

Correlated Flow Cytometric Analysis of H-ras p21 and Nuclear DNA in Multiple Myeloma

By Hirouki Tsuchiya, Joshua Epstein, Peter Selvanayagam, John R. Dedman, Gary Gallick, Raymond Alexanian, and Bart Barlogie

Correlated analysis of the H-ras oncogene product (p21) and of nuclear DNA content was performed by flow cytometry (FCM) in patients with DNA-aneuploid multiple myeloma (MM). Bone marrow cells from normal donors and MM patients in remission served as controls. Seventy-four percent of 23 patients with active MM had higher p21 fluorescence in aneuploid tumor cells than were observed in normal donor or myeloma remission bone marrows; 39% of the 23 patients also showed high H-ras p21 expression

in diploid cells. There was an inverse relationship between p21 levels and the presence of trisomy 11; especially high p21 levels were noted in patients without trisomy 11. The frequent elevation of p21 protein in aneuploid plasma cells suggests the involvement of the H-ras oncogene in the pathophysiology of MM, which is further supported by a shorter survival among patients with high p21 levels.
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RECENT ADVANCES in molecular biology have revealed the involvement of cellular oncogenes in neoplasia.¹⁻³ In hematologic tumors they are frequently activated by chromosomal translocations with breakpoints at or near their sites.¹ In contrast to extensive cytogenetic and molecular studies in lymphomas and leukemias, such investigations are limited in multiple myeloma (MM). This slowly proliferating B-cell tumor poses major difficulties to obtaining metaphase spreads for chromosomal analysis. In the absence, so far, of MM-specific cytogenetic anomalies,^{4,7} molecular studies could not be guided by morphological aberrations. Review of the available cytogenetic literature and the authors' own studies suggested a frequent involvement of chromosomes 1 and 11, which are the sites of the N-ras and H-ras 1 oncogenes, respectively. This observation and the role of the ras family oncogenes in inducing the malignant phenotype in DNA transfection experiments¹⁻³ led to the current systematic evaluation of ras oncogene expression in MM cells. The commonest mechanism of ras-gene activation in human neoplasia is gene mutation, although DNA amplification has also been reported.⁸ The ras-gene product is a 21-Kd protein (p21) that is located at the inner plasma membrane.⁹ Its role in normal growth and differentiation is still being investigated.

Given the heterogeneity of human neoplasia, much effort has been expanded to in situ examination of oncogene expression, using either autoradiographic or more recently flow cytometry (FCM) studies.^{10,11} Thus Andreeff et al¹⁰ have recently demonstrated the feasibility of quantitating by FCM ras-protein content in single cells. Since MM exhibits an abnormal nuclear DNA content in at least 80% of cases,¹² the authors elected to examine ras p21 expression in relationship to DNA aneuploidy using FCM of doubly stained bone marrow cells from 23 patients with MM. While preliminary studies with a pan-ras antibody (Y13-259) had revealed high levels of ras p21 in MM cells, recent studies in malignant lymphoma showing high messenger RNA (mRNA) levels of H-ras prompted the authors to focus on H-ras p21 in MM as well.¹³ Compared to normal bone marrow cells, high levels of H-ras p21 were present in aneuploid plasma cells and seemed to confer a poor prognosis.

MATERIALS AND METHODS

Preparation of anti-H-ras p21 antibody. The anti-H-ras p21 antibody was prepared using synthetic peptide corresponding to a unique sequence of H-ras protein (amino acid no. 160-179),¹⁴ which was purchased from Peninsula Laboratories (San Carlos, CA). The method followed the previous report,¹⁵ except for using a sheep rather than a rabbit as the recipient animal. Briefly, the synthetic peptide was coupled to keyhole limpet hemocyanin and injected subcutaneously with Freund's adjuvant into a sheep. The selected amino acid sequence is common to both c- and v-H-ras p21.¹⁴ The reactivity of the generated antiserum with the synthetic peptide was determined by enzyme-linked immunosorbent assay (ELISA), with a titer of greater than 1:10,000. Antiserum was purified by CN-Br-activated Sepharose column (Pharmacia, Uppsala, Sweden) and was termed HAS2. Using Kirsten or Harvey murine sarcoma virus-transformed normal rat kidney cell lines (Ki- or Ha-NRK), the specificity of HAS2 was confirmed by immunoblotting with the biotin-avidin rabbit antishrimp peroxidase system (Vector Laboratories, Burlingame, CA) and DNA/p21 FCM described below. The reactivity and localization of HAS 2 to H-ras p21 was also determined by fluorescence microscopy using NRK, Ki-NRK, and Ha-NRK cell lines as well as a human myeloma cell line (ARH-77¹⁶). Cells were stained according to a three-step indirect immunofluorescence assay described below.

