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Tumorigenesis and Neoplastic Progression

An Extensive Tumor Array Analysis Supports Tumor Suppressive Role for Nucleophosmin in Breast Cancer

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Nucleophosmin (NPM) is a multifunctional protein involved in a complex network of interactions. The role of NPM in oncogenesis is controversial. The NPM gene (*NPM1*) is mutated or rearranged in a number of hematological disorders, but such changes have not been detected in solid cancers. However, experiments with cultured *NPM*-null cells and with mice carrying a single inactivated *NPM* allele indicate a tumor suppressor function for NPM. To resolve the role of NPM in solid cancers, we examined its expression and localization in histologically normal breast tissue and a large array of human breast carcinoma samples (n = 1160), and also evaluated its association with clinicopathological variables and patient survival. The intensity and localization (nucleolar, nuclear, cytoplasmic) of NPM varied across clinical samples. No mutations explaining the differences were found, but the present findings indicate that expression levels of NPM affected its localization. Our study also revealed a novel granular staining pattern for NPM, which was an independent prognostic factor of poor prognosis. In addition, reduced levels of NPM protein were associated with poor prognosis. Furthermore, luminal epithelial cells of histologically normal breast displayed high levels of NPM and overexpression of NPM in the invasive MDA-MB-231 cells abrogated their growth in soft agar. These results support a tumor suppressive role for NPM in breast cancer. ***(Am J Pathol 2011, 179:1004–1014; DOI: 10.1016/j.ajpath.2011.04.009)***

Nucleophosmin (NPM) is a ubiquitously expressed multifunctional nucleolar phosphoprotein. It localizes mainly to the nucleoli, but also shuttles in and out of the nucleolus, and between the nucleus and the cytoplasm.[1,2] NPM belongs to the nucleoplasmin family of nuclear chaperone proteins. It is involved in a complex network of interactions and has multiple functions. In addition to its chaperone activity,[3,4] NPM is involved in centrosome duplication,[5] ribosome biogenesis,[6,7] and environmental stress responses.[8,9] NPM also regulates the tumor suppressor proteins p53[10–12] and p14^{ARF}.[13–15] Moreover, NPM protein is post-translationally modified by acetylation,[16] sumoylation,[17,18] ubiquitinylation,[19] and phosphorylation.[20–23]

NPM has been heavily implicated in cancer pathogenesis, but its actual role in oncogenesis is controversial.[24] *NPM1* is mutated or rearranged in a number of hematological disorders,[25] and it is the most frequently mutated gene in acute myeloid leukemia. In addition, NPM protein is reported to be overexpressed in cancer cells, and it was originally proposed as a proto-oncogene. However, rapidly proliferating tumor cells could show elevated NPM levels, simply because NPM expression increases rapidly in early G1 phase during mitosis.[26] On the other hand, inactivation of *NPM1* in the germline leads to embryonic lethality.[27,28] Moreover, experiments with cultured *NPM*-null cells[27,28] and mice carrying a single inactivated *NPM* allele indicate a tumor suppressor function for NPM.[27] *NPM1*

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function is required for the maintenance of genomic stability,[27–29] and *NPM1* acts as a haploinsufficient tumor suppressor in the hematopoietic compartment.[29]

To shed light on the role of NPM in solid cancers, we investigated NPM expression levels and localization in a large array of human breast carcinoma samples ($n = 1160$) and evaluated its association with clinicopathological variables, patient survival, and molecular subtypes of breast cancer. We identified granular staining as a novel staining pattern for NPM. Both reduced NPM levels and granular staining pattern were associated with poor prognosis.

Materials and Methods

Cell Culture

All media were supplemented with 10% fetal calf serum, 1% glutamine, and penicillin-streptomycin, except as stated otherwise. MDA-MB-231 and MCF-7 cells were maintained in RPMI 1640 medium, MDA-MB-436 cells in Leibovitz's L-15 medium with 16 μg/mL glutathione, 10 μg/mL insulin, and MDA-MB-361 cells in Leibovitz's L-15 medium with 20% fetal calf serum.

Immunofluorescence and IHC

We used a monoclonal mouse-anti-NPM antibody (Zymed Laboratories, South San Francisco, CA). The specificity of the antibody has been described previously.[8] For immunofluorescence staining, cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% NP-40 in PBS, blocked using 3% bovine serum albumin in PBS, and incubated with primary and secondary (Molecular Probes goat anti-mouse Alexa Fluor 594; Invitrogen, Carlsbad, CA) antibodies. DNA was visualized with DAPI (Vector Laboratories, Burlingame, CA). For the immunohistochemical (IHC) analyses, 4-μm sections were prepared and deparaffinized. After antigen retrieval (120°C autoclave, 2 minutes in 10 mmol/L sodium citrate buffer, pH 6.0), blocking of endogenous peroxidase activity (0.7% hydrogen peroxidase in methanol, 30 minutes), and blocking of unspecific antibody binding sites (normal horse serum in 0.3% bovine serum albumin-PBS, 30 minutes), sections were incubated with anti-NPM antibody (0.3% bovine serum albumin-PBS, overnight, 4°C). NPM antibody was detected with peroxidase staining (biotinylated horse anti-mouse immunoglobulins 1:200 and Vectastain ABC complex; Vector Laboratories) visualized with 3,3′-diaminobenzidine (Vector Laboratories). The sections were counterstained with Mayer's hematoxylin. c-Kit staining was performed using a polyclonal rabbit-anti-CD117 antibody (1:300, A4502; Dako, Carpinteria, CA) similarly to the NPM staining, with the following exceptions: 98°C water bath for 15 minutes was used in antigen retrieval and a PowerVision Novocastra preantibody blocking solution (Leica Microsystems, Wetzlar, Germany) was used for primary antibody incubation. Staining was detected using a PowerVision + Poly-HRP histostaining kit (DPVB + 110DAB; ImmunoVision Technologies, Daly City, CA; Springdale, AR) according to the manufacturer's instructions. Cytokeratin staining was performed with a Ventana Discovery IHC slide stainer (Ventana Medical Systems, Tucson, AZ) and a Ventana 3,3′-diaminobenzidine tetrahydrochloride biotin avidin detection kit using a polyclonal rabbit-anti-cytokeratin 7 antibody (1:100, ab52870; Abcam, Cambridge, UK).

Virus Production and Creation of Stable Cell Lines

The *NPM1* gene was cloned into a lentiviral pBOB\cag\green fluorescent protein (GFP) expression vector (a kind gift from Dr. Ylä-Herttuala, University of Eastern Finland, Kuopio, Finland) and was transfected into human embryonic kidney cells (293FT; 4×10^6) using Lipofectamine 2000 (Invitrogen). Virus-containing supernatants were collected at 72 hours after transfection. To remove cell debris, the medium was centrifuged briefly (2 minutes, $160 \times g$) and filtered through a 0.45-μm filter. The supernatant was concentrated with an Optima L-80 XP ultracentrifuge supplied with a swinging bucket rotor (SW28) (both Beckman Coulter, Brea, CA). MDA-M-231 and MDA-MB-361 cells (50% confluent) were transduced with concentrated NPM-GFP and GFP encoding viruses. We sorted the moderately transgene-positive cells by using a FACSAria fluorescence-activated cell sorter (purity mode; BD Biosciences, San Jose, CA). Expression of transgenes was verified using Western blot analysis. Efficiency of transduction was determined using immunofluorescence microscopy by counting the percentage of transgene-expressing cells.

17β-Estradiol Treatment

Cells grown to 60% to 70% confluency were starved for 48 hours in Phenol Red-free RPMI (Gibco, California, USA) supplemented with 2% charcoal-dextran filtrated fetal calf serum (HyClone; Thermo Scientific, Logan, UT), 1% glutamine, and penicillin-streptomycin. After starvation, cells were treated with 10 nmol/L 17β-estradiol diluted in dimethyl sulfoxide (DMSO) for 3 days, followed by either fixation for the immunofluorescence analysis or cell lysis in NP-40 buffer (1% NP-40, 50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl) for 10 minutes at 4°C. DMSO-treated cells served as control. Percentage of cells displaying even nuclear staining (no visible nucleoli) and nucleolar staining was determined from the microscopic images. NPM expression was quantified with ImageJ software version 1.45h (NIH, Bethesda, MD) against β-tubulin, detected from the same membranes.

