Nerve growth factor induces the re-expression of functional androgen receptors and p75\textsuperscript{NGFR} in the androgen-insensitive prostate cancer cell line DU145

Sandra Sigala, Nadia Tognazzi, Maria Cristina Rizzetti, Isabella Faraoni\textsuperscript{1}, Cristina Missale, Enzo Bonmassar\textsuperscript{1} and PierFranco Spano

Section of Pharmacology, Department of Biomedical Sciences and Biotechnologies, Brescia University Medical School, Via Valsabbina 19, 25123 Brescia, Italy and \textsuperscript{1}Department of Neurosciences, University of Rome ’Tor Vergata’. Via di Tor Vergata 135, 00133 Roma, Italy

(Correspondence should be addressed to Sandra Sigala; Email: sigala@med.unibs.it)

Abstract

Background: One of the paracrine/autocrine factors regulating prostate growth and differentiation is nerve growth factor (NGF). The role of NGF and its receptors in the prostate, however, remains controversial. We have shown that NGF treatment of human prostate cancer cell lines reduced their tumorigenicity, both in vitro and in vivo.

Objective: To investigate the involvement of NGF as a differentiation factor in prostate cancer cells.

Design: We exposed the androgen-independent/androgen receptor (AR)-negative prostate cancer cell line DU145 to NGF to study whether this neurotrophin could revert DU145 cells to a less malignant phenotype.

Methods: DU145 cells were treated with NGF, then ARs and NGF receptor p75\textsuperscript{NGFR} expression and telomerase activity were studied. Finally, we investigated whether re-expression of ARs could restore the androgen sensitivity in this cell line.

Results and Conclusions: NGF treatment induced a reversion of DU145 cells to a less malignant phenotype, characterized by the re-expression of ARs and p75\textsuperscript{NGFR} NGF receptors. Re-expression of ARs restored the androgen sensitivity, as suggested by the fact that exposure to dihydrotestosterone stimulated the growth of NGF-treated DU145 cells. This effect was blocked by androgen antagonist drugs, such as hydroxyflutamide and cyproterone acetate, which also induced apoptotic death of NGF-treated cells. The hypothesis that a differentiation pathway is activated by exogenous NGF in DU145 cells is also supported by findings indicating that NGF-treated DU145 cells expressed a low telomerase activity, as a result of a decrease in human telomerase reverse transcriptase transcription.

Introduction

In the prostate, cellular growth and differentiation are finely regulated by a complex interaction between stromal and epithelial cells under the control of both autocrine and paracrine regulatory factors (1). It is well known that a pivotal role is played by androgens, which are required for the physiological development of the prostate and for the maintenance of prostatic size and function during adulthood (1). The effect of androgen in the target cells is mediated by androgen receptors (ARs) which, after binding of androgens, are transformed into their transactivating form and interact with hormone-responsive elements located on the regulatory sequence of the androgen-dependent genes (2). In prostate cancer cells, molecular changes occur that induce a progression from an androgen-dependent to an androgen-independent state; however, the mechanisms responsible for the androgen-independence remain unclear (3).

Beside androgens, peptide growth factors that are involved as autocrine and paracrine prostate regulators have been identified (4): one particular paracrine/autocrine factor that regulates prostate growth and differentiation is nerve growth factor (NGF). NGF and its receptors, the trkA proto-oncogene and the 75kDa glycoprotein p75\textsuperscript{NGFR} (5), are present in the prostate of several species, localized both in epithelial and in stromal cells (6–12). In neuronal cells, trkA and p75\textsuperscript{NGFR} interact in a competitive manner in modulating cell growth or apoptosis; hence, the choice between survival and death among NGF-responsive cells seems to be determined by the balance of different signaling pathways.
activated by trkA and p75NGFR (13, 14). In the prostate, p75NGFR is progressively lost during tumorigenesis (15), and it has been proposed that disappearance of this NGF receptor could represent a marker of malignancy of prostate cancer (16). An imbalance between trkA- and p75NGFR-mediated signals could, thus, be involved or cooperate in the progression of prostate cancer cells. In this context, we have shown that human prostate cancer cell lines are responsive to NGF, and that exposure to NGF markedly inhibits telomerase activity and strongly induces the re-expression of the KAI1 metastasis suppressor gene and p75NGFR mRNAs, with no modification of trkA mRNA levels (12). These molecular effects are accompanied by a reduction in the cell proliferation rate both in vitro and in vivo, and by abrogation of the invasive potential in vitro (12).

