exhibiting Sézary syndromes suggested that the cell population involved in the *rab2* protein overexpression belongs to the lymphocyte lineage, expresses the CD2 differentiation antigen, and may not display the CD3–TCR complex at their surface [12]. These features are to be confirmed in patients with solid tumours.

High variation rates in the *rab2* expression levels were observed when blood samples were prospectively harvested while treatment was administrated. In chemotherapy-treated patients, these results could reflect the cytotoxic effect of the drugs on the cell population involved in the *rab2* overexpression. However, such an hypothesis cannot be applied to patients treated with immunotherapy. Interleukin 2 (IL2) has been shown to affect PBM cells in several ways, including decreased proliferative response to soluble antigens [13–15], increased density of IL2 receptor-positive lymphocytes and monocytes [16], and increased numbers of CD2 and CD56 cells [14, 17]. This latter property is noteworthy, regarding the presumed immunophenotype of the cells involved in the *rab2* overexpression. However, we did not observe any clear-cut relationship between treatment duration and overexpression rates, and no firm conclusion can be drawn. There was also no correlation between the *rab2* expression levels before or after treatment and the status of the disease at the end of therapy. Whether a prognostic value could be ascribed to the *rab2* expression remains to be determined.

Our results suggest evidence for the implication of a small GDP/GTP binding protein in immunological events associated with neoplastic diseases. Impaired immunity is known to be associated with the development of cancers. Lymphocytopenia and disturbances in the network of cells concerned with immunoregulation have been shown to contribute to the depressed immunity of patients with solid tumours. Studies to evaluate disturbances in the regulation network by quantifying circulating leucocyte subpopulations have demonstrated altered numbers in various cellular subsets. Such abnormalities are most readily detected in patients with advanced diseases, and mainly involved an increase in relative numbers of monocytes with a variable decrease in numbers of T lymphocytes [18]. Quantitative immunophenotyping of peripheral blood T cells from pati-

ents with tumours of various origins gave divergent results. Regarding urological tumours, Kaver and colleagues recently demonstrated that, in patients with infiltrating bladder carcinomas, the CD4 subset is often reduced with a concomitant inversion in the CD4/CD8 ratio and impairment in the T cell function, as determined by the ability to proliferate upon phytohaemagglutinin and concavalin stimulation. Conversely, patients with prostatic cancer have a higher mean CD4/CD8 ratio than the control group. Patients with superficial bladder tumours or renal cell carcinomas have an immune profile similar to that of the control group [19]. In breast cancer, peripheral blood natural killer activity with untreated stage I was shown to be significantly higher than in healthy controls. However, the possible intervention of therapy has been stressed in the sense that cytotoxic drugs selectively decline the cytolytic lymphocyte killer cell pool whereas endocrine therapy did not [20].

The reason why immunoregulatory disturbance in solid neoplasms could result in an overexpression of the *rab2* protein can only be speculated upon. A modulated expression of low molecular weight GDP/GTP binding proteins has already been described in immune reactive cells. The expression of the *rab* proteins is increased during phagocyte maturation [9] and the transcription levels of the *rac2* gene have been shown to be enhanced during T cell activation [21]. Cumulative evidence now supports the involvement of the *rab* gene products in the secretory pathway [6]. Specifically, the *rab2* protein was shown to be associated with a structure having the characteristics of an intermediate compartment between the endoplasmic reticulum and the Golgi apparatus [11]. Moreover, mutations in the guanine nucleotide-binding domains of the *rab2* proteins result in potent inhibition of vesicular transport from the endoplasmic reticulum to the Golgi complex [22]. Whether the overexpression of the *rab2* protein correlates with an activation state of a CD2+ lymphocytes subset requiring increased secretion of yet unknown molecules remains to be determined.

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