The antibody HAS2 is especially suitable for DNA-correlated FCM, since ethanol can be used as a fixative that provides higher resolution of DNA measurements than methanol. In contrast, methanol fixation is required when using the Y13-259 monoclonal antibody (MoAb), which reacts with all p21 species (ie, N-, H-, and K-ras p21).

From the University of Texas System Cancer Center, M.D. Anderson Hospital and Tumor Institute, Departments of Hematology (Hirouki Tsuchiya, Joshua Epstein, Peter Selvanayagam, Raymond Alexanian, Bart Barlogie) and Tumor Biology (Gary Gallick), and The University of Texas Health Science Center, Department of Physiology and Cell Biology (John R. Dedman), Houston.

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Address reprint requests to Bart Barlogie, MD, M.D. Anderson Hospital and Tumor Institute, 1515 Holcombe Blvd, Box 30, Houston, TX 77030.

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Study samples. Following informed consent, heparinized bone marrow cells were obtained from 23 patients with DNA-aneuploid MM (untreated, seven; primary resistant, five; relapse, 11; Table 1) as well as from eight MM patients in remission and eight healthy donors. Aneuploid MM samples were selected on the basis of abnormalities of DNA/RNA (acridine orange) or DNA-clg patterns (propidium iodide/fluorescein-isothiocyanate).^{17,18} In questionable cases normal blood lymphocytes were directly mixed with the patient sample. Remission marrow was defined by the presence of less than 0.5% DNA-aneuploid cells in patients who had achieved more than 75% cytorreduction.¹⁹ Donors for allogeneic bone marrow transplantation (BMT) were used as normal controls. The human T-cell-acute lymphocytic leukemia cell line (Molt-4) expresses high H-ras p21 protein level and was used as a positive control in each experiment.

DNA/p21 flow cytometry. Marrow aspirates were subjected to Ficoll-Hypaque (FH) gradient separation (SG 1.077), and interphase cells were collected and washed twice in phosphate-buffered saline (PBS). A small amount of cells was used for confirmation of DNA aneuploidy. The remaining sample was fixed in 70% ethanol at 4°C for at least 24 hours. Cells were then processed for 2-parameter FCM analysis by a three-step indirect immunofluorescence method with subsequent propidium iodide reaction for DNA counterstaining. Fifty microliters of HAS2 antibody at a concentration of 500 µg/mL were added to 10⁶ cells in 50 µL of incubation buffer; incubation buffer consisted of 4% inactivated normal human serum with 0.5% Tween 20 (Sigma, St. Louis) in PBS. As nonspecific control, 50 µL of purified normal sheep IgG at a concentration of 500 µg/mL was used (Organon Teknika-Cappel, Malvern, PA). In several experiments HAS2 was preincubated with the synthetic peptide coupled to sepharose and the supernatant used as an additional control (blocked HAS2). Cells were then incubated successively with 100 µL of biotinylated rabbit anti-sheep IgG (Vector Laboratories) at a dilution of 1:50 and with 100 µL of fluorescein isothiocyanate (FITC)-labeled streptavidin (BRL, Gaithersburg, MD) at a dilution of 1:100. Each incubation step was carried out at 4°C for 30 minutes. Following each step, cells were washed twice with 0.5% Tween 20 in PBS. Finally, cells were incubated with 70 µg of ribonuclease A (Cooper-biomedical, Malvern, PA) in 1 mL of PBS at 37°C for ten minutes and DNA

counterstained with 40 µL of propidium iodide (Sigma, 500 µg/mL in PBS) for five minutes at room temperature. Five thousand cells were analyzed with a FACS II flow cytometer (Becton Dickinson, Mountain View, CA). Data were further analyzed with a Terak 8600 computer (Terak Co, Scottsdale, AZ) using the GRAF3D and CUBE3D programs (Coulter Electronics, Hialeah, FL).