Human prostate adenocarcinomas are telomerase-positive tumors (17–19). Studies of the structure and function of telomerase have led to the molecular cloning of several components of the holoenzyme (for a review see (20)). The human telomerase catalytic subunit is an RNA-dependent DNA polymerase (hTERT) that uses part of its RNA moiety (hTR) as a template for the synthesis of telomeric DNA sequences that are usually lost at each cell division, thus contributing to the maintenance of telomeres. Subunits hTR and hTERT seem to form the essential complex needed for telomerase activity, as suggested by results indicating that in vitro expression of these components leads to a detectable telomerase activity (21).

Data presented here suggest that NGF treatment induces a reversion of the androgen-independent/AR-negative prostate cancer cell line DU145 (22, 23) to a less malignant, more differentiated phenotype with low telomerase activity, characterized by the re-expression of ARs and p75NGFR. It should be emphasized that ARs restore the androgen sensitivity, as suggested by the fact that exposure of NGF-treated DU145 cells to the androgen hormone dihydrotestosterone (DHT) stimulated cell growth, and that this effect was blocked by the anti-androgen drugs, hydroxyxylutamide (HF) and cyproterone acetate (CPA).

Materials and methods

Cell culture and treatment

DU145 and LNCaP cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cell lines were grown according to the ATCC instructions. PC12 cells were kindly provided by Professor Pietro Calissano (Neurobiology Institute, CNR, Rome, Italy) and grown in RPMI 1640 medium supplemented with 10% horse serum, 5% fetal calf serum, 4 mmol/l glutamine and 100 U/ml penicillin/streptomycin. Media and all supplements for cell culture were purchased from Biochrom, Berlin, Germany. DU145 cells were treated with 50 ng/ml 2.5S NGF (Boehringer Mannheim, Italie, Milan, Italy) for 8 days, as previously described (12). At the end of treatment, in most experiments, as specifically indicated, untreated and NGF-treated DU145 cells were switched to a serum-free medium and divided into groups for analysis of the specific effect of the AR agonist DHT (Sigma, Italie, Milan, Italy) and the AR antagonists HF and CPA (Sigma).

Cell proliferation studies

Untreated and NGF-treated DU145 cells were plated at the concentration of 50 × 10³ cells/well in 24-well plates and treated with 10 nmol/l DHT or 10 nM DHT + 10 μmol/l HF or CPA for 3 days. Cellular growth was then assessed by total cell count and by [³H]thymidine incorporation. For cell counting, cells were detached and counted using a hemocytometer. [³H]Thymidine incorporation was determined as described by Sigala et al. (12).

Measurement of telomerase activity

Telomerase activity of untreated and NGF-treated DU145 cells was evaluated using the Telomeric Repeat Amplification Protocol (TRAP) assay (24), as described by Sigala et al. (12). PCR products were electrophoresed on a 10% acrylamide gel; after fixation, the gel was immediately exposed to Kodak-X-Omat film.

RNA extraction and hybridization conditions

Total RNA was extracted using the guanidinium thiocyanate method (25) from untreated and NGF-treated cells (10⁶ cells for each sample). Twenty micrograms of total RNA were resolved on 1% agarose–formaldehyde gel and blotted onto nylon membranes (Schleicher and Schuell, Berlin, Germany). Blots were then hybridized with the full-length hTERT cDNA (kindly provided by Dr R Weinberg, Whitehead Institute for Biomedical Research, MIT, Cambridge, MA, USA), with the hTR probe, obtained from Geron Corp (Menlo Park, CA, USA), and with a GAPDH probe (a generous gift from Dr R Dalla Favera, Department of Pathology, Columbia University, New York, NY, USA) labeled with phosphorus-32 using a multiprime labeling system (Amersham, Milan, Italy). Hybridization was performed in 50% formamide, 5 × SSC, 10% dextran-sulfate at 42°C overnight and filters were washed twice for 15 min to a stringency of 0.1 × SSC/0.1% SDS at 55°C. Blots were then exposed to Kodak-X-Omat film.