Immunoblotting

Samples were analyzed using SDS-PAGE and transferred to Immobilon-P membrane (Millipore, Billerica, MA) for immunoblotting. We used the following primary antibodies: anti-GFP (Molecular Probes A6455; Invitrogen), anti-NPM (Zymed Laboratories; Cell Signaling Technology, Danvers, MA), and anti-β-tubulin (BD Biosciences). Proteins were visualized with a SuperSignal West Pico kit (Pierce; Thermo Scientific, Rockford, IL).

Patients and Preparation of the TMA

Histologically normal breast samples from reduction surgery were obtained from the Helsinki University Central Hospital. The Finprog breast cancer database used in the cancer tissues analysis contains samples from women diagnosed with breast cancer within five well-defined geographical regions in Finland in 1991 and 1992; approximately 50% of the Finnish population lives in these five regions.[30] Patients with *in situ* carcinoma, distant metastases at the time of diagnosis, synchronous or metachronous bilateral breast cancer, malignancy other than breast cancer in history (except for basal cell carcinoma or cervical carcinoma *in situ*), or women who did not undergo breast surgery were excluded from the study series, leaving a total of 2032 patients eligible. Tissue array core biopsies with an interpretable staining for NPM were available for 1160 of these patients. The median follow-up time for patients alive and without recurrence at the end of follow-up was 9.5 years. Adjuvant chemotherapy (typically a combination of cyclophosphamide, methotrexate, and 5-fluorouracil) was given to 160 of the 1160 patients (13.8%), adjuvant hormonal therapy (typically tamoxifen) to 278 patients (24%), and combined chemotherapy and hormonal therapy to 6 patients (0.5%). TMAs were prepared as described previously.[31]

Evaluation of NPM Immunostaining

Expression and localization of NPM was examined under a multiheaded Leica DM LB microscope by two independent observers (P.-R.K. and P.L.). All evaluations were performed with blinding to the clinical data.

The expression level scoring of the TMA was performed as follows: 0, all tumor cells negative; 1, low expression in tumor cells (5% to 100%); 2, moderate expression in ≥50% of tumor cells; and 3, high expression in ≥50% of tumor cells. Nucleolar localization, nucleoplasmic staining pattern, and cytoplasmic staining of NPM were scored independently of the expression analysis. The following criteria were used for the nucleolar NPM staining: 0 (negative), no staining in the nucleoli or the nucleoplasmic intensity much higher than that found in the nucleoli; 1 (an intermediate staining), equal staining intensities in the nucleolus and in the nucleoplasm; and 2, staining only in the nucleoli. Nucleoplasmic staining pattern was scored as granular if the staining was observed at the nuclear periphery and appeared speckled. Cytoplasmic staining was scored as negative, low, or high. Because of nonrepresentative sample material, the nucleolar and the cytoplasmic localization could not be analyzed in 10% (112/1160) and in 4% (47/1160) of the tumors, respectively. Image acquisition was performed through an Olympus DP50 color camera and with Olympus Studio Lite software version 1.0. Immunofluorescence staining of cells was visualized with an Axioplan 2 epifluorescence microscope (Zeiss, Jena, Germany) with appropriate filters (Chroma Technology, Rockingham, VT). Image acquisition was performed using a Zeiss digital AxioCam camera and AxioVision software version 4.5.

Soft Agar Assays

Soft agar assays were performed with MDA-MB-231 human breast carcinoma cells infected with lentiviruses encoding NPM-GFP. Cells infected with lentiviruses encoding GFP served as control. Cells (5×10^3 cells per 35-mm well) were resuspended in complete medium containing 0.35% agarose. Cells were grown on tissue culture dishes containing a 2-mL layer of solidified 0.7% agar in a complete medium. After 14 days, number of colonies was quantified using ImageJ software from two randomly taken micrographs per well (original magnification, ×20). For visualization, foci were methanol-fixed and stained with 0.005% crystal violet.

Cell Proliferation Assays

Cells (5×10^3) were grown on 96-well plates for 2 or 3 days, after which 10 μL MTT (5 mg/mL) was added. After incubation for 2 hours, cells were lysed (10% SDS, 10 mmol/L HCl) overnight. Absorbance was measured at 540 nm using Multiskan Ascent software version 2.6 (Thermo Labsystems, Vantaa, Finland).

NPM Sequence Analysis from the Patient Samples

Total RNA was isolated from paraffin-embedded sections with a High Pure RNA paraffin kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. A QuantiTect reverse transcription kit (Qiagen, Hilden, Germany) was used for the cDNA synthesis. A 206-bp C-terminal fragment was amplified with FastStart TaqDNA polymerase (Roche Diagnostics) using the following primers: 5′-CTTCCCAAAGTGGAAGCC-3′ and 5′-GGAAAGTTCTCACTCTGC-3′.[32] The amplified fragment was sequenced using the 5′-GGAAAGTTCTCACTCTGC-3′ primer and the Dye Terminator kit version 3.1 with an ABI 3100 genetic analyzer (Applied Biosystems, Foster City, CA).

qPCR Analysis

Expression of NPM in the histologically normal breast tissue was investigated from cDNA samples by quantitative PCR (qPCR), using a DyNAmo HS SYBR Green qPCR kit (Finnzymes, Vantaa, Finland). The cDNA used as a template was prepared from the total RNA extracts as described above. Presence of NPM cDNA was detected using primers 5′-GCGCCAGTGAAGAAATCTATACG-3′ and 5′-GAAGGATTCTTGTCCTTTTGATCTTG-3′ and was normalized against the cDNA level of glyceraldehyde-3-phosphate dehydrogenase as the reference gene, which was detected using primers 5′-GAAGGTGAAGGTCGGAGTCAAC-3′ and 5′-CAGAGTTAAAAGCAGCCCTGGT-3′. Relative quantification of expression levels between the samples was performed according to the Pfaffl method.[33]

Statistical Analysis

The χ^2 test was used to test for associations between factors and the odds ratio to examine the strength of the relationships. Life tables were calculated according to the Kaplan-Meier method. Distant disease-free survival (DDFS) was calculated from the date of diagnosis to the occurrence of either metastases outside the locoregional area or death from breast cancer. Survival curves were compared using the log-rank test. Multivariate survival analyses were performed with the Cox proportional hazards model, entering the following covariates: NPM intensity as a categorical variable (with the highest intensity value as the reference) and the number of metastatic lymph nodes, tumor size in centimeters, grade (well differentiated versus moderately versus poorly differentiated), estrogen receptor (ER) and progesterone receptor (PgR) status, and the HER2 test results as binary variables (negative versus positive). Cox regression was performed using a backward stepwise selection of variables and a P value of 0.05 was adopted as the limit for inclusion of a covariate. Separate Cox models were fitted with NPM staining pattern (granular versus not granular) instead of NPM intensity and with adjuvant chemotherapy, as well as adjuvant hormonal therapy, as covariates (no adjuvant versus adjuvant). To test for interaction, product terms between NPM staining pattern and chemotherapy and hormonal therapy, respectively, were added to the models. The assumption of proportional hazards was ascertained with complementary log plots.

Results

Expression and Localization of NPM in Histologically Normal Mammary Gland

NPM expression level and localization varies among different normal organs and between embryonic and adult tissues.[34] We therefore analyzed expression of NPM in 14 histologically normal breast tissue samples originating from breast reduction surgery. NPM was expressed at high levels (scored 3) in the luminal epithelial cells of all samples. Two different locations were observed: uniform nuclear localization (10/14, 71%; Figure 1A) and nucleolar localization (4/14, 29%; Figure 1B). c-Kit[35] (Figure 1, C and D) and cytokeratin-7 (Figure 1F) staining verified the luminal epithelial type of the NPM-positive cells.

The two different localizations correlated with age at time of surgery (≤42 versus >42 years, $P = 0.001$), suggesting a possible hormonal regulation of NPM expression. We therefore quantified NPM expression levels from the histologically normal breast tissue samples using qPCR analysis. We were able to obtain RNA for this analysis from 7/14 samples; of these, 4 samples were from women ≤42 years of age and 3 samples were from women >42 years of age (Figure 1G). The qPCR analysis revealed that the younger women expressed approximately twofold more NPM than the older women (Figure 1G). This difference in expression was detected also at the protein level in the IHC stainings (Figure 1, A and B).

