The interferon-inducible gene, Ifi204, acquires malignant transformation capability upon mutation at the Rb-binding sites

Marco De Andrea[a], Monica Ravotto[a], Emanuela Noris[a], Guo-Guang Ying[a], Daniela Gioia[b], Barbara Azzimonti[b], Marisa Gariglio[b], Santo Landolfo[a,c,*]

[a] *Department of Public Health and Microbiology, Medical School, Via Santena 9, 10126 Turin, Italy*
[b] *Department of Medical Sciences, Medical School, Via Solaroli 17, 28100 Novara, Italy*
[c] *Immunogenetics and Experimental Oncology Section, C.N.R., 10126 Turin, Italy*

Received 5 November 2001; revised 15 January 2002; accepted 5 February 2002

First published online 26 February 2002

Edited by Masayuki Miyasaka

Abstract p204 overexpression in retinoblastoma (Rb)−/− mouse embryo fibroblasts or transfection of p204 mutated at both Rb-binding sites confer growth advantages, resulting in a significantly higher number of foci in a cell focus assay. To investigate the possibility that mutated p204 acquires malignant transformation capability, NIH3T3 cells were stably transfected with the expression vector pRcRSV204 double-mutant (p204dm) harboring both the C-terminal deletion up to amino acid 568 and the point mutation from glutamic acid to lysine at position 427, and analyzed for markers typical of cell immortalization and transformation. We detected a greater abundance of cell colonies in soft agar with p204dm-expressing cells than vector control cells. The p204dm-transfected cells also displayed two other characteristics associated with malignant transformation, i.e. growth under low-serum conditions and formation of tumors in athymic nude mice. Moreover, their telomerase activity was significantly higher than in the vector control cells. It would thus seem that p204, devoid of functional Rb-binding motifs, can become oncogenic.

Key words: Interferon-inducible gene; Ifi204; Retinoblastoma; Oncogenic activity; p204 mutant

1. Introduction

To exert their antiproliferative activity, interferons (IFNs) activate a family of transcription factors designated signal transducers and activators of transcription and interferon regulatory factors, respectively, that translocate into the nucleus and stimulate the expression of the IFN-stimulatable genes [22,23]. One of the first downstream targets was identified as the c-myc gene since its mRNA expression in hematopoietic cells was suppressed at the transcriptional level by IFN-α [6]. Moreover, its disruption by ectopic expression of deregulated c-myc partially suppressed the ability of IFN-α to arrest cells specifically in the G0/G1 phase [14]. The pRb protein was then identified as another independent target of the IFN-α signaling capable to complement the partial cell cycle arrest mediated by c-myc inhibition through the suppression of its phosphorylation [20]. The cyclin D3 and cdc25A genes have since been shown to be primary targets of the IFN-α signaling cascade. Suppression of cdc25A phosphatase is indeed the main pathway for inhibition of cyclin E- and cyclin A-associated kinase activities by IFN-α [6,24].

Of the several genes induced by IFNs, the mouse Ifi200 genes [17,19] and their human homologues, myeloid nuclear differentiation antigen [2] and Ifi16 [12], are transcriptionally activated, both in vivo and in vitro. Ifi204, a member of the Ifi200 gene cluster, encodes a 72 kDa phosphoprotein (p204) that contains two partially conserved 200 amino acid segments, designated type a and type b, bearing an LXCXE motif, known to be a potential site for binding to the retinoblastoma (Rb)-family proteins [4]. Overexpression of p204 in C2C12 myoblasts induced their fusion to myotubes suggesting that it may be involved in muscle differentiation [5]. In vivo the Ifi204 is constitutively expressed in myeloid cells and selectively induced in the monocytes by IFNs, likely driving their differentiation to macrophages [7]. p204 overexpression in mouse embryo fibroblasts (MEF) retarded their proliferation, delayed G1 progression into S-phase and accumulated cells with a DNA content equivalent to cells arrested in late G1 [18]. These effects on cell cycle progression were strictly dependent on its association with the Rb protein [11]. Altogether these studies demonstrated that p204–pRb interaction is physiologically relevant to growth control and cell differentiation, and changes in this pathway confer growth advantages. Moreover, these findings suggested that p204 mutated at the Rb-binding sites can acquire cell transformation capability, as shown for the mutated form of the PKR gene, another IFN-inducible gene involved in growth control [16]. The aim of the present study, therefore, was to investigate the oncogenic activity of mutated p204 expression. For this purpose, NIH3T3 cells were transfected with the expression construct containing the mutated 204 cDNA and several aspects associated with cell immortalization or malignant transformation, namely growth under low-serum conditions, amplification of telomerase activity (TERT), anchorage-independent growth and the ability to form tumors in nude mice were evaluated.

