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Altered Profiles in Nuclear Matrix Proteins Associated with DNA *in Situ* during Progression of Breast Cancer Cells[1]

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ABSTRACT

Nuclear matrix proteins (NMPs) show promise as informative biomarkers in following the pathogenesis of breast cancer. The nuclear matrix is a dynamic RNA-protein network involved in the organization and expression of chromatin. Cisplatin, which preferentially cross-links nuclear matrix proteins to DNA *in situ*, may be used to identify NMPs that organize and/or regulate the processing of DNA. In this study, we analyzed the nuclear matrix proteins from an estrogen receptor-positive breast cancer cell line panel consisting of MCF-7, MIII, LCC1, and LCC2 cell lines. This cell line panel reflects the stages of malignant progression in breast cancer. Proteins isolated from nuclear matrices and proteins cross-linked to nuclear DNA *in situ* with cisplatin were analyzed by two-dimensional gel electrophoresis. Specific changes in nuclear matrix proteins bound to nuclear DNA were identified. In concordance with estrogen independence and antiestrogen insensitivity, a loss in cisplatin cross-linking of specific NMPs to nuclear DNA was observed. Our results suggest that progression of breast cancer is accompanied by a reorganization of chromosomal domains, which may lead to alterations in gene expression.

INTRODUCTION

The most common form of cancer among women is breast cancer. Although it is the second leading cause of cancer mortality among females, the pathogenesis of the disease remains unclear (1, 2). Most human breast cancers originate as hormonally dependent tumors, and ~60% of ER[3]-positive human breast tumors respond to antiestrogen or other endocrine therapies (3, 4). In the malignant progression of breast cancer, breast tumors progress from hormone-dependent growth to a more aggressive phenotype characterized by hormone-independent growth, resistance to endocrine therapy, and a frequently widespread metastases (5, 6). Current evidence suggests that ER-positive breast cancer cells do not acquire an ER-negative phenotype during breast cancer progression; rather, ER is a stable phenotype in breast cancer cells (7). Over 30% of all human breast tumors expressing both ERs and progesterone receptors fail to regress after antiestrogen treatment. This would suggest that loss of hormone dependence in breast cancer cells is a crucial step in the development of breast cancer (8).

A cellular landmark in the pathological diagnosis of cancer is the nucleus. Abnormal nuclear shape is used as a pathological marker for a transformed cell (9). The nuclear matrix has a role in determining nuclear shape (10). The nuclear matrix is a dynamic RNA-protein network that organizes chromosome territories (11) and provides a platform for the assembly of protein machines (*e.g.*, replication and transcription factories) involved in the processing of the genetic information (12, 13). Proteins and enzymes involved in transcription, chromatin modification, replication, and RNA splicing are targeted to specific nuclear matrix sites (14–18). The organization and composition of the nuclear matrix are dynamic, changing with nuclear activity (19–21). NMPs, some of which are tissue and cell type specific, are altered with transformation and state of differentiation. They have been identified as informative markers of disease states (22–24). Informative NMPs have been identified for bladder, breast, colon, prostate, and head and neck cancers (22–27). In an analysis of NMPs from human breast cancer cell lines, specific changes were identified in the NMPs of hormone-dependent and hormone-independent human breast cancer cells (28).

Previously, we used the cross-linker cisplatin to identify NMPs bound to nuclear DNA *in situ*. Cisplatin preferentially cross-links NMPs to nuclear DNA (29). In contrast, the abundant nucleosomal histones are poorly cross-linked to DNA with cisplatin (30, 31). Thus, the cisplatin cross-linking protocol is useful in the identification of NMPs involved in the organization and function of nuclear DNA (29, 32). Our studies have revealed nuclear matrix-bound transcription factors that are bound to DNA *in situ* and have shown that the intermediate filament protein, cytokeratin, is associated with nuclear DNA in breast cancer cells (32, 33). Through application of this cross-linking protocol, NMPs that were differentially associated with nuclear DNA in ER-positive/hormone-dependent and ER-negative/hormone-independent human breast cancer cells were identified (34).

In this study, we analyzed the NMPs and proteins cross-linked to DNA *in situ* in a cell line panel that reflects the stages of progression in breast cancer (35). The cell line series consists of MCF-7 (parent), MIII, LCC1, and LCC2. MIII and LCCI human breast cancer cells are ER positive, invasive, metastatic, estrogen independent, estrogen responsive, and antiestrogen sensitive. LCC2 is ER positive, invasive, metastatic, estrogen independent, estrogen responsive, tamoxifen resistant, and ICI 182,780 sensitive (35, 36). Using this panel of cells as a model for progression in breast cancer, the purpose of this study was to identify NMPs and DNA-associated NMPs that are informative in breast cancer progression.