PCR and hybridization conditions

Two micrograms total RNA from untreated and NGF-treated DU145 cells was transcribed in cDNA using
murine leukemia virus reverse transcriptase (Promega, Italie, Milan, Italy) and oligo-dT 15–18 as a primer. PCR amplification and hybridization of trkA and p75NGFR were conducted as described by Sigala et al. (12). Blots were exposed to Kodak-X-Omat films and quantified using the Analytic Imaging Station software (Imaging Research Inc., St Catherines, Ontario, Canada).

**Immunocytochemistry**

DU145 cells were plated (10⁶ cells/well) in tissue-culture Petri dishes (3.5 mm diameter) containing sterile poly-l-lysine-treated coverslips, and treated with 50 ng/ml NGF for 8 days. Coverslips were then fixed for 5 min at −20ºC in cold methanol and washed in phosphate buffer solution (PBS). Endogenous peroxidases were inactivated for 30 min with 1% hydrogen peroxide solution. Cells were permeabilized and incubated with a 1:100 dilution of p75NGFR antibody (NeoMarkers, Fremont, CA, USA) or 1:100 dilution of AR antibody (Santa Cruz Biotechnology, Heidelberg, Germany). After an extensive wash, biotinylated secondary antibodies (Dako SpA, Milan, Italy) were applied and cells were treated according to the supplier’s instructions for the ABC kit (Dako SpA).

**Western blot**

Untreated and NGF-treated DU145 cells (5 × 10⁶ cells) were detached from plates and homogenized in 500 μl ice-cold lysis buffer (pH 7.4) containing 20 mmol/l Tris-HCl, 5 mmol/l EDTA, 2 mmol/l EGTA, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40 and protease inhibitors (0.5 mmol/l phenylmethylsulfonyl fluoride, 10 μmol/l pepstatin, 4 μmol/l aprotinin, 80 μmol/l leupeptin and 5 mmol/l benzamidine). Homogenates were centrifuged for 20 min at 4 ºC at 10 000 g and supernatant collected. Equal amounts of protein estimated by the BioRad Protein Assay were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% SDS-PAGE and electroblotted to a nitrocellulose membrane. The membrane was washed and incubated with the same antibodies raised against AR and p75NGFR that were described for the immunocytochemical experiment presented in Fig. 1 (top row). TrkA receptors were present in untreated DU145 cells and NGF exposure did not modify their expression. DU145 cells do not express p75NGFR (15) and our results were in agreement with this (Fig. 1e). Exposure of DU145 cells to NGF resulted in the re-expression of the NGF receptor p75NGFR, as suggested by the induction of a measurable relative density, with no changes in trkA mRNA levels.

**Statistical analysis**

One-way analysis of variance followed by Student’s t-test was used for statistical evaluation of differences.

**Results**

**Expression of NGF receptors in DU145 cell line**

Table 1 shows the densitometric analysis of hybridization bands obtained by RT-PCR followed by Southern blot of NGF receptors. DU145 cells expressed mRNA encoding for the NGF receptor trkA, but virtually lacked p75NGFR mRNA. Exposure of DU145 cells to NGF resulted in the re-expression of the NGF receptor p75NGFR, as suggested by the induction of a measurable relative density, with no changes in trkA mRNA levels.

**NGF receptor mRNAs are translated into their proteins**

The presence of trkA protein is shown in the immunocytochemical experiment presented in Fig. 1 (top row). TrkA receptors were present in untreated DU145 cells and NGF exposure did not modify their expression. DU145 cells do not express p75NGFR (15) and our results were in agreement with this (Fig. 1e). Exposure of DU145 cells to NGF induced the re-expression of this receptor, as shown in Fig. 1 (bottom row): a positive signal could be detected in NGF-treated DU145 cells.