2. Materials and methods

2.1. Cell cultures and transfection

NIH3T3 cells were grown as monolayers in Dulbecco's modified

*Corresponding author. Fax: (39)-11-6966977.
E-mail address: santo.landolfo@unito.it (S. Landolfo).

Abbreviations: IFN, interferon; Rb, retinoblastoma; MEF, mouse embryo fibroblast; TERT, telomerase activity; DHFR, dihydrofolate reductase; PCNA, proliferating cell nuclear antigen

PII: S0014-5793(02)02431-6

Eagle's medium (DMEM; Gibco) supplemented with 10% donor calf serum (DCS). Transfections were performed using Lipofectamine Plus (Gibco) according to the manufacturer's protocol. 2 μg of expression construct DNA pRcRSV204dm (for brevity p204dm), containing the Ifi204 cDNA mutated at both Rb-binding sites with an in-frame fusion at the C-terminus with the hemagglutinin (HA) epitope as described by Hertel et al. [10], were used for approximately 3×10^5 cells. Stable transfectants were selected in the presence of 800 μg/ml G418 in the culture medium. Pools of at least 100 colonies were harvested and maintained in G418 selection. Expression of the exogenous protein was evaluated by Western blotting using the anti-HA-probe monoclonal antibody (Boehringer). The morphology of the cells was examined using an Olympus microscope.

2.2. Telomerase assay

Cells were washed in PBS, lysed, and TERT activity in the indicated amount of total cell extracts was measured with the TeloTAGGG Telomerase PCR ELISA^{PLUS} kit (Roche Molecular Biochemicals) according to the manufacturer's protocol [3]. The telomerase PCR ELISA is an extension of the original described TRAP protocol [13] and allows for the highly specific amplification of telomerase-mediated elongation products, combined with non-radioactive detection following an ELISA protocol. Each assay was performed with an extract-free sample that contained only the reaction mixture to detect PCR amplification of primer dimers, and one in which extracts were treated with 1 U of RNase A as a control for telomerase specificity. Only assays in which all control lanes showed the expected results were included in this study.

2.3. Cell lysis and Western blotting

Cells were lysed in 3% SDS lysis buffer as previously described [11]. Insoluble material was removed by centrifugation. Protein concentration was determined by the Bio-Rad Dc Protein Assay (Bio-Rad Laboratories). Total cell extracts were separated on SDS–8.5% polyacrylamide gel and transferred onto PVDF membrane (Amersham). Membranes were blocked in blocking solution (10 mM Tris–HCl, pH 7.5, 0.1 M NaCl, 0.1% Tween 20, 5% [w/v] non-fat dry milk) and incubated in the same buffer with primary antibodies for 3 h at room temperature, followed by a 1 h incubation with the appropriate horseradish-peroxidase-conjugate secondary antibody and then detected by enhanced chemoluminescence (Amersham). pRb was detected using the Rb control antibody from Santa Cruz Biotechnology (C-15/sc-50) at a dilution of 1:4000. The phosphorylation status of pRb at Ser-780 was detected with the phospho-Rb (Ser-780) antibody from Cell Signaling Technology at a dilution of 1:4000. Monoclonal anti-actin antibodies (Boehringer) were used as internal control. Densitometry was performed by scanning the radiographs and then analyzing the bands with the software Quantity One (Bio-Rad).

2.4. Soft agar and in vivo tumorigenicity assay

After transfection, cells were grown in the presence of G418 for 2.5 weeks. Pools of stable transfectants were harvested and aliquots were added to 60-mm dishes in duplo containing 0.5% (w/v) SeaPlaque agarose (FMC BioProducts) in complete culture medium on top of a layer of 1% (w/v) SeaPlaque agarose. As a positive control, transfectants with a construct containing an activated ras allele (Kirstein) were included. Cells transfected with the empty vector pRcRSV were included as negative controls. Aliquots of 1000, 10 000 and 100 000 cells were plated in duplo. After 16 days, 25 squares of 1 cm^2 were counted. Transfected cells were subcutaneously inoculated into the right flank of 6–8-week-old female athymic nude mice (Charles River Laboratories) at the indicated concentrations. Each group consisted of six animals. After inoculation, the incidence and growth of tumors were evaluated at least twice a week. Neoplastic masses were measured with calipers in the two perpendicular diameters for at least 40 days. The mice were sacrificed when tumor growth started to interfere with the health of the animals.