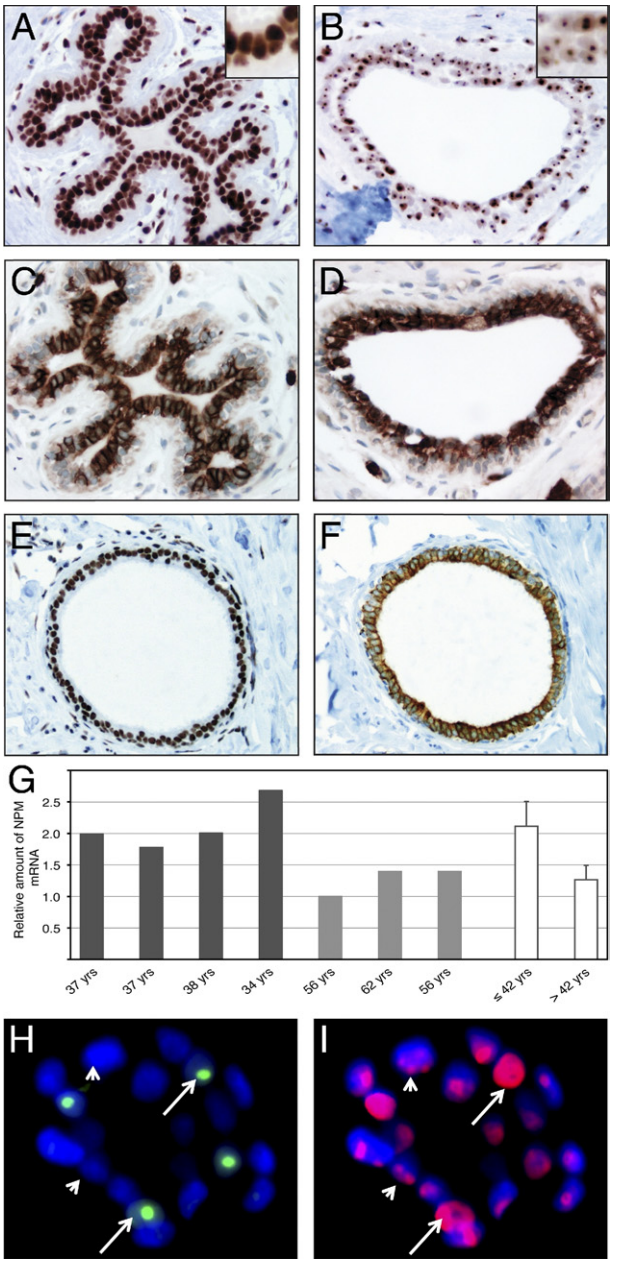

Figure 1. NPM is expressed at high levels in luminal epithelial cells of histologically normal breast tissue. **A:** Very high levels of NPM are expressed and distributed evenly throughout the nucleus in luminal epithelial cells of histologically normal breast samples from women ≤42 years of age at the time of surgery. **B:** In contrast, in luminal epithelial cells of histologically normal breast samples from women >42 years of age, NPM was detected mostly in the nucleolus. **Insets, A** and **B:** Digitally enlarged view of NPM staining. **C** and **D:** c-Kit staining was used to verify the luminal identity of the NPM-positive cells. **E** and **F:** To further verify the luminal identity of the NPM-expressing cells, histologically normal breast samples were stained for NPM (**E**) and cytokeratin-7 (**F**). **G:** qPCR analysis of NPM expression in histologically normal breast tissue, shown as relative NPM mRNA levels in seven women. NPM levels were normalized against the amount of glyceraldehyde-3-phosphate dehydrogenase in each sample. Black: individual samples from four women <42 years of age. Gray: individual samples from three women >42 years or age. White: mean ± SD for the two groups. **H** and **I:** Overexpression of NPM-GFP (green) (**arrows, H**) by the aid of lentiviruses in the MDA-MB-361 cells resulted in even distribution of endogenous NPM (red) throughout the nuclei (**arrows**, **I**), whereas in the noninfected cells (**arrowheads**) NPM was detected mainly in the nucleoli. Nuclei were visualized with DAPI (blue). Original magnification, ×400 (**A–F**, **H**, **I**).

To further study whether the expression level of NPM could affect its localization, we infected MDA-MB-361 breast carcinoma cells, which normally show nucleolar NPM staining, with lentiviruses encoding NPM-GFP. NPM overexpression in these cells affected its localization. Cells that overexpressed NPM displayed a uniform NPM staining throughout the nuclei (Figure 1, H and I), supporting the idea that the nucleolar localization of NPM in the histologically normal breast samples might be due to reduced NPM protein levels.

NPM Expression Is Regulated by Estrogen

To investigate whether the change in NPM expression in the luminal epithelial cells of histologically normal breast tissue was due to estrogen regulation, we treated serum-starved ER^+ (MDA-MB-361 and MCF7) and ER^- (MDA-MB-231 and MDA-MB-436) human breast cancer cells with 10 nmol/L 17β-estradiol for 3 days, followed by extraction with NP-40 buffer and analysis of NPM protein levels using Western blot. NP-40 extraction solubilizes cytoplasmic and nucleoplasmic but not nucleolar NPM, and allows analysis of extranucleolar NPM levels.

The 17β-estradiol treatment led to approximately 2.5-fold and 2.0-fold up-regulation of NPM protein levels in the ER^+ MDA-MB-361 and MCF-7 cells, respectively. No effect was detected in the ER^- cell lines (MDA-MB-231 and MDA-MB-436), indicating regulation of NPM protein levels by estrogen (Figure 2, A and B). In agreement with results obtained from the qPCR analysis and with NPM overexpression in the MDA-MB-361 cells, increased NPM levels in the 17β-estradiol-treated MCF-7 and MDA-MB-361 cells showed more NPM immunoreactivity in the nucleoplasm, compared with the vehicle DMSO-treated control cells (Figure 2, C–F). We determined the number of cells displaying even nuclear staining (no visible nucleoli) and nucleolar staining from the microscopic images of MCF-7 and MDA-MB-361 cells. Even nuclear staining was significantly more frequent in the 17β-estradiol-treated cells (62.6% in MCF-7 and 60% in MDA-MB-361 cells) than in the vehicle (DMSO) treated control cells (25.4% in MCF-7 and 40% in MDA-MB-361 cells) (χ^2 test; $P < 0.0001$ and $P < 0.001$, respectively).

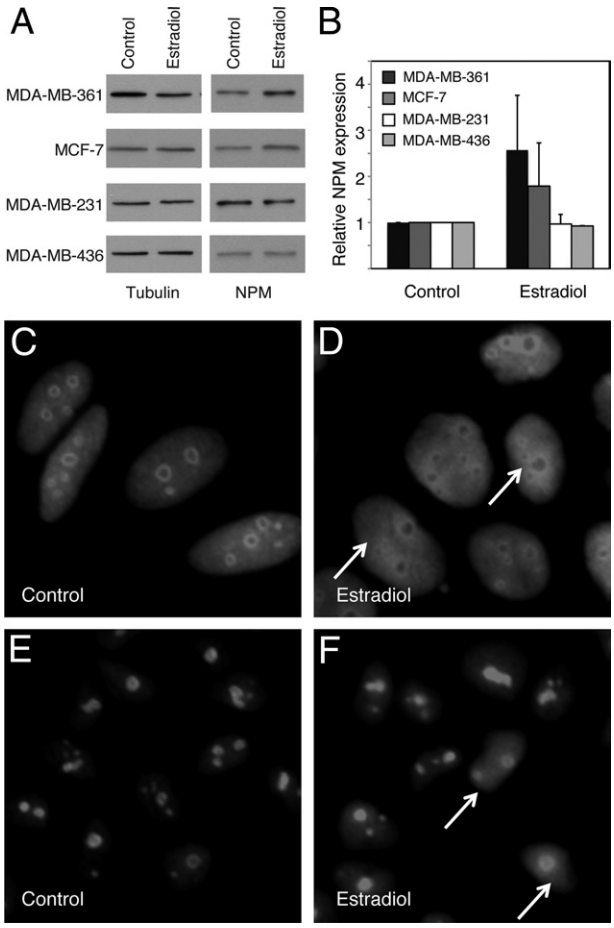

Figure 2. NPM expression is regulated by estrogen. 17β-estradiol treatment of ER^+ MDA-MB-361 and MCF-7 cells resulted in up-regulation of NPM protein levels, but no effect was detected in ER^- MDA-MB-231 and MDA-MB-436 cells. **A:** The NP-40 soluble cell fraction containing extranucleolar NPM was analyzed on a 12% SDS-PAGE, followed by Western blot analysis. Representative gels from at least two independent experiments are shown. **B:** Relative NPM expression levels in 17β-estradiol and control vehicle (DMSO) treated cell extracts, after quantification of NPM and β-tubulin signals using ImageJ software; values were normalized to DMSO control samples, which were set as 1. **C–F:** ER^+ MCF-7 (**C** and **D**) and MDA-MB-361 (**E** and **F**) cells were treated with either DMSO as control (**C** and **E**) or 17β-estradiol (**D** and **F**) for 3 days. NPM was visualized using anti-NPM antibody. 17β-estradiol-induced up-regulation of NPM protein levels led to increased extranucleolar localization of NPM (**arrows**). Original magnification, ×200.