Fluorescence intensities were analyzed independently for aneuploid and diploid G₀/G₁ cell compartments and were expressed as mean channel numbers. The mean channel number for H-ras p21 was obtained from gated G_{1/0} areas shown in Figs 1 A through C, thus eliminating any influence of cycle stage-related differences in ras expression. The H-ras p21-specific fluorescence was derived by subtracting nonspecific fluorescence (obtained from normal sheep IgG staining) from total fluorescence (obtained from HAS2 staining). To avoid interexperimental variation, H-ras p21-specific fluorescence was always standardized using the fluorescence intensity of Molt-4 cells.

Statistical analysis. The relationship between H-ras p21 fluorescence intensity and other markers was examined by Student's *t* and chi-square tests, and Kaplan-Meier plots were used for survival analysis.

RESULTS

Specificity of HAS2 antibody. Immunoblotting experiments with HAS2 at a concentration of 1:100 revealed strong reactivity in Ha-NRK cells. In contrast, Ki-NRK cells, which showed high levels of p21 when developed with the pan-ras MoAb Y13-253 (results not shown) did not react with HAS-2. Ha-NRK revealed two discrete bands corresponding to H-ras p21 and its phosphorylated form with a

Table 1. Characteristics of Patients With DNA Aneuploid Myeloma High v Low H-ras p21 Levels

H-ras p21*	Disease Status†			Ig Type‡					Serum B2M§		Chromosome 11 Trisomy	
	µ	rel	res	K	L	G	A	D	High	Low	Yes	No
≥9	3	5	4	8	4	7	3	1	5	5	1	5
<9	4	6	1	8	3	9	1	1	1	10	5	0

Abbreviations: µ, untreated; rel, relapsed; res, primary resistance; K, kappa; L, lambda.

*H-ras p21 fluorescence was calculated by the methods described in "Materials and Methods." Value 9 corresponds to the median H-ras p21 level in aneuploid cells from 23 patients with aneuploid myeloma (Fig 4).

†Disease status at time of study.

‡Immunoglobulin typing by immunoelectrophoresis and cytoplasmic Ig analysis using FCM. One patient did not produce the heavy chain.

§Serum beta-2-microglobulin levels were measured by radioimmunoassay.²⁶ Data were available for 21 patients. High denotes ≥6mg/L and low <6mg/L. *P* < .1.

||Patients with chromosome 11 trisomy. Data were available for 11 patients.

¶One patient with 11 tetrasomy and another patient with chromosome 11 trisomy plus del (11) (q22) were included.

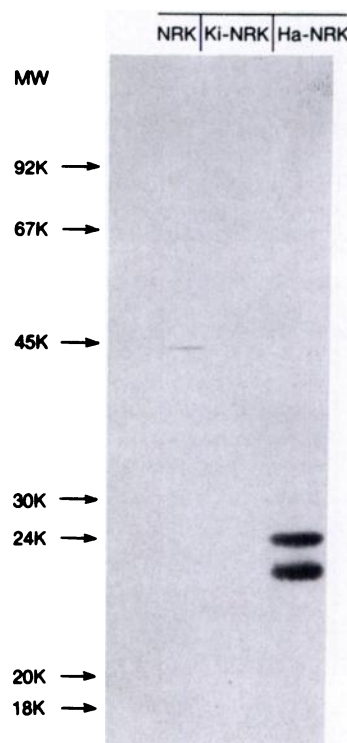


Fig 1. Specificity of anti-H-ras p21 antibody HAS2 was confirmed by immunoblotting using NRK, Ki-NRK, and Ha-NRK cells. Immunoblotting was performed with the biotin-avidin rabbit anti-sheep peroxidase system.

slower migration, as described previously²⁰ (Fig 1). Specificity of HAS2 was also confirmed by DNA/p21 FCM; the H-ras p21 specific fluorescence level of Ha-NRK was 2.7 times as high as that of NRK. Microscopic examination showed strong cytoplasmic staining of Ha-NRK cells (originally infected with v-H-ras) and strong membrane staining of ARH-77 myeloma cells, but weak reactivity with Ki-NRK and NRK cells (data not shown; Fig 2). In additional control