MATERIALS AND METHODS

Cell Culture. The human breast carcinoma cell lines used in this study were MCF-7, MIII, LCC1, and LCC2 (35). All cell lines were maintained at 37°C (humidified atmosphere, 5% CO_2/95% air) on 150 × 20-mm tissue culture dishes (Nunc). MCF-7 was cultured in medium containing DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 1% (v/v) L-glutamine, 1% (v/v) glucose, 1% (v/v) penicillin/streptomycin, and 5% (v/v) FCS (Life Technologies, Inc.). MIII, LCC1, and LCC2 were cultured in medium containing DMEM-phenol red free (Sigma Chemical Co., St. Louis, Missouri), 5% (v/v) twice charcoal-stripped FCS and supplemented as mentioned above. At ~90% confluence, cells were scraped from the plates and frozen as pellets containing 1×10^7 cells at −70°C.

Isolation and Analysis of NMPs and Proteins Cross-Linked to DNA *in Situ*. Nuclear matrices were isolated from the breast cancer cell lines as described previously (28). Intermediate filament proteins were removed from the NMP preparation (37). Analysis of the NMPs of each cell line was done at least three times. DNA-protein cross-linking was performed as described previously (33). Briefly, MCF-7 cells at a density of 1×10^6 cells/ml were

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[3] The abbreviations used are: ER, estrogen receptor; NMP, nuclear matrix protein; XLP, proteins cross-linked to DNA *in situ* with cisplatin; hnRNP, human nuclear ribonucleoprotein.

resuspended in Hanks' buffer containing sodium acetate instead of NaCl at the same concentration. The cells were incubated with 1 mM cisplatin at 37°C for 2 h with gentle shaking. After this incubation, cells were treated with lysis buffer (5 M urea, 2 M guanidine-HCl, 2 M NaCl, and 0.2 M potassium phosphate, pH 7.5). Hydroxylapatite (4 g/20 A_{260} units of lysate; Bio-Rad, Richmond, CA) was then added. The hydroxylapatite resin was washed with lysis buffer to remove RNA and proteins not cross-linked to DNA. To reverse the cross-linking, the hydroxylapatite was incubated in lysis buffer containing 1 M thiourea instead of 5 M urea. By doing so, the proteins were released from hydroxylapatite, whereas the DNA remained bound. Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad) with BSA as a standard.

Two-Dimensional Gel Electrophoresis. Two-dimensional PAGE was performed as described previously (38). Gels were stained with silver using the Pharmacia Silver Stain kit (Amersham Pharmacia Biotech) and then dried between sheets of gel drying film (Promega Corp.) at room temperature. Stained gels were scanned using a PDI 325OE densitometer (PDI, Huntington Station, NY), and the data were analyzed with Image Master software (Amersham Pharmacia Biotech). Molecular masses and isoelectric points were determined using two-dimensional SDS-PAGE standards (Bio-Rad and Amersham Pharmacia BioTech) and carbamylated carbonic anhydrase (Amersham Pharmacia BioTech). All studies were carried out using three preparations from each cell line.

RESULTS

NMPs and proteins cross-linked to DNA *in situ* were isolated from MCF-7, MIII, LCC1, and LCC2 cells. The proteins were analyzed by two-dimensional gel electrophoresis. To compare the proteins in the various gel patterns, several exogenous proteins were used to align protein patterns. Carbamylated carbonic anhydrase (30 kDa; pI 4.8–6.7) served as a reference for determining the molecular mass and isoelectric point of the proteins. Two-dimensional SDS-PAGE standards were used to determine the molecular mass of the proteins.

Representative silver-stained two-dimensional gel patterns of NMPs and proteins cross-linked to DNA *in situ* with cisplatin (XLP) isolated from the various cell lines are shown in Figs. 1 and 2, respectively (see Fig. 3 for a schematic of the two-dimensional gel pattern data). Many of the proteins were common to the NMP and XLP preparations both within each cell line and between the four cell lines. For example, hnRNP K, which was identified according to its molecular mass and pI coordinates, was found in all patterns. However, differences in the protein patterns were noted.