2.5. Expression analysis

Total cellular RNA was isolated by using the total RNA extraction solution EUROzol (EUROclone). Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) was carried out using 1 μg of total RNA as previously described [21]. Briefly, total RNA was reversed-transcribed into mRNA-dependent cDNA by using Moloney murine leukemia virus RT (Ambion Inc.). 1-μg aliquot for each sample served as non-RT control to control for genomic contamination in subsequent PCR reactions. Equivalent amounts of cDNA were then subjected to PCR analysis performed in a Hybaid PCR Express Thermal cycler. The Ifi204 primers (sense [5′-AAAGAGACAACCAAGAGCAATACACC-3′] and antisense [5′-GAATGTTAGATGAAGCCGAAGATGAG-3′]) amplified a 567 bp fragment. The β-actin primers (sense [5′-TGGAATCCTGTGGCATCCATGAAA-3′] and antisense [5′-TAAAACGCAGCTCAGTAACAGTCCG-3′]) amplified a 348 bp fragment. For amplification of the DNA fragments, a cycle number which was in the middle of the linear amplification range (with a minimum of 25 and a maximum of 35 cycles) was used. The reaction mixtures for each sample were added to a tube containing 50 pmol each of the oligonucleotide primers for amplification of both Ifi204 and β-actin. PCR was carried out as follows: 5-min denaturation step at 95°C, followed by 30 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 30 s, followed by 72°C for 5 min to allow for complete extension. Under these conditions, amplification of Ifi204 and β-actin product is in the exponential phase and the assay is linear with respect to the amount of input RNA. Water controls in which all components of the RT-PCR reaction were present, except RNA, were also included. PCR products were electrophoresed on 1.5% agarose and stained with ethidium bromide for visualization.

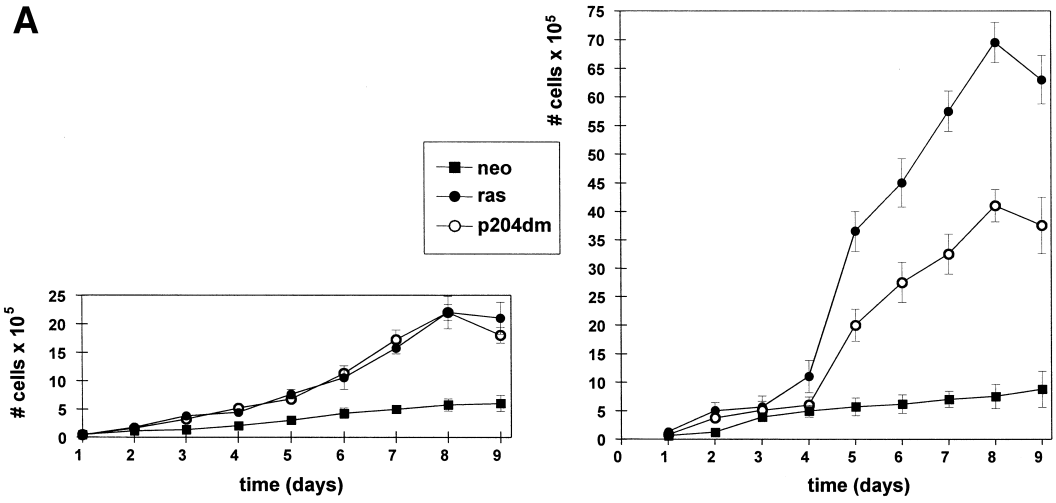
3. Results and discussion

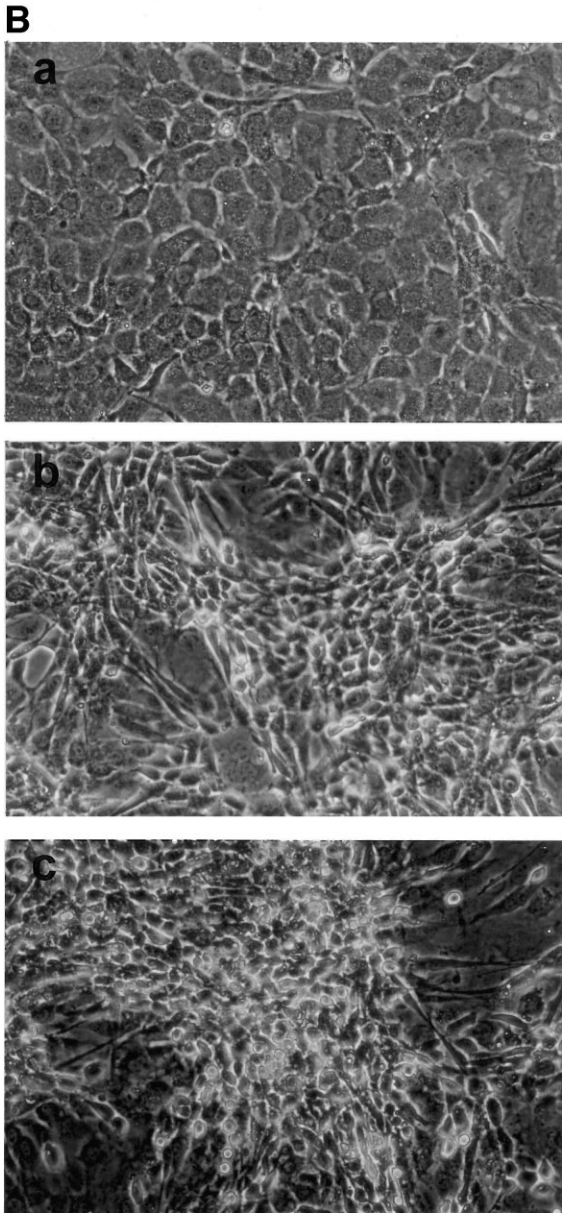
As a first attempt to verify whether the p204 mutant displays any oncogenic activity, NIH3T3 cells were first transfected with the expression vector pRcRSV204 double mutant (for brevity p204dm) harboring both the C-terminal deletion up to amino acid 568 and the point mutation from glutamic acid to lysine at position 427, which means that the protein is devoid of any functional Rb-binding domain. Cells transfected with an activated ras allele (226 cells) or the empty vector (neo) were included as positive and negative controls. We chose NIH3T3 cells because this cell line has been used successfully to demonstrate the phenotypic effects of a number of proto-oncogenes [25]. After G418 selection for 2.5 weeks, pooled populations of neomycin-resistant clones were harvested and used for further analysis. Expression of the transfected mutant form was assessed by Western blotting by using anti-HA monoclonal antibodies as previously described (data not shown) [10].