Expression and Localization of NPM in the Clinical Breast Cancer Specimens

To examine the clinical relevance of NPM, we analyzed NPM expression level and localization in a large array of patient material ($n = 1160$), from the Finprog breast cancer database.[31] Only 11% (121/1160) of the samples displayed levels of NPM as high as the histologically normal breast tissue (see Supplemental Figure S1A at *http://ajp.amjpathol.org*). The majority of samples (85%) demonstrated moderate (55%, 639/1160) or low (30%, 353/1160) levels of NPM (see Supplemental Figure S1, B and C, respectively, at *http://ajp.amjpathol.org*), and 4% (47/1160) of the tumors showed no NPM immunoreactivity (see Supplemental Figure S1D at *http://ajp.amjpathol.org*).

Analysis of NPM localization revealed that 14% (141/1048) of the tumors showed NPM only in the nucleoli (Figure 3, A and F), 55% (580/1048) showed NPM in equal amounts in the nucleoli and nucleoplasm (Figure 3, B and G), and 31% (327/1048) showed NPM only in nucleoplasm, with no detectable nucleolar NPM (Figure 3, C and H). Cytoplasmic NPM was rarely detected: 93% (1035/1113) of the samples were negative, 4% (42/1113) showed low levels, and 3% (36/1113) showed high levels of cytoplasmic NPM (Figure 3, D and I). A very distinct, speckled staining pattern was revealed in 14% (147/1073) of the samples, a pattern hereafter referred to as granular. The granular pattern typically showed NPM staining at the nuclear periphery and as speckles in the nucleoplasm (Figure 3, E and J). In contrast to histologically normal breast tissue, no correlation between the

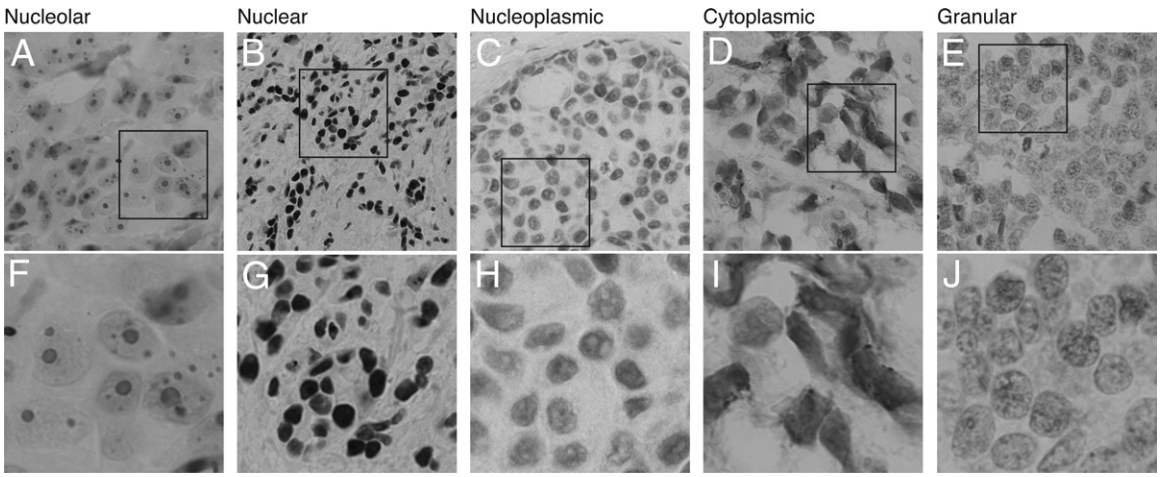

Figure 3. Localization of NPM in human breast cancer specimens. Human breast cancer tissue array sections were stained with anti-NPM antibody. Primary antibody was detected with biotinylated secondary antibodies, followed by Vectastain ABC complex. Sections were counterstained with Mayer's hematoxylin. We detected five different localizations for NPM. **A** and **F:** Nucleolar localization (score 2). **B** and **G:** Equal staining in the nucleoli and in the nucleoplasm (score 1). **C** and **H:** Nucleoplasmic localization with negative nucleolar staining (score 0). **D** and **I:** Cytoplasmic staining. **E** and **J:** Granular staining pattern. **F–J:** Digitally enlarged views of the corresponding boxed areas in **A–E**. Original magnification, ×630.

NPM localization and age was detected in the tumor specimens ($P = 0.67$).

Our findings with histologically normal breast samples and with breast cancer cell lines suggested that NPM localization within the cell is dependent on its expression levels, although in hematological cancers the cytoplasmic localization is caused by mutations in the last exon of NPM.[25] To investigate whether the differential NPM localization in tumor samples was due to NPM mutations in its C-terminus, we isolated total RNA from paraffin sections from 52 patients displaying different NPM locations, then prepared cDNA and amplified a 206-bp region containing the exons that harbor the reported NPM mutations using PCR.[32] We detected no mutations in any of the samples exhibiting different NPM expression levels and cellular localizations (see Supplemental Table S1 at *http://ajp.amjpathol.org*).

Association of NPM with the Clinicopathologic Variables and Molecular Subtypes of Breast Cancer

To evaluate the significance of NPM in breast cancer, we analyzed association of NPM protein level and the granular NPM staining pattern with clinical variables in the Finprog database.[31] The frequency of different NPM protein levels (scored as 0, 1, 2, or 3) did not vary significantly for different clinicopathological variables ($P > 0.05$ for all comparisons; see Supplemental Table S2 at *http://ajp.amjpathol.org*). On the other hand, the granular staining was significantly more frequent in large (≥2 cm) tumors, in axillary lymph node-positive tumors, in ductal tumors, in high-grade tumors, and in PgR^- tumors; granular staining was not detected in samples with high NPM levels (see Supplemental Table S2 at *http://ajp.amjpathol.org*).

Recently, gene expression profiling[36,37] and IHC[38] were used to define five molecular subtypes of breast cancer: i) luminal A (ER^+ and/or PR^+, HER-2^-); ii) luminal B (ER^+ and/or PR^+, HER-2^+); iii) basal-like (ER^-, PR^-, HER-2^-, CK5/6^+ and/or HER-1^+); iv) HER-2^+/ER^- (ER^-, PR^-, $HER2^+$); and v) unclassified. Classification of subtypes in the Finprog material has been described by Sihto et al.[39] Frequency of different NPM protein levels did not significantly vary among the molecular subtypes. However, granular staining was significantly more frequent in the basal-like and unclassified molecular subtype tumors ($P < 0.05$; see Supplemental Table S2 at *http://ajp.amjpathol.org*).

Reduced NPM Expression Is an Independent Prognostic Marker of Poor Survival in the Luminal A Molecular Subgroup of Breast Cancer

Our study revealed a clear trend for association of reduced NPM expression and poor survival of patients. The 10-year DDFS of patients was 84% [95% confidence interval (CI) = 69.7 to 98.4] with high staining intensity, 73% (95% CI = 65.7 to 80.4) with moderate staining intensity, 71% (95% CI = 60.1 to 82.0) with low staining intensity, and 60% (95% CI = 16.8 to 102.5) when no NPM immunoreactivity was detected (Figure 4A). Because all of the analyzed histologically normal breast tissue samples showed high NPM expression level (scored as 3), we used that as a reference expression level in further analyses. When patients with reduced NPM expression (no, low or moderate NPM staining intensity; $n = 1039$) were combined into a single group, the 10-year DDFS was 72% (95% CI = 65.7 to 77.8), compared with 84% (95% CI = 69.7 to 98.4) for those with high staining intensity (hazard ratio HR = 1.71, 95% CI = 1.02 to 2.73, $P = 0.02$) (see Supplemental Table S3 at *http://ajp.amjpathol.org*). The subgroup analysis suggested that reduced NPM protein levels were associated significantly with a poor outcome in the subgroups of ER^+, $p53^-/^{low}$, and Her-2^- breast carcinomas (see Sup-