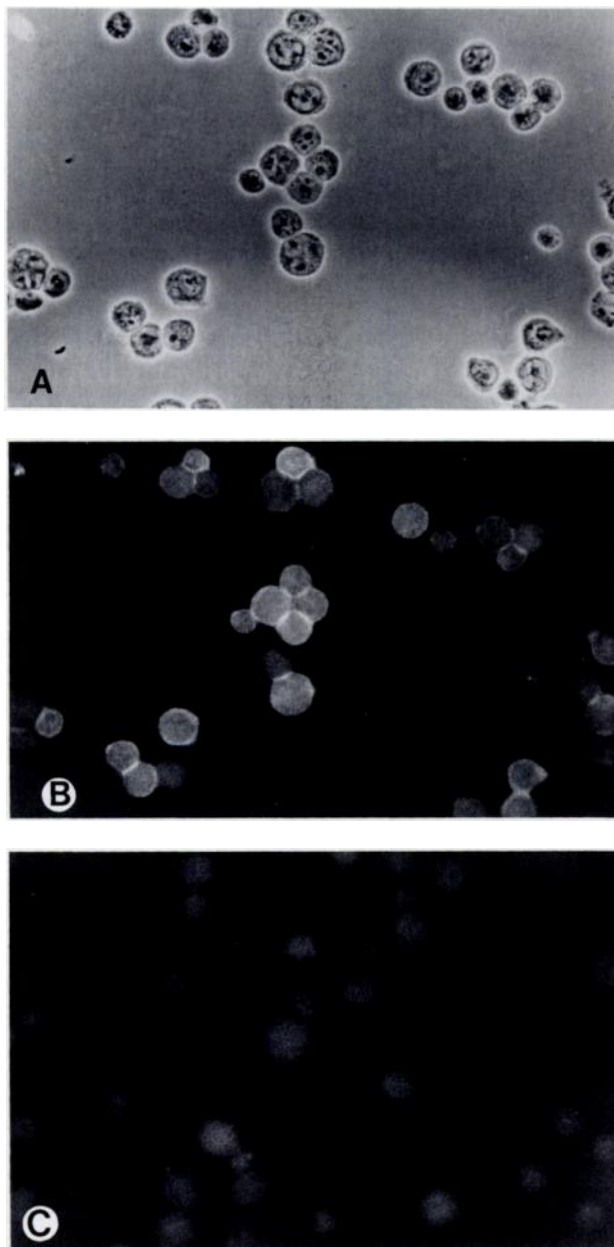


Fig 2. Microphotographs of ARH-77 human myeloma cells. Phase contrast (A) and immunofluorescence (B) of the same area stained for H-ras p21 with HAS2 antibody and background control stained with normal sheep IgG (C). Cells were stained with HAS2 or normal sheep IgG as a primary antibody, then incubated with biotinylated rabbit anti-sheep IgG and fluorescein isothiocyanate-labeled streptavidin.

experiments, the fluorescence level of cells reacted with blocked HAS-2 was indistinguishable from background fluorescence.

DNA/p21 FCM analysis. Representative examples of DNA/p21 FCM distributions for an aneuploid MM and morphologically normal bone marrow specimens from a healthy donor and a MM patient in remission are depicted in Fig 3. Markedly higher H-ras p21 fluorescence is observed in hyperdiploid plasma cells of the myeloma patient compared to seemingly normal diploid hematopoietic cells (Fig 3 A) and by comparison with morphologically uninvolved bone marrow (Figs 3 B and C). Among the 23 patients with aneuploid MM, high H-ras p21 fluorescence levels greater than 5 (upper limit of H-ras p21 level in normal bone marrow) were present in 74% of aneuploid and in 39% of diploid subpopulations (Fig 4). With one exception, the latter had lower p21 levels than the corresponding aneuploid tumor cells. Lowest p21 levels were noted in the eight patients with MM in remission (all ≤ 2), whereas four of the eight normal donors had values between 3 and 5.

Clinical relevance of p21 expression. No relationship was noted between H-ras p21 levels and immunoglobulin isotypes (Table 1) or plasma cell phenotype analyzed by FCM (surface beta 2 microglobulin, B2, J5, R1-3 and B4).²¹ Serum beta-2-microglobulin levels weakly correlated with H-ras p21 levels (Table 1; $P < .1$). Of 11 patients with available cytogenetic data, six had trisomy 11 with or without additional numeric or structural aberrations (Table 1). Such patients had significantly lower p21 levels than the remaining five without chromosome 11 involvement ($12.1 \text{ v } 5.5$; $P < .01$).