Since growth in the absence of exogenous growth factors is a characteristic of immortalized, transformed cells, the transfected cells were tested for their ability to grow under low-serum conditions (1 and 5% DCS). As can be seen in Fig. 1 (panel A), p204dm transfectants grew faster and to higher densities compared to neo cells, which invariably displayed lower growth rates and density. A similar growth pattern was observed with 226 cells transformed by a c-Ki-ras gene. Differences in the numbers of cells when reaching confluence between the p204dm transfectants on one hand and the neo cells on the other hand was mainly due to their ability to grow to higher densities. Moreover, the morphologies of NIH3T3

→

Fig. 1. A: Growth curves of NIH3T3-transfected cells under low-serum conditions. Counts of the cells cultured in medium supplemented with 1% DCS (left panel), or 5% DCS (right panel) are shown. Each point represents the mean ± standard deviation of data obtained in three independent experiments. B: Morphological changes in NIH3T3 expressing the empty vector (a), p204dm (b), and oncogenic ras (c). Cells were cultured in 10% DCS–DMEM, allowed to grow until confluence, and photographed with an inverted microscope.


A
neo
ras
p204dm
cells x 10^5
time (days)
cells x 10^5
time (days)


B
a
b
c

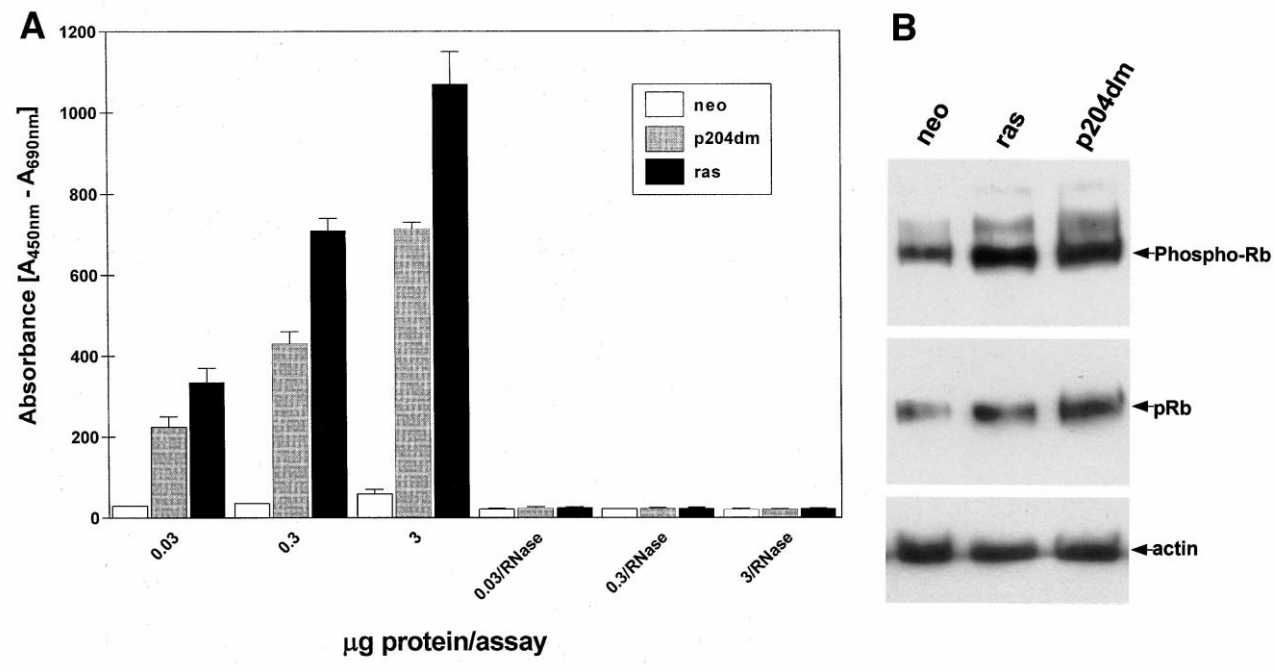


Fig. 2. A: Effect of p204dm overexpression on TERT detected by ELISA. Different amounts of total protein extracts from neo, p204dm and ras cells pretreated or not with RNase were assayed for TERT using telomerase PCR ELISA assay. Absorbance values are reported as the $A_{450\,nm}$ reading against blank control (reference wavelength $A_{690\,nm}$). Each point represents the mean ± standard deviation of data obtained in three independent experiments. B: Effect of p204dm overexpression on pRb phosphorylation. Total cell extracts (50 μg) were size-fractionated by 8.5% SDS–PAGE, transferred to PVDF membrane, and subjected to Western blotting for detection of pRb expression (pRb) and phosphorylation (phospho-Rb). Actin immunodetection was performed as internal control.

cells transfected with p204dm or oncogenic ras were clearly different from those of the neo cells. After approximately 8–10 days, both p204dm and ras-transfected cells were more refractile, elongated, and piled up when they reached confluence, each other, whereas neo cells showed epithelial-like morphology and formed elongated, rounded and neatly aligned structures (Fig. 1, panel B).