Proteins displaying different levels in the two-dimensional gel patterns were catalogued into three groups: those found in NMP but not in XLP patterns; those detected in NMP and XLP patterns; and those seen in XLP but not NMP patterns. Few NMPs were placed into group 1 because most NMPs were also found in the XLP patterns. The sole group 1 entry NMP1, which has a molecular mass of 79 kDa and pI of 4.85, was present in MCF-7 and MIII cells but not in LCC1 or LCC2 cells.

Among the group 2 proteins were those showing differences in both NMP and XLP patterns, those displaying differences in NMP but not XLP patterns, and those showing variations in the XLP but not NMP patterns. In the NMP fractions, NMP8 was at higher abundance in LCC1 than in MCF-7, MIII, or LCC2. However, the abundance of NMP8 did not vary significantly in the XLP patterns (Table 1). NMP5 showed a progressive decrease in abundance in the NMP and XLP preparations from MIII, LCC1, and LCC2, respectively (Table 1). Among the XLP patterns, NMP10 and NMP15 were found to progressively decline in abundance in the MIII, LCC1, and LCC2 preparations (see Fig. 2 and Table 1). However, in NMP patterns, NMP10 and NMP15 could only be detected in the LCC1 cell line. NMPs 12 and 14 were at higher concentrations in the XLP preparations from MIII than in those of MCF-7, LCC1, and LCC2. Furthermore, NMP6 and NMP7 displayed a decrease in abundance in the LCC2 pattern when compared with MIII and LCC1 preparations. The levels of NMPs 2, 3, and 4 were greatest in the XLP pattern of LCC2 compared with those of MIII and LCC1. Furthermore, NMP13 showed a progressive increase in abundance over the cell line panel.

The third group of proteins, which were detected in the XLP patterns but not in the NMP patterns, consisted of five proteins (Table 2). Fig. 2 shows that XLP1 was prominent in the MIII and LCC2 XLP

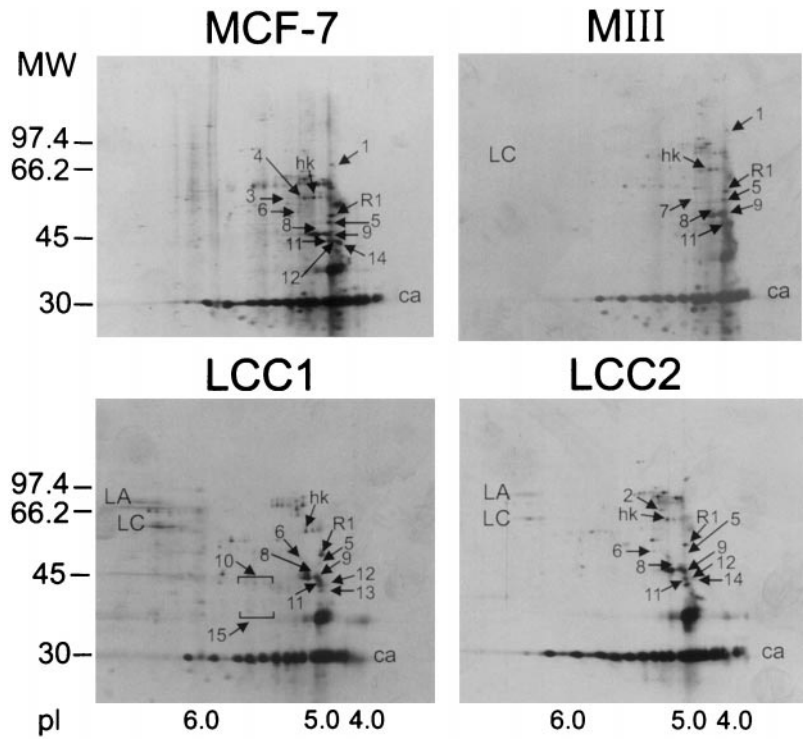

Fig. 1. NMPs of MCF-7, MIII, LCC1, and LCC2 breast cancer cell lines. NMPs (90 μg) were electrophoretically resolved on two-dimensional gels. The gels were stained with silver. *ca*, position of the carbamylated forms of carbonic anhydrase. The position of the molecular mass standards (kDa) is shown to the *left* of the two-dimensional gel patterns. *LA* and *LC*, positions of lamins A and C, respectively. *hK*, position of transcription factor hnRNP K. *R1*, protein displaying similar levels among the four cell lines studied.