**Measurement of apoptosis**

Untreated and NGF-treated DU145 cells (10⁶ cells/well) were plated in 3.5 mm tissue-culture Petri dishes containing sterile poly-l-lysine-treated coverslips, maintained in serum-free medium and treated with 10 nmol/l DHT or 10 nmol/l DHT + 10 μmol/l HF or CPA for 3 days. Coverslips were washed in PBS and fixed in 4% w/v paraformaldehyde for 30 min at room temperature. For the detection of apoptotic nuclei, coverslips were placed in Hoechst 3342 dye (2 μg/ml) for 2 min, washed in PBS and mounted under glass coverslips. All reagents were purchased from Sigma. For the detection of DNA fragmentation using the TUNEL assay, coverslips were incubated for 90 min at 37 ºC with 50 μl of a terminal transferase (TdT) reaction mixture containing 4 units TdT (Roche Diagnostics, Milan, Italy) and 0.25 nmol/l 12 dUTP-labeled fluorescein isothiocyanate (Roche Diagnostics). Coverslips were then washed and mounted.

**Table 1** Densitometric analysis of hybridization signals obtained by semiquantitative RT-PCR of p75NGFR and trkA in the DU145 cell line.

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<th>Untreated DU145 cells</th>
<th>NGF-treated DU145 cells</th>
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<tbody>
<tr>
<td>TrkA</td>
<td>0.41 ± 0.03</td>
<td>0.49 ± 0.05</td>
</tr>
<tr>
<td>P75NGFR</td>
<td>0.75 ± 0.07</td>
<td>nd</td>
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Data are the means ± S.E. of three different blots. Blots were exposed to Kodak-X-Omat films and autoradiograms were scanned using Analytic Imaging Station software as described in Materials and methods. Relative density was expressed as arbitrary units. nd, not detectable.
(Fig. 1f). No signal was found in immunocytochemistry experiments when the primary antibodies for trkA and p75NGFR were omitted (Fig. 1a, d). The NGF-mediated p75NGFR re-expression was then studied in time-course experiments. Western blot analysis (Fig. 2A) showed that antibody against p75 NGFR did not recognize any band in untreated DU145 cells, whereas a band of 75 kDa was clearly revealed in NGF-treated cells. In particular, the band was already detectable after 1 day of exposure to NGF, and its intensity increased according to the duration of NGF treatment. Cell extracts obtained from PC12 cells were used as internal positive control. These observations were further supported by results obtained with the immunocytochemical experiments presented in Fig. 2B.

Telomerase activity of the DU145 cell line

The untreated DU145 cell line had high telomerase activity, as demonstrated by the signal obtained with the TRAP assay, shown in Fig. 3A. DU145 cells exposed to 50 ng/ml NGF for 8 days exhibited a remarkable reduction in telomerase activity (Fig. 3A), which correlated well with the strong reduction in hTERT mRNA levels (Fig. 3B). Expression of the hTR was not modified by NGF treatment (Fig. 3B), as shown by the superimposable intensity of the hybridization bands in both cell samples. GADPH mRNA levels, measured as internal control, revealed that there were no significant differences between samples (not shown).

NGF treatment induces the AR re-expression and restores androgen dependency in DU145 cells