TERT has been demonstrated in a high percentage of extracts from most tumor types [13]. Cell lines, immortalized either spontaneously or after transformation by oncogenic viruses, such as simian virus 40 and human papillomavirus types 16 or 18, are usually telomerase-positive [15]. Moreover, TERT should be regarded as a biomarker of cell proliferation rather than malignant transformation [1]. Since the above experiments demonstrated that introduction of p204dm confers growth characteristics typical of immortalized cells, neo, p204dm and ras cells were compared for TERT activity. As shown in Fig. 2, panel A, TERT was barely detectable in neo cells, but significantly increased (10-fold) in p204dm cells. As expected, ras cells displayed very high levels of TERT activity, possibly associated with the growth properties of the Ki-ras oncogene.

Altogether these results demonstrate that mutation at both Rb-binding motifs wipes out the antiproliferative activity of the Ifi204 gene and endows it with growth-promoting activity.

We previously reported that as a consequence of p204 overexpression and growth arrest, the Rb protein is almost completely converted to the rapidly migrating hypophosphorylated status [18]. To investigate the effect of p204dm overexpression on the Rb phosphorylation status, total cell extracts were obtained from exponentially growing neo and p204dm transfectants. As control, extracts from ras-transformed cells were included. As shown in Fig. 2, panel B, a significant increase of pRb phosphorylation (2.5-fold) was detected in p204dm extracts, by using a phosphospecific Rb antibody, in comparison to the neo cells. As expected, ras-transformed cells showed high levels of phosphorylated pRb (four-fold induction). As a consequence of pRb phosphorylation, the E2F transcription factors are released and activate the expression of the E2F-dependent genes required for

Table 1
Microscopic counts and plating efficiencies in soft agar assay

Construct	1×10 cells		1×10^4 cells		1×10^3 cells	
	Colonies/cm^2	Plating eff. (%)[a]	Colonies/cm^2	Plating eff. (%)	Colonies/cm^2	Plating eff. (%)
Neo	5 (4–6)[b]	0.005	2 (2–2)	0.02	–	–
p204dm	58 (52–64)	0.05	32 (31–33)	0.3	12 (10–14)	1.2
ras	76 (72–80)	0.05	49 (46–52)	0.4	21 (20–22)	2.1

[a]Plating efficiency in the number of colonies ×100 divided by the number of cells plated.
[b]The values for the duplos are given separately between brackets.