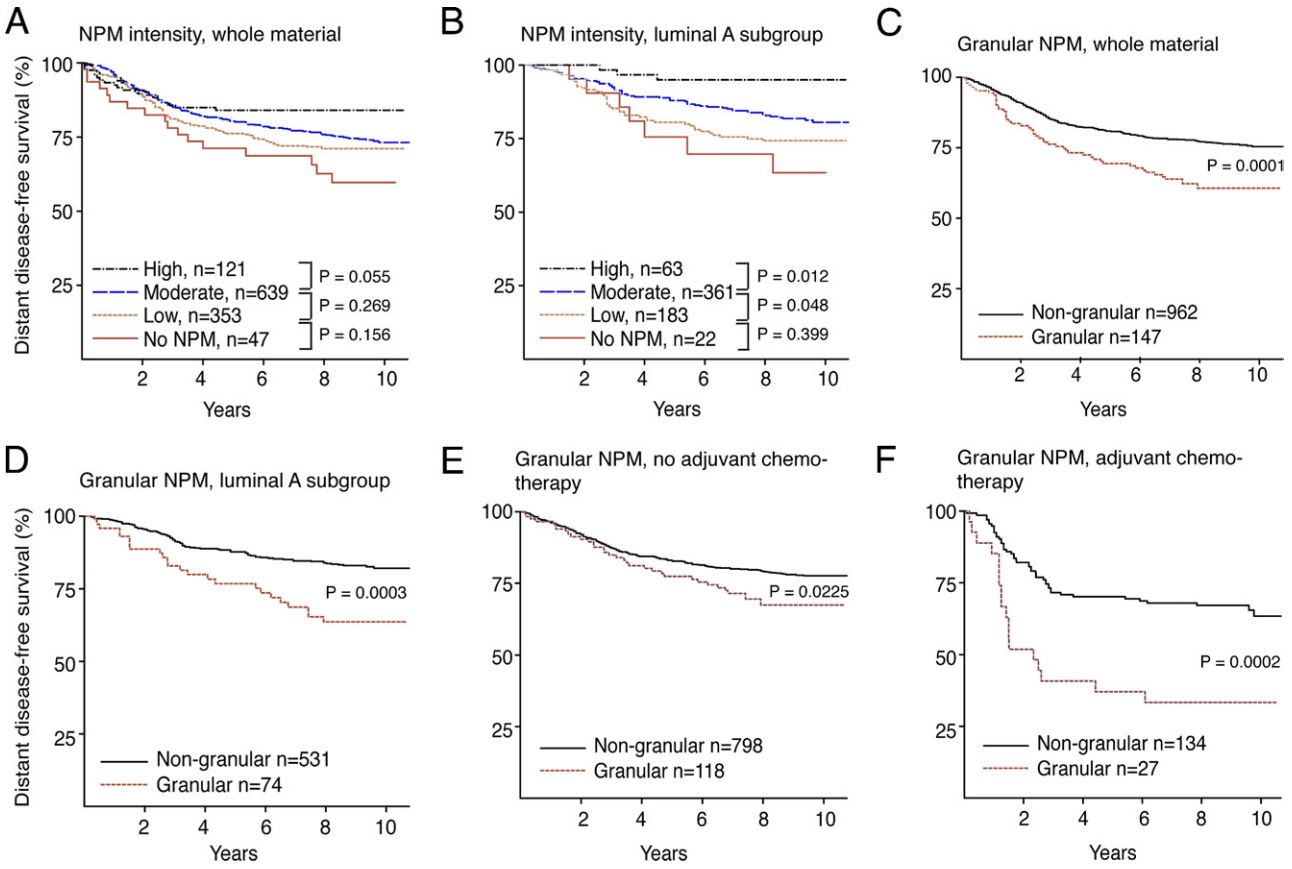

Figure 4. Association of NPM with distant disease-free survival of breast cancer patients. **A** and **B:** The 10-year DDFS of patients according to the intensity of NPM expression in the entire series from the Finprog breast cancer material (**A**) and in the luminal A molecular subtype (**B**). **C** and **D:** The 10-year DDFS of patients with granular NPM staining pattern versus nongranular NPM in the entire series (**C**) and in the luminal A molecular subtype (**D**). **E** and **F:** The 10-year DDFS of patients treated (**F**) or not treated (**E**) with adjuvant chemotherapy according to granular and nongranular NPM. *P* values in **A** and **B** were calculated between the individual scorings (ie, high versus moderate; moderate versus low, and low versus no NPM expression).

plemental Table S3 at *http://ajp.amjpathol.org*), which are otherwise subgroups associated with better prognosis. When the subgroup analysis was performed within molecular subtypes, reduced NPM protein levels were associated significantly with prognosis only in the luminal A subtype ($P = 0.004$; Figure 4B; see also Supplemental Table S3 at *http://ajp.amjpathol.org*). In a multivariate analysis with the Cox proportional hazards model, the intensity of NPM staining was not an independent prognostic factor in the whole series overall (Table 1), but appeared as an independent prognostic factor in the luminal A subgroup (Table 2).

Granular Staining Pattern of NPM Is an Independent Prognostic Marker of Poor Survival

The 10-year DDFS of women with granular NPM staining was 61% (95% CI = 41.9 to 80.5), whereas a 75% (95% CI = 69.5 to 81.2) DDFS was observed in the absence of granular staining (HR = 1.78, $P = 0.0001$; Figure 4C; see also Supplemental Table S4 at *http://ajp.amjpathol.org*). To further characterize the role of granular NPM staining, we analyzed certain subgroups and molecular subtypes with respect to survival. Granular NPM correlated with poor outcome in several subgroups (see Supplemental Table S4 at *http://ajp.amjpathol.org*), including luminal A (Figure 4D). Moreover, granular staining was identified as an independent prognostic factor with a Cox proportional hazards model (Table 3, $P = 0.04$). Granular staining was also a stronger prognostic factor for poor outcome in patients who received adjuvant chemotherapy (HR = 2.73, $P = 0.0002$), compared with those who did not (HR = 1.51, $P = 0.02$) (Figure 4, E and F). In accord with these findings, chemotherapy and granular staining showed a significant interaction in a Cox regression model ($P = 0.02$; Table 4). Adjuvant hormonal therapy did not show any significant interaction with granular NPM staining ($P = 0.77$; Table 4).

Neither cytoplasmic nor nucleolar localization of NPM showed any significant prognostic value in the present study. The 10-year DDFS was 74% (95% CI = 67.9 to 79.6) for patients with no detectable cytoplasmic NPM, and 68% (95% CI = 42.8 to 93.0) for patients with low or high cytoplasmic NPM immunostaining. The 10-year DDFS was 72% (95% CI = 61.7 to 81.8) when no nucleolar NPM expression could be detected, 76% (95% CI = 67.9 to 84.2) when NPM was present in equal amounts in the nucleoli and nucleoplasm, and 70% (95% CI = 55.9 to 83.8) when only nucleolar staining was observed.

Table 1. Cox Proportional Hazards Regression of DDFS in the Whole Finprog Series Including NPM Expression Level (n = 759)

Variable	HR	CI 95%	*P* value
Tumor size, cm	1.19	1.08–1.30	<0.0001
Number of positive lymph nodes	1.16	1.11–1.20	<0.0001
Histologic grade			
1	1.00	Ref	
2	2.19	1.33–3.60	0.002
3	2.17	1.27–3.70	0.004
Method of detection			
Screen detected	1.00	Ref	
Outside of screen	1.78	1.14–2.79	0.01
Age at diagnosis			
35–69 years	1.00	Ref	
<35 years	1.25	0.63–2.48	0.52
≥70 years	0.93	0.66–1.31	0.69
NPM expression			
High	1.00	Ref	
Moderate	1.17	0.69–1.99	0.57
Low	1.22	0.70–2.12	0.49
Negative	1.90	0.92–3.89	0.08

CI, confidence interval; DDFS, distant disease-free survival; HR, hazard ratio; NPM, nucleophosmin; Ref, reference value.

Overexpression of NPM Abolished the Growth of Breast Cancer Cells in Soft Agar

To investigate whether NPM expression is solely a marker of survival or whether it has a role in tumor progression, we overexpressed the full-length protein as a GFP fusion by the aid of lentiviruses in the MDA-MB-231 cells, and then analyzed the anchorage-independent growth of these cells in a soft agar assay. Cells infected with lentiviruses encoding GFP served as a control. Expression of the NPM-GFP transgene was verified using Western blot analysis (see Supplemental Figure S2 at *http://ajp.amjpathol.org*) and with immunofluorescence (data not shown). Overexpression of NPM significantly reduced the number of colonies of MDA-MB-231 cells in soft agar (Figure 5, A and B), but had no effect on the proliferation of these cells (Figure 5C). Similar results were obtained both with a non-sorted heterogeneous cell population (~40% of cells expressing the transgene) and with sorted cells (fluorescence-activated cell sorting) moderately expressing the transgene. These results show that NPM overexpression suppressed the anchorage-independent growth of MDA-MB-231 cells, supporting the data obtained with the tumor microarray.