Western blot analysis of cell extracts (50 µg/lane) obtained from untreated and NGF-treated cells was performed and the results indicated that the expression of ARs was nearly undetectable in untreated DU145 cells, whereas exposure to NGF remarkably enhanced the AR protein concentration (Fig. 4A). LNCaP cell extracts (50 µg/lane) were used as an internal positive control (23). The immunocytochemistry experiment performed in DU145 cells, presented in Fig. 4B, indicates that, in NGF-treated DU145 cells, ARs are mostly localized in the nuclei, suggesting that, in standard cell culture conditions, androgens in the medium bound their receptors and were translocated.
from the cytoplasm to the nucleus. The NGF-mediated re-expression of ARs restored the androgen-dependency of DU145 cells. To assay the effect of DHT selectively, we switched NGF-treated cells to a serum-free medium and 10 nmol/l DHT was added daily. The results of the immunocytochemistry experiment shown in Fig. 4C suggest that the binding of DHT to ARs induced their activation and their translocation from the cytoplasm to the nucleus (Fig. 4Ca). Interestingly, when NGF-treated AR-positive DU145 cells were cultured in androgen-free medium, in the majority of cells the AR-positive signal was localized mainly in the cytoplasm, in the perinuclear area (Fig. 4Cb). The DHT-induced translocation of ARs was accompanied by a significant time-dependent increase in cell proliferation rate, measured both by direct cell counting (Fig. 4D) and by [3H]thymidine incorporation (not shown): up to 70% after 3 days of DHT treatment. No effects were seen when untreated DU145 cells were cultured in serum-free medium and then exposed to 10 nmol/l DHT (not shown). Stimulation of the rate of proliferation of NGF-treated DU145 cells induced by 10 nmol/l DHT was partially prevented by the addition of 10 μmol/l of the anti-androgen HF to the culture...
medium (Fig. 4D). NGF-treated, AR-positive DU145 cells underwent apoptosis after the pharmacological ablation of androgen by the exposure of the cells to 10 μmol/l HF in the presence of 10 nmol/l DHT in serum-free medium, evaluated both by Hoechst 3342 dye and by the TUNEL assay. As shown in Fig. 5, no apoptotic cells were present in NGF-treated cells (A, D) or in NGF-treated cells exposed to 10 nmol/l DHT for 3 days (B, E). When the effect of DHT was antagonized with 10 μmol/l HF, the presence of a significant number of apoptotic cells was revealed both by Hoechst 3342 dye (42 ± 4% compared with NGF-treated cells; Fig. 5C) and by the appearance of fluorescent TUNEL-positive cells (Fig. 5F). Interestingly, similar results were obtained when NGF-treated AR-positive DU145 cells were exposed for 3 days to 10 nmol/l DHT in the presence of 10 μmol/l of the anti-androgen CPA (not shown). No effects were seen when NGF-treated DU145 cells were exposed to 10 μmol/l HF or 10 μmol/l CPA, or when untreated DU145 cells were maintained in serum-free medium and exposed for 3 days to 10 nmol/l DHT or 10 μmol/l HF or 10 nmol/l DHT and 10 μmol/l HF (not shown).

**Discussion**

In the light of these findings, we suggest that NGF treatment may be able to redirect the androgen-independent/AR-negative DU145 prostate cancer cell line to a more dependent state on androgen signaling.

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**Figure 4** NGF treatment induced the re-expression of AR and restored the androgen dependency in DU145 cells. (A) **Western blot.** Cell extracts (50 μg/lane) from untreated (lane 1) and NGF-treated (50 ng/ml, 8 days, lane 2) DU145 cells were analyzed by western blot using a rabbit polyclonal antibody (0.1 μg/mL) raised against the -COOH terminus of the human AR, which detected a band of about 110 kDa. The membrane was then incubated with a secondary horseradish peroxidase conjugated anti-rabbit IgG and, after washing of secondary antibody, the signal was visualized using the ECL detection system. Extracts from LNCaP cells (50 μg) were loaded in lane 3 and used as internal positive control. (B) **Immunocytochemistry.** DU145 cells were treated with 50 ng/ml NGF for 8 days in tissue-culture Petri dishes containing sterile poly-lysine-treated coverslips. Cells were then fixed for 5 min at −20°C in cold methanol, permeabilized and incubated with a 1:100 dilution of the same polyclonal antibody raised against the human AR described for the Western blot analysis. After an extensive wash, biotinylated secondary antibodies were applied and the cells were then treated according to the supplier’s instructions for the ABC kit, and revealed with diaminobenzidine. Panel 1: untreated DU145 cells; panel 2: NGF-treated DU145 cells. Original magnification: 200 x. (C) **Immunocytochemistry.** NGF-treated DU145 cells were divided in two Petri dishes, one of which was treated with 10 nmol/l DHT for 3 days, while the other was maintained in serum-free medium. Cells were then washed and fixed in cold methanol. AR expression was studied with the immunocytochemistry method described above, using a polyclonal antibody raised against the -COOH terminus of the human AR. Panel a: NGF-treated cells in serum-free medium+10 nmol/l DHT; panel b: NGF-treated cells in serum-free medium. Original magnification: 200 x. (D) **Cell proliferation.** NGF-treated DU145 cells were plated at the density of 50 × 10^3 in 24-well plates for both direct cell counting and [3H]thymidine incorporation. NGF-treated cells were maintained in serum-free medium or treated for 3 days with 10 nmol/l (nM) DHT, or 10 nmol/l DHT + 10 μmol/l (μM) HF. For direct counting with the hemocytometer, five fields for each treatment condition were counted, and counts were made in at least three separate experiments. Data are the means ± S.E. from three different experiments run in quadruplicate.
cell line towards a differentiation pathway, as indicated by the fact that NGF-treated DU145 cells strongly reduced their telomerase activity and regained the ability to express AR and p75NGFR proteins. Most notably, ARs were functionally active and restored a DHT sensitivity to NGF-treated DU145 cells. When cells were exposed to DHT, ARs translocated to the nucleus and this effect was accompanied by a remarkable induction of the rate of cell proliferation, which was blocked by anti-androgens such as HF and CPA, which also induced apoptotic death of NGF-treated DU145 cells.