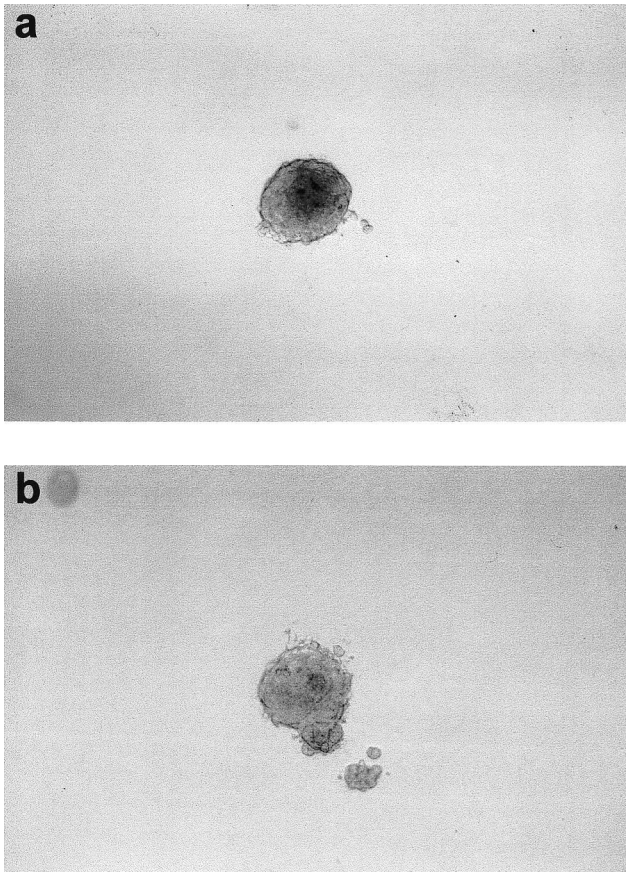


Fig. 3. Morphology of soft agar colonies formed by p204dm (a) or oncogenic ras (b) transfected NIH3T3 cells. The colony size and morphological changes were detected by microscope observation. Representative photomicrographs are shown. Magnification: 10×.

S-phase entry. Consistent with this hypothesis, we have previously shown that overexpression of wild type p204 but not p204dm leads to a significant decrease of both proliferating cell nuclear antigen (PCNA) and dihydrofolate reductase (DHFR) protein levels, known to be regulated in a E2F dependent manner [11].

Since anchorage-independent growth is one of the characteristics of malignantly transformed cells, 1000, 10 000 and 100 000 transfected cells were plated in duplo onto soft agar dishes. After 16 days, macroscopic colonies were present in all dishes, including those of the negative controls and the ras-transformed cells. Typical colony images are shown in Fig. 3. The numbers of colonies and plating efficiencies are displayed in Table 1. When compared to neo cells, p204dm transfectants yielded a number of colonies and a plating efficiency significantly higher. As expected, the positive control (ras) clearly demonstrated a better colony-forming efficiency than any other transfectants. Microscopic examination also revealed that the morphology of the p204dm colonies resembled that of ras colonies (Fig. 3). These results indicated that overexpression of p204dm in NIH3T3 cells enhanced the cellular proliferation rate and conferred anchorage-independent characteristics.

To establish whether p204dm transfectants form tumors in vivo, cells were inoculated at the indicated concentrations into the right flank of nude mice. Tumor size was examined twice a week and palpable tumors were measured on two perpendicular axes. Tumor volume was calculated by assuming spherical growth, using the standard formula. All mice injected with p204dm-transfected cells developed tumors in an inverse dose–time-dependent manner within 10–25 days (Fig. 4, upper panel). The mean tumor size in p204dm-transplanted nude mice was approximately 4 cm^3 at day 20 after transplantation (with 10^6 cells/mouse); whereas, the mean tumor volume in nude mice injected with neo cells (10^6 cells/mouse) only reached 1 cm^3 at day 35 after transplantation (Fig. 4, lower panel). As expected, all the ras-inoculated mice bore large tumors (2 cm^3) as early as 2 weeks after inoculation. These results indicate that p204dm not only promoted tumor growth, but also enhanced the tumor incidence rate in nude mice. After dissection, the tumors were analyzed by RT-PCR for expression of the Ifi204dm and the β-actin gene as control. The results, presented in Fig. 5, showed comparable expression levels of the mutated 204 cDNA in the tumor extracts derived from the p204dm group inoculated with the highest cell concentration. By contrast, tumor extracts derived from mice inoculated with 1×10^6 neo-cells were negative.

The results of this study demonstrate that expression of the p204dm inactivated by mutation at both LXCXE motifs increases cell proliferation along with TERT activity and anchorage independence. In addition, when p204dm-transfected NIH3T3 were transplanted into nude mice, they also enhanced tumor size and incidence. It has been demonstrated that Rb and the related p107 and p130 primarily inhibit growth by targeting the E2F family transcription factors [9]. We previously observed that the Rb protein is an essential mediator that links the IFN-inducible 204 gene to cell-cycle regulation [8,11]. FACS analysis together with BrdU incorporation and expression of DHFR and PCNA proteins in Rb+/+ MEF transfected with wild type p204 demonstrated indeed that interaction of p204 with Rb represses E2F transcriptional activity. Although the effect of p204 binding on pRb function remains to be explored, we can conclude that the transdominant negative p204dm increases pRb phosphorylation and relieves E2F to activate transcription of target genes, such as DHFR, involved in cell growth stimulation.

Acknowledgements: We thank Mirella Giovarelli, Turin, for helpful discussion and statistical analysis of in vivo data. This work was supported by grants from Associazione Italiana per la Ricerca sul Cancro (AIRC), and MURST-CNR ('Biotechnology Program L95/95' and 'Program 40%').