Table 2. Cox Proportional Hazards Regression of DDFS in Women with Breast Cancer of the Luminal A Type (n = 395)

Variable	HR	CI 95%	*P* value
Tumor size, cm	1.15	0.97–1.35	0.10
Number of positive lymph nodes	1.19	1.11–1.27	<0.0001
Histologic grade			
1	1.00	Ref	
2	2.34	1.17–4.68	0.016
3	3.25	1.44–7.32	0.004
Method of detection			
Screen detected	1.00	Ref	
Outside of screen	1.54	0.77–3.06	0.22
Age at diagnosis			
35–69 years	1.00	Ref	
<35 years	2.63	0.92–7.53	0.07
≥70 years	1.08	0.63–1.86	0.78
NPM expression			
High	1.00	Ref	
Moderate	2.49	0.76–8.14	0.13
Low	3.24	0.97–10.78	0.06
Negative	10.16	2.48–41.61	0.001

CI, confidence interval; DDFS, distant disease-free survival; HR, hazard ratio; NPM, nucleophosmin; Ref, reference value.

Table 3. Cox Proportional Hazards Regression of DDFS in the Whole Finprog Series Including Granular NPM Staining (n = 725)

Variable	HR	CI 95%	*P* value
Tumor size, cm	1.18	1.08–1.30	<0.0001
Number of positive lymph nodes	1.17	1.13–1.22	<0.0001
Histologic grade			
1	1.00	Ref	
2	2.29	1.36–3.87	0.002
3	2.21	1.27–3.85	0.005
Method of detection			
Screen detected	1.00	Ref	
Outside of screen	1.73	1.09–2.76	0.02
Age at diagnosis			
35–69 years	1.00	Ref	
<35 years	1.36	0.69–2.69	0.38
≥70 years	1.01	0.71–1.43	0.96
NPM expression			
Nongranular	1.00	Ref	
Granular	1.47	1.01–2.16	0.04

CI, confidence interval; DDFS, distant disease-free survival; HR, hazard ratio; NPM, nucleophosmin; Ref, reference value.

Table 4. Cox Proportional Hazards Regression of DDFS In Women with Breast Cancer, Including a Test of Interaction between Chemotherapy and Granular NPM (n = 712)

Variable	HR	CI 95%	*P* value
Tumor size, cm	1.18	1.07–1.30	0.001
Number of positive lymph nodes	1.16	1.11–1.21	<0.0001
Histologic grade			
1	1.00	Ref	
2	2.32	1.37–3.93	0.002
3	2.21	1.26–3.85	0.005
Method of detection			
Screen detected	1.00	Ref	
Outside of screen	1.74	1.09–2.79	0.02
Age at diagnosis			
35–69 years	1.00	Ref	
<35 years	1.38	0.69–2.75	0.36
≥70 years	1.08	0.74–1.56	0.70
NPM expression			
Nongranular	1.00	Ref	
Granular	1.10	0.68–1.80	0.70
Chemotherapy			
No	1.00	Ref	
Yes	0.94	0.61–1.44	0.77
NPM granular × Cx interaction term	2.54	1.13–5.70	0.02

CI, confidence interval; Cx, chemotherapy; DDFS, distant disease-free survival; HR, hazard ratio; NPM, nucleophosmin; Ref, reference value.

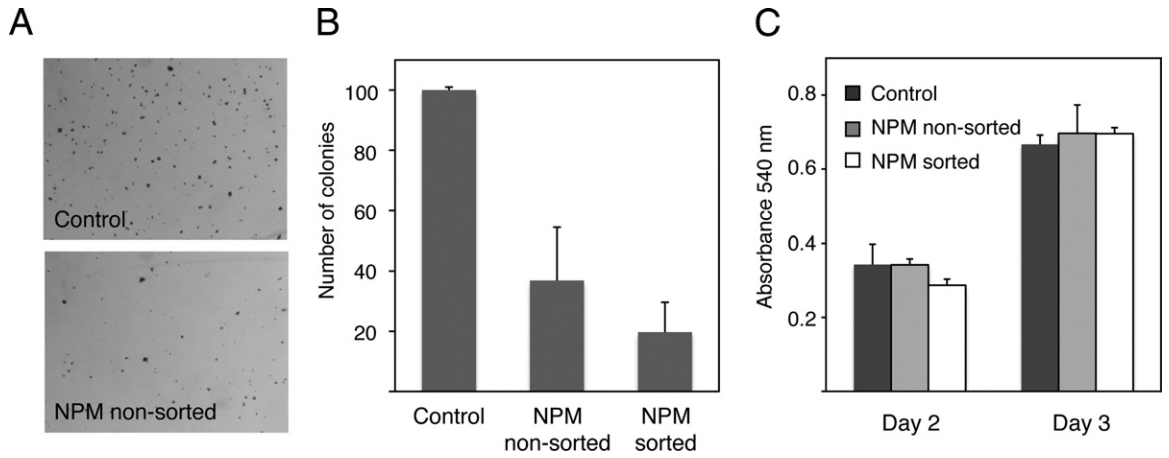

Figure 5. NPM expression in invasive MDA-MB-231 breast cancer cells suppressed their growth in the soft agar. NPM-GFP was expressed in the MDA-MB-231 cells with the aid of lentiviruses. The experiment was performed both with a heterogeneous non-sorted cell population (~40% of cells expressing the transgene) and sorted cells (fluorescence-activated cell sorting) expressing moderate levels of NPM-GFP. Cells expressing GFP served as a control. Cells were grown in soft agar for 14 days prior to quantification of the number of colonies using ImageJ software. **A:** Visualization of colonies with crystal violet staining of GFP-expressing cells (control) and NPM-GFP-expressing cells (NPM non-sorted). Original magnification, ×20. **B:** Number of colonies of NPM-GFP-expressing cells compared with the GFP-expressing control cells from two (sorted) or three (non-sorted) independent experiments performed in triplicate. Control cells were set as 100%, and the number of colonies of NPM-expressing cells are reported as percent of control. **C:** NPM-GFP-expressing and GFP-expressing control cells (5×10^3) were grown in 96-well plates for 2 or 3 days, after which 10 μL MTT (5 mg/mL) was added. On day 2 or day 3, absorbance was measured at 540 nm after incubation overnight. Experiments were performed in triplicate.

Discussion

We report here an extensive analysis of expression levels and localization of NPM in histologically normal breast tissue ($n = 14$) and in tissue from Finprog, a large breast cancer database ($n = 1160$). Notably, in addition to the intensity of NPM immunoreactivity, NPM localization varied in the clinical samples. We detected NPM staining solely in the nucleolus, evenly distributed throughout the nucleus, and only in the nucleoplasm. We also observed cytoplasmic staining for NPM in the patient material.

NPM is considered a nucleolar protein shuttling between nucleoli and nucleoplasm, and nucleus and cytoplasm. NPM moves to the nucleoplasm after serum starvation[8] and after treatment with various DNA damaging or cytotoxic agents[40]; however, the role of this translocation is unknown. Kurki et al[11] reported that interaction between MDM2 (a negative regulator of p53) and nucleoplasmic NPM, translocated from nucleoli due to cellular stress, led to stabilization and possibly also activation of p53. We detected moderate to high levels of nucleoplasmic NPM in 51% (594/1160) of samples, but only 12% (71/594) of these samples showed high levels of p53, an indication of p53 stabilization. This suggests that the presence of NPM in the nucleoplasm *per se* is not sufficient to stabilize p53. Very high nucleoplasmic levels of NPM in the luminal epithelial cells of histologically normal breast samples indicate a normal non-stress induced nucleoplasmic localization for NPM in these cells. Probably additional effectors (eg, DNA damage) are required for the stabilization and activation of p53.

In the present study, patients with reduced NPM levels relative to histologically normal breast samples had poor prognosis. Notably, this prognostic value was detected especially in subgroups that conventionally have good prognosis (ER^+, $Her2^-$, and $p53^{-/low}$ tumors). Moreover, in the Cox proportional hazards model, NPM staining intensity showed independent prognostic value in the luminal A breast cancer subgroup (ER^+ and/or PrR^+, $HER\text{-}2^-$ cancers). In the future, NPM staining might serve as a useful marker to pinpoint patients with less favorable prognosis within these subgroups.