Although serial studies are lacking to determine whether p21 expression changes during the disease course, the clinical relevance of high p21 expression at any time was examined. Patients were divided into two groups according to the level of p21 expression; patients with lower than the median level of p21 ($n = 11$) and those with p21 levels $\geq$ median (median = 9, Fig 4). There was no difference in the median times from diagnosis to the date of p21 analysis between these two groups. None of the 11 patients with low p21 expression has died compared to only six survivors among the 12 patients with high levels, with a projected median survival of 46 months (Fig 5; $P = .03$).

DISCUSSION

FCM has already emerged as a valuable tool in studying the cellular heterogeneity in MM. Thus drug resistance was closely associated with the presence of DNA hypodiploidy and low plasma cell RNA content.²² A novel common acute lymphoblastic leukemia antigen (CALLA)⁺/cIg⁺ phenotype has been defined in aneuploid myeloma, generally conferring a favorable prognosis.²¹ Similarly, specific therapy-related measurements of anthracycline drug uptake²³ and glucocorticoid receptor expression have been conducted.²⁴ The authors have now demonstrated the feasibility of using FCM for quantitation of H-ras p21 expression using the HAS2 antibody. Counterstaining with propidium iodide for nuclear DNA content permitted correlated analysis sepa-

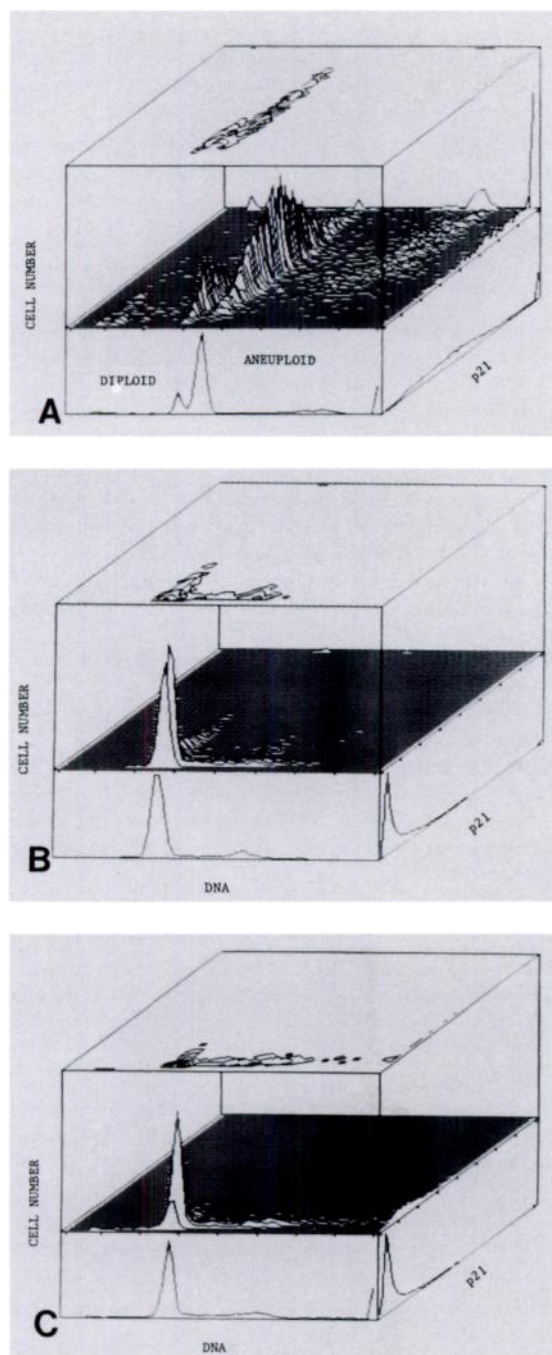


Fig 3. Correlated DNA/H-ras p21 FCM analysis of cells from aneuploid myeloma (A), normal bone marrow (B), and myeloma in remission (C). Cells were processed for 2-parameter FCM analysis by a three-step indirect immunofluorescence and propidium iodide counterstaining method. Five thousand cells were analyzed for each sample and displayed in CUBE3D mode. The hyperdiploid nature of MM cells (A) was ascertained from DNA-clg FCM revealing monoclonal clg confined to hyperdiploid cells (not shown).^{17,18} In each experiment MOLT-4 cells served as a positive standard.

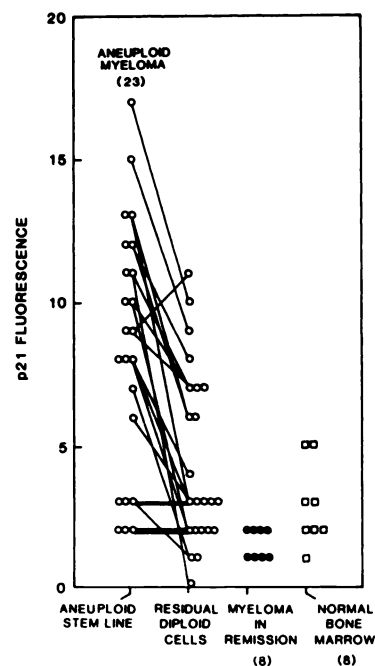


Fig 4. H-ras p21 expression in aneuploid and diploid cells of patients with active myeloma, myeloma in remission, and normal bone marrow. Solid lines connect data derived from the same patients. Numbers in parentheses are the numbers of subjects studied. H-ras p21 fluorescence was calculated as described in "Materials and Methods."

rately for aneuploid tumor and diploid cells. Since the amino acids involved most commonly in ras mutations (nos. 12, 13, or 61) and those recognized by the HAS2 antibody (nos. 160-179) do not coincide, the mechanism underlying high H-ras p21 expression in MM remains yet to be elucidated. High p21 expression by aneuploid tumor cells in almost 75% of MM patients suggests an involvement of H-ras in MM. Elevated p21 levels in presumably normal DNA-diploid cells

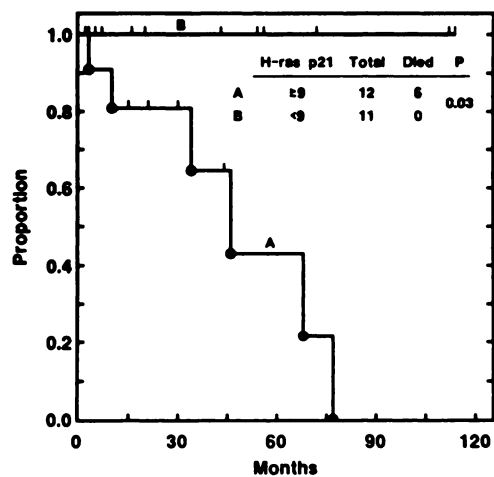


Fig 5. Kaplan-Meier survival analysis in myeloma according to high (≥ 9) or low (< 9) H-ras p21 expression in aneuploid tumor cells. Tick marks indicate living patients; closed circles deceased patients.

in nine patients raises the question of disease involvement of these cells, analogous to previous observations with CALLA and cIg.²⁰ The more favorable clinical course of patients with low p21 expression is presently unexplained but is reminiscent of observations in gastric cancer.²⁵ Although weak, the correlation between cellular p21 expression and serum beta-2-microglobulin might suggest that high p21 expression is associated with a more aggressive clinical course with higher tumor stage.²⁶ Of the six patients with trisomy 11, one had high p21 expression, while five had low levels of H-ras p21. Molecular studies are underway to define the mechanism of H-ras expression in MM and its possible relationship to the number of chromosomes 11. High ras expression in MM has not been reported previously. Shen et al²⁷ reported high p21 levels in 75% of patients with acute leukemias. Point mutations,²⁷ rare allelic restriction fragments,²⁸ and elevated

mRNA levels^{13,29} of H-ras gene have been demonstrated in human lymphocytic leukemia and lymphoma.

Studies in cell lines and clinical samples from MM patients failed to reveal a relationship between p21 expression and cellular proliferation as studied by bromodeoxyuridine incorporation and Ki-67 MoAb reactivity. Multiparameter FCM analysis is ideally suited to examine the phenotypic and cytokinetic characteristics of tumor cells with high oncogene expression, thus opening entirely new avenues of cell biological investigation of human neoplasia.

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