References

[1] Belair, C.D., Yeager, T.R., Lopez, P.M. and Reznikoff, C.A. (1997) Proc. Natl. Acad. Sci. USA 94, 13677–13682.
[2] Briggs, R.C., Kao, W.Y., Dworkin, L.L., Dessypris, E.N. and Clark, J. (1994) J. Cell. Biochem. 56, 559–567.
[3] Cherry, A.M., Bangs, C.D., Oei, Y., Bodnar, A., Bronstein, A., Chiu, C.-P. and Scott, H.G. (1999) J. Biol. Chem. 274, 26141–26148.
[4] Choubey, D. and Lengyel, P. (1992) J. Cell. Biol. 116, 1333–1341.
[5] Cj, L., Wang, H., Zhao, Z., Yu, S., Lu, Y.B., Chatterjee, G., Deschamps, S., Roe, B.A. and Lengyel, P. (2000) Mol. Cell. Biol. 20, 7024–7036.
[6] Einat, M., Resnitzky, D. and Kimchi, A. (1985) Nature (London) 313, 597–600.
[7] Gariglio, M., De Andrea, M., Lembo, M., Ravotto, M., Zappador, C., Valente, G. and Landolfo, S. (1998) J. Leuk. Biol. 64, 608–614.

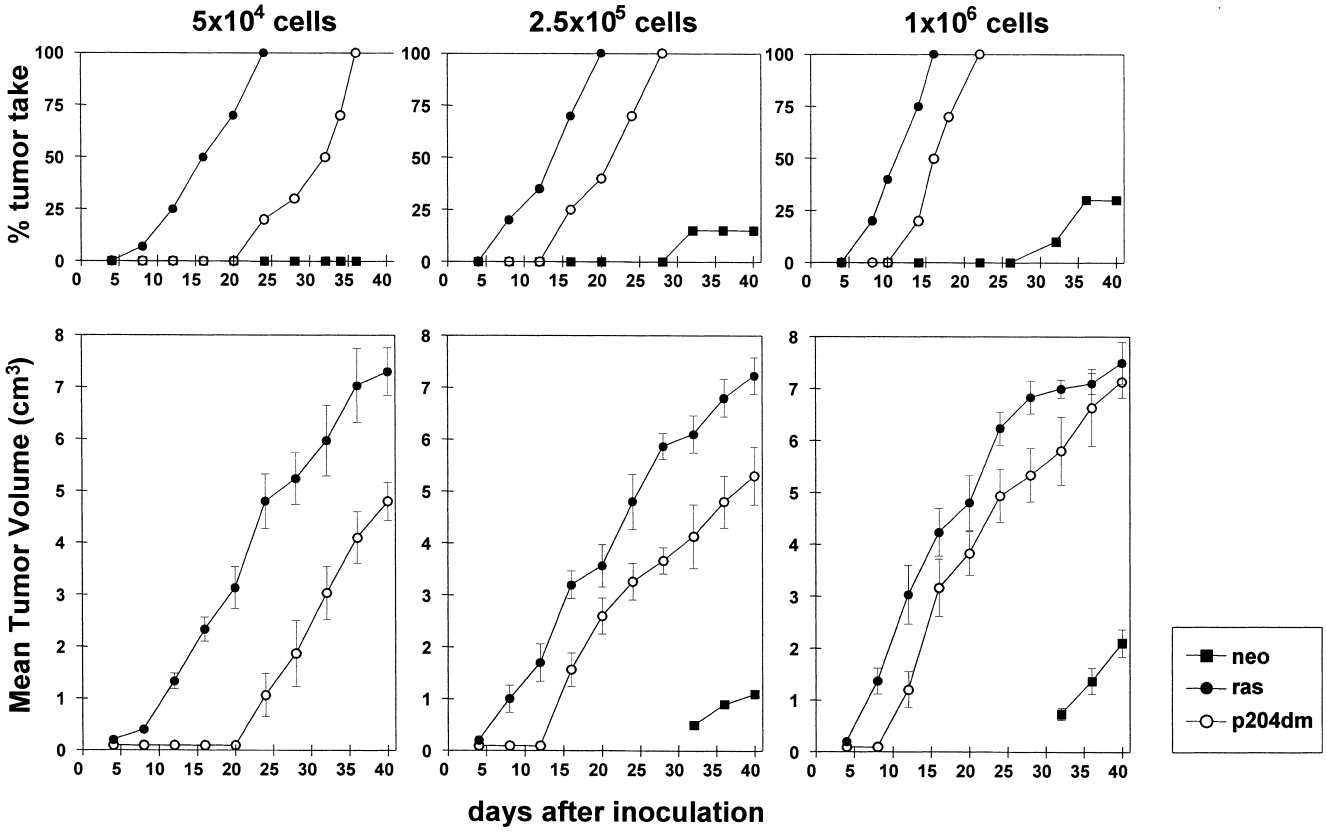


Fig. 4. Effect of p204dm overexpression on tumor growth in vivo. Percentage of tumor take (upper panel) and mean tumor volume (lower panel) of palpable tumors arising in nude mice (six mice/group) after inoculation of 5×10^4, 2.5×10^5, 1×10^6 NIH3T3 cells expressing the empty vector, p204dm and oncogenic ras.