The present study also revealed a novel granular staining pattern for NPM, which appeared to be an independent prognostic factor of poor prognosis in the whole series of material. Of note, the granular staining pattern was also associated with known markers of poor prognosis (large tumor size, positive axillary lymph nodes, ductal histology, high grade, PgR negativity, and basal-like molecular subtype). Intriguingly, the granular staining pattern was a stronger prognostic factor in patients who received adjuvant chemotherapy, and it interacted significantly with chemotherapy in the Cox regression model. Taken together, these findings indicate that patients displaying granular staining pattern of NPM appear less likely to benefit from chemotherapy, suggesting a role for the normal NPM distribution in the chemotherapeutic response pathway.

Nuclear localization of NPM in the luminal epithelial cells of histologically normal breast samples was significantly different in women ≤42 versus >42 years of age. In the younger women, NPM showed higher expression and equal distribution throughout the nucleus; in the older women, NPM levels were lower, and were detected mostly in the nucleoli. NPM staining intensity was also an independent prognostic marker in patients with luminal A breast cancer subtype (ER^+ and/or PR^+, $HER\text{-}2^-$). Moreover, the granular staining pattern was associated significantly with survival in the hormone receptor-positive cancers. This suggests a hormonal contribution to the expression of NPM. In agreement with these observations, *NPM1* was among genes whose transcription was regulated by estrogen in breast cancer cells.[41,42] In the

present study, our *in vitro* results confirm the up-regulation of NPM protein by estrogen treatment in ER^+ breast cancer cells. We also found that increased NPM expression induced either by estrogen or lentiviruses affected NPM subcellular localization. In accord with the present findings, UV-induced up-regulation of NPM expression changes its localization from nucleolar to even nuclear distribution.[43] Nonetheless, there might be other determinants that affect localization of NPM, especially in cancer cells.

The role of NPM in oncogenesis is controversial, and both oncogenic and tumor suppressor functions have been implicated.[24] *NPM1* is translocated and mutated[25] or deleted[44] in many hematological disorders, making the NPM gene one of the most frequently genetically altered genes in these malignancies.[24] On the other hand, no amplifications of *NPM1* have been detected in human tumors, but deletion of the gene has been observed in non-small cell lung carcinomas.[45] Moreover, NPM is indispensable during development. Complete loss of NPM in mice is lethal at midgestation, and the embryos display genomic instability, widespread apoptosis, and activation of p53,[43,44] findings that uncover an essential developmental role for *NPM1* and implicate its functional loss in tumorigenesis. $NPM^{-/-}$ primary fibroblasts (mouse embryonic fibroblasts) show centrosome amplification, polyploidy, activation of p53, growth arrest, and senescence.[24,46] More importantly, NPM haploinsufficiency in $NPM^{+/-}$ mouse embryonic fibroblasts that mimic cancer cells harboring chromosomal rearrangements/deletions at the *NPM1* locus show an immortal phenotype with noticeably high proliferation rates.[47] Furthermore, in the $p53^{-/-}$ background, $NPM^{+/-}$ mouse embryonic fibroblasts have higher proliferation rates than $NPM^{+/+}$ mouse embryonic fibroblasts, and they are more susceptible to transformation by activated oncogenes, indicating that loss of one *Npm1* allele cooperates with oncogenes in transformation and tumorigenesis both *in vitro* and *in vivo*.[46] $Npm1^{+/-}$ mice show higher susceptibility for development of malignancies, especially of hematological and lymphoid origin, than their wild-type counterparts, indicating NPM as a haploinsufficient tumor suppressor.[29]

Here we show that reduced NPM protein expression was associated with poor prognosis and that over-expression of NPM in the MDA-MB-231 breast cancer cells abrogated their growth in soft agar, supporting a tumor suppressive function for NPM in breast cancer. Previously, high NPM expression has been associated with poor prognosis or recurrence in Ewing's sarcoma,[48] hepatocellular carcinoma,[34] bladder cancer,[49] prostate cancer,[28] colon carcinoma,[50] and gastric carcinoma.[51] Intriguingly, where data on NPM expression in the corresponding normal tissue was available in these studies,[28,34,50,51] it was markedly lower than in the cancer tissue (NPM expression in normal tissue was not reported for bladder cancer and Ewing's carcinoma). In the present study, high NPM levels were detected in the histologically normal luminal epithelial cells of the breast. Taken together, these results suggest that the expression level of NPM in non-neoplastic tissue might depict whether either high levels or low levels/absence of NPM in the cancer tissue correlates with the poor prognosis of patients, indicating a context-dependent role for NPM in neoplasia.

In summary, we have evaluated the role of NPM for the first time in a large series of breast cancer patients. Our results show that reduced NPM protein levels are associated with poor prognosis, supporting a role for NPM as a tumor suppressor in breast cancer. Our data also indicate a hormonal contribution to NPM expression and localization. Moreover, we have identified a completely novel staining pattern for NPM, a granular staining, which appears to be an independent indicator of poor prognosis in breast cancer. Notably, the granular staining pattern and chemotherapy showed interaction in the Cox regression model. Further study of NPM as a potential predictive factor is warranted.

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References

1. Borer RA, Lehner CF, Eppenberger HM, Nigg EA: Major nucleolar proteins shuttle between nucleus and cytoplasm. Cell 1989, 56:379–390
2. Wang D, Umekawa H, Olson MO: Expression and subcellular locations of two forms of nucleolar protein B23 in rat tissues and cells. Cell Mol Biol Res 1993, 39:33–42
3. Okuwaki M, Matsumoto K, Tsujimoto M, Nagata K: Function of nucleophosmin/B23, a nucleolar acidic protein, as a histone chaperone. FEBS Lett 2001, 506:272–276
4. Szebeni A, Olson MO: Nucleolar protein B23 has molecular chaperone activities. Protein Sci 1999, 8:905–912
5. Okuda M: The role of nucleophosmin in centrosome duplication. Oncogene 2002, 21:6170–6174
6. Herrera JE, Savkur R, Olson MO: The ribonuclease activity of nucleolar protein B23. Nucleic Acids Res 1995, 23:3974–3979
7. Savkur RS, Olson MO: Preferential cleavage in pre-ribosomal RNA by protein B23 endoribonuclease. Nucleic Acids Res 1998, 26:4508–4515
8. Chan PK, Aldrich M, Busch H: Alterations in immunolocalization of the phosphoprotein B23 in HeLa cells during serum starvation. Exp Cell Res 1985, 161:101–110
9. Kurki S, Peltonen K, Laiho M: Nucleophosmin. HDM2 and p53: players in UV damage incited nucleolar stress response. Cell Cycle 2004, 3:976–979
10. Colombo E, Marine JC, Danovi D, Falini B, Pelicci PG: Nucleophosmin regulates the stability and transcriptional activity of p53. Nat Cell Biol 2002, 4:529–533
11. Kurki S, Peltonen K, Latonen L, Kiviharju TM, Ojala PM, Meek D, Laiho M: Nucleolar protein NPM interacts with HDM2 and protects tumor suppressor protein p53 from HDM2-mediated degradation. Cancer Cell 2004, 5:465–475
12. Maiguel DA, Jones L, Chakravarty D, Yang C, Carrier F: Nucleophosmin sets a threshold for p53 response to UV radiation. Mol Cell Biol 2004, 24:3703–3711
13. Bertwistle D, Sugimoto M, Sherr CJ: Physical and functional interactions of the Arf tumor suppressor protein with nucleophosmin/B23. Mol Cell Biol 2004, 24:985–996