The relevance of the re-expression of functionally active ARs is underlined by the part played by ARs in the prostate. Mechanisms by which prostate cancer cells escape androgen ablation and become independent of the need for androgens are not yet well understood. It has been proposed that development of the androgen independency can occur by a variety of mechanisms, such as environmental adaptation, multifocal origin or genetic instability (26). The vast majority of androgen-independent prostate tumors express ARs highly; many paradigms, indeed, relate the finding of AR overexpression to the development of hormone resistance, and it has been suggested that amplifications or mutations, or both, of ARs may play a part in altering sensitivity to androgens (27). However, 20–30% of advanced metastatic hormone-independent prostate cancer is characterized by a significant loss of AR expression, as a result of transcriptional silencing that does not comprise either deletion or mutational mechanisms (27). Both in clinical tumors and in prostate cancer cell lines, the transcriptional silencing of ARs involves hypermethylation of the AR promoter and histone deacetylation, known as epigenetic mechanisms (28).

Results reported here lead to the hypothesis that NGF treatment could modulate gene expression in the DU145 prostate cancer cell line through the epigenetic regulation of transcription. This suggestion is supported by the finding that this phenomenon also occurs in PC12 cells, in which NGF-mediated differentiation induces downregulation of the enzyme catalyzing the DNA methylation reaction, namely DNA methyltransferase (29). Furthermore, the inhibition of this enzyme restores androgen sensitivity in DU145 cells, and it has been proposed that this effect could be due to a partial demethylation of the AR (30). A possible role of NGF in the epigenetic mechanisms is also supported by results obtained in the study of telomerase activity. NGF-treated DU145 cells had a strongly inhibited telomerase activity through the reduction of hTERT mRNA, with no modifications of hTR levels. In a recent paper by Suenaga et al. (31), it was reported that hTERT mRNA expression and telomerase activity could be strongly decreased in prostate cancer cell lines when exposed to drugs that inhibit histone deacetylation.
without an effect on hTR level. These results were obtained in PC3 and LNCaP cell lines, and we have found that both DU145 and PC3 cell lines are responsive to NGF treatment, inducing inhibition of telomerase activity (12), and also AR re-expression in the PC3 cell line (unpublished results). The possible role of NGF as a modulator of epigenetic mechanisms in prostate cancer cell lines clearly deserves further investigation.

As already mentioned, NGF treatment induces re-expression of the NGF receptor p75NGFR in the DU145 cell line. We would like to stress the relevance exists between expression of p75NGFR and the malignant progression of prostate epithelial cells (12, 15, 16). On this basis, p75NGFR has been proposed as a tumor suppressor gene for the prostate (16). Furthermore, our data give support to increasing evidence suggesting that a complex interaction and reciprocal modulation does indeed occur between trkA and p75NGFR, and that the type of response to NGF may be strictly dependent on the pattern of NGF receptors expressed (32–34). Taken together, the results presented here indicate a differentiating role for NGF in a cell line derived from an androgen-insensitive/AR-negative metastatic human prostate adenocarcinoma, as shown by the findings that: (a) NGF induced the re-expression of functionally active ARs and restored the androgen sensitivity of DU145 cells; (b) NGF strongly reduced the telomerase activity of DU145 cells, through the inhibition of hTERT at the transcriptional level; (c) NGF-treated DU145 cells re-expressed p75NGFR, the progressive loss of which has been proposed as a marker of malignant degeneration of prostate epithelial cells (16).

Acknowledgements

We would like to thank the Italian Association for Cancer Research and Progetto Strategico Oncologia, MURST-CNR 2000 for their financial support, Drs Daniela Uberti and Alessandra Valerio for helpful suggestions and Nicoletta Guerra for her excellent technical support.

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Received 29 January 2002
Accepted 30 May 2002