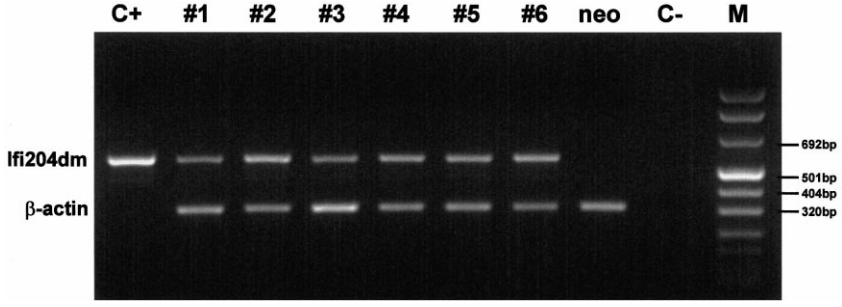


Fig. 5. Expression levels of Ifi204dm and control β-actin in tumors from nude mice injected with 1×10^6 cells as measured by semiquantitative RT-PCR. The marker (M) shown is the 100 bp ladder VIII (Roche Biochemicals). Amplification was performed by Ifi204 sense and antisense primers originating a 567 bp fragment. The β-actin (348 bp fragment) was coamplified as an internal control. C− contains a water control for the RT-PCR. Neo corresponds to RT-PCR amplification of total RNA obtained from tumor developed after neo cell injection. C+ corresponds to RT-PCR amplification of total RNA obtained from the pooled population of p204dm-transfected cells used to inject mice.

[8] Gribaudo, G., Riera, L., De Andrea, M. and Landolfo, S. (1999) FEBS 456, 31–36.
[9] Harbour, J.W. and Dean, D.C. (2000) Genes Dev. 14, 2393–2409.
[10] Hertel, L., De Andrea, M., Azzimonti, B., Rolle, S., Gariglio, M. and Landolfo, S. (1999) Virology 262, 1–8.
[11] Hertel, L., Rolle, S., De Andrea, M., Azzimonti, B., Osello, R., Gribaudo, G. and Gariglio, M. (2000) Oncogene 19, 3598–3608.
[12] Johnstone, R.W. and Trapani, J.A. (1999) Mol. Cell. Biol. 19, 5833–5838.
[13] Kim, N.W., Piatyszek, M.A., Prowse, K.R., Harley, C.B., West, M.D., Ho, P.L.C., Coviello, G.M., Wright, W.E. and Weinrich, S.L. (1994) Science 266, 2011–2015.
[14] Kimchi, A., Resnitzky, D., Ber, R. and Gat, G. (1988) Mol. Cell. Biol. 8, 2828–2836.
[15] Klingelhutz, A.J., Barber, S.A., Smith, P.P., Dyer, K. and McDougall, J.K. (1994) Mol. Cell. Biol. 14, 961–969.
[16] Koromilas, A.E., Roy, S., Barber, G.N., Katze, M.G. and Sonenberg, N. (1992) Science 257, 1685–1689.
[17] Landolfo, S., Gariglio, M., Gribaudo, G. and Lembo, D. (1998) Biochimie 80, 721–728.
[18] Lembo, M., Sacchi, C., Zappador, C., Bellomo, G., Gaboli, M., Pandolfi, P.P., Gariglio, M. and Landolfo, S. (1998) Oncogene 16, 1543–1551.
[19] Lengyel, P., Choubey, D., Li, S.-J. and Datta, B. (1995) Sem. Virol. 6, 203–213.

[20] Resnitzky, D., Tiefenbrun, N., Berissi, H. and Kimchi, A. (1992) Proc. Natl. Acad. Sci. USA 89, 402–406.
[21] Rolle, S., De Andrea, M., Gioia, D., Lembo, D., Hertel, L., Landolfo, S. and Gariglio, M. (2001) Virology 286, 249–255.
[22] Stark, G.R., Kerr, I.M., Williams, B.R.G., Silverman, R.H. and Schreiber, R.D. (1998) Annu. Rev. Biochem. 67, 227–264.
[23] Taniguchi, T., Ogasawara, K., Takaoka, A. and Tanaka, N. (2001) Annu. Rev. Immunol. 19, 623–655.
[24] Tiefenbrun, N., Melamed, D., Levy, N., Resnitzky, D., Hoffmann, I., Reed, S.I. and Kimchi, A. (1996) Mol. Cell. Biol. 16, 3934–3944.
[25] Weinberg, R.A. (1989) Cancer Res. 49, 3713–3721.