14. Brady SN, Yu Y, Maggi LB Jr, Weber JD: ARF impedes NPM/B23 shuttling in an Mdm2-sensitive tumor suppressor pathway. Mol Cell Biol 2004, 24:9327–9338
15. Itahana K, Bhat KP, Jin A, Itahana Y, Hawke D, Kobayashi R, Zhang Y: Tumor suppressor ARF degrades B23, a nucleolar protein involved in ribosome biogenesis and cell proliferation. Mol Cell 2003, 12:1151–1164
16. Swaminathan V, Kishore AH, Febitha KK, Kundu TK: Human histone chaperone nucleophosmin enhances acetylation-dependent chromatin transcription. Mol Cell Biol 2005, 25:7534–7545
17. Liu X, Liu Z, Jang SW, Ma Z, Shinmura K, Kang S, Dong S, Chen J, Fukasawa K, Ye K: Sumoylation of nucleophosmin/B23 regulates its subcellular localization, mediating cell proliferation and survival. Proc Natl Acad Sci USA 2007, 104:9679–9684
18. Tago K, Chiocca S, Sherr CJ: Sumoylation induced by the Arf tumor suppressor: a p53-independent function. Proc Natl Acad Sci USA 2005, 102:7689–7694
19. Sato K, Hayami R, Wu W, Nishikawa T, Nishikawa H, Okuda Y, Ogata H, Fukuda M, Ohta T: Nucleophosmin/B23 is a candidate substrate for the BRCA1-BARD1 ubiquitin ligase. J Biol Chem 2004, 279: 30919–30922
20. Beckmann R, Buchner K, Jungblut PR, Eckerskorn C, Weise C, Hilbert R, Hucho F: Nuclear substrates of protein kinase C. Eur J Biochem 1992, 210:45–51
21. Okuda M, Horn HF, Tarapore P, Tokuyama Y, Smulian AG, Chan PK, Knudsen ES, Hofmann IA, Snyder JD, Bove KE, Fukasawa K: Nucleophosmin/B23 is a target of CDK2/cyclin E in centrosome duplication. Cell 2000, 103:127–140
22. Peter M, Nakagawa J, Doree M, Labbe JC, Nigg EA: Identification of major nucleolar proteins as candidate mitotic substrates of cdc2 kinase. Cell 1990, 60:791–801
23. Pfaff M, Anderer FA: Casein kinase II accumulation in the nucleolus and its role in nucleolar phosphorylation. Biochim Biophys Acta 1988, 969:100–109
24. Grisendi S, Mecucci C, Falini B, Pandolfi PP: Nucleophosmin and cancer. Nat Rev Cancer 2006, 6:493–505
25. Falini B, Nicoletti I, Bolli N, Martelli MP, Liso A, Gorello P, Mandelli F, Mecucci C, Martelli MF: Translocations and mutations involving the nucleophosmin (NPM1) gene in lymphomas and leukemias. Haematologica 2007, 92:519–532
26. Feuerstein N, Spiegel S, Mond JJ: The nuclear matrix protein, numatrin (B23), is associated with growth factor-induced mitogenesis in Swiss 3T3 fibroblasts and with T lymphocyte proliferation stimulated by lectins and anti-T cell antigen receptor antibody. J Cell Biol 1988, 107:1629–1642
27. Feuerstein N, Chan PK, Mond JJ: Identification of numatrin, the nuclear matrix protein associated with induction of mitogenesis, as the nucleolar protein B23. Implication for the role of the nucleolus in early transduction of mitogenic signals. J Biol Chem 1988, 263:10608–10612
28. Subong EN, Shue MJ, Epstein JI, Briggman JV, Chan PK, Partin AW: Monoclonal antibody to prostate cancer nuclear matrix protein (PRO: 4–216) recognizes nucleophosmin/B23. Prostate 1999, 39:298–304
29. Sportoletti P, Grisendi S, Majid SM, Cheng K, Clohessy JG, Viale A, Teruya-Feldstein J, Pandolfi PP: Npm1 is a haploinsufficient suppressor of myeloid and lymphoid malignancies in the mouse. Blood 2008, 111:3859–3862
30. Lundin J, Lundin M, Isola J, Joensuu H: A web-based system for individualised survival estimation in breast cancer. BMJ 2003, 326:29
31. Joensuu H, Isola J, Lundin M, Salminen T, Holli K, Kataja V, Pylkkänen L, Turpeenniemi-Hujanen T, von Smitten K, Lundin J: Amplification of erbB2 and erbB2 expression are superior to estrogen receptor status as risk factors for distant recurrence in pT1N0M0 breast cancer: a nationwide population-based study. Clin Cancer Res 2003, 9:923–930
32. Calvo KL, Ojeda MJ, Ammatuna E, Lavorgna S, Ottone T, Targovnik HM, Lo-Coco F, Noguera NI: Detection of the nucleophosmin gene mutations in acute myelogenous leukemia through RT-PCR and polyacrylamide gel electrophoresis. Eur J Haematol 2009, 82:69–72
33. Pfaffl MW: A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001, 29:e45
34. Yun JP, Miao J, Chen GG, Tian QH, Zhang CQ, Xiang J, Fu J, Lai PB: Increased expression of nucleophosmin/B23 in hepatocellular carcinoma and correlation with clinicopathological parameters. Br J Cancer 2007, 96:477–484
35. Simon R, Panussis S, Maurer R, Spichtin H, Glatz K, Tapia C, Mirlacher M, Rufle A, Torhorst J, Sauter G: KIT (CD117)-positive breast cancers are infrequent and lack KIT gene mutations. Clin Cancer Res 2004, 10:178–183
36. Sørlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Eystein Lønning P, Børresen-Dale AL: Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci USA 2001, 98:10869–10874
37. Sørlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, Deng S, Johnsen H, Pesich R, Geisler S, Demeter J, Perou CM, Lønning PE, Brown PO, Børresen-Dale AL, Botstein D: Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci USA 2003, 100:8418–8423
38. Carey LA, Perou CM, Livasy CA, Dressler LG, Cowan D, Conway K, Karaca G, Troester MA, Tse CK, Edmiston S, Deming SL, Geradts J, Cheang MC, Nielsen TO, Moorman PG, Earp HS, Millikan RC: Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. JAMA 2006, 295:2492–2502
39. Sihto H, Lundin J, Lehtimäki T, Sarlomo-Rikala M, Bützow R, Holli K, Sailas L, Kataja V, Lundin M, Turpeenniemi-Hujanen T, Isola J, Heikkilä P, Joensuu H: Molecular subtypes of breast cancers detected in mammography screening and outside of screening. Clin Cancer Res 2008, 14:4103–4110
40. Yang C, Maiguel DA, Carrier F: Identification of nucleolin and nucleophosmin as genotoxic stress-responsive RNA-binding proteins. Nucleic Acids Res 2002, 30:2251–2260
41. Cicatiello L, Scafoglio C, Altucci L, Cancemi M, Natoli G, Facchiano A, Iazzetti G, Calogero R, Biglia N, De Bortoli M, Sfiligoi C, Sismondi P, Bresciani F, Weisz A: A genomic view of estrogen actions in human breast cancer cells by expression profiling of the hormone-responsive transcriptome. J Mol Endocrinol 2004, 32:719–775
42. Skaar TC, Prasad SC, Sharareh S, Lippman ME, Brünner N, Clarke R: Two-dimensional gel electrophoresis analyses identify nucleophosmin as an estrogen regulated protein associated with acquired estrogen-independence in human breast cancer cells. J Steroid Biochem Mol Biol 1998, 67:391–402
43. Wu MH, Yung BY: UV stimulation of nucleophosmin/B23 expression is an immediate-early gene response induced by damaged DNA. J Biol Chem 2002, 277:48234–48240
44. Westbrook CA, Hsu WT, Chyna B, Litvak D, Raza A, Horrigan SK: Cytogenetic and molecular diagnosis of chromosome 5 deletions in myelodysplasia. Br J Haematol 2000, 110:847–855
45. Mendes-da-Silva P, Moreira A, Duro-da-Costa J, Matias D, Monteiro C: Frequent loss of heterozygosity on chromosome 5 in non-small cell lung carcinoma. Mol Pathol 2000, 53:184–187
46. Colombo E, Bonetti P, Lazzerini Denchi E, Martinelli P, Zamponi R, Marine JC, Helin K, Falini B, Pelicci PG: Nucleophosmin is required for DNA integrity and p19Arf protein stability. Mol Cell Biol 2005, 25:8874–8886
47. Grisendi S, Bernardi R, Rossi M, Cheng K, Khandker L, Manova K, Pandolfi PP: Role of nucleophosmin in embryonic development and tumorigenesis. Nature 2005, 437:147–153
48. Kikuta K, Tochigi N, Shimoda T, Yabe H, Morioka H, Toyama Y, Hosono A, Beppu Y, Kawai A, Hirohashi S, Kondo T: Nucleophosmin as a candidate prognostic biomarker of Ewing's sarcoma revealed by proteomics. Clin Cancer Res 2009, 15:2885–2894
49. Tsui KH, Juang HH, Lee TH, Chang PL, Chen CL, Yung BY: Association of nucleophosmin/B23 with bladder cancer recurrence based on immunohistochemical assessment in clinical samples. Acta Pharmacol Sin 2008, 29:364–370
50. Nozawa Y, Van Belzen N, Van der Made AC, Dinjens WN, Bosman FT: Expression of nucleophosmin/B23 in normal and neoplastic colorectal mucosa. J Pathol 1996, 178:48–52
51. Tanaka M, Sasaki H, Kino I, Sugimura T, Terada M: Genes preferentially expressed in embryo stomach are predominantly expressed in gastric cancer. Cancer Res 1992, 52:3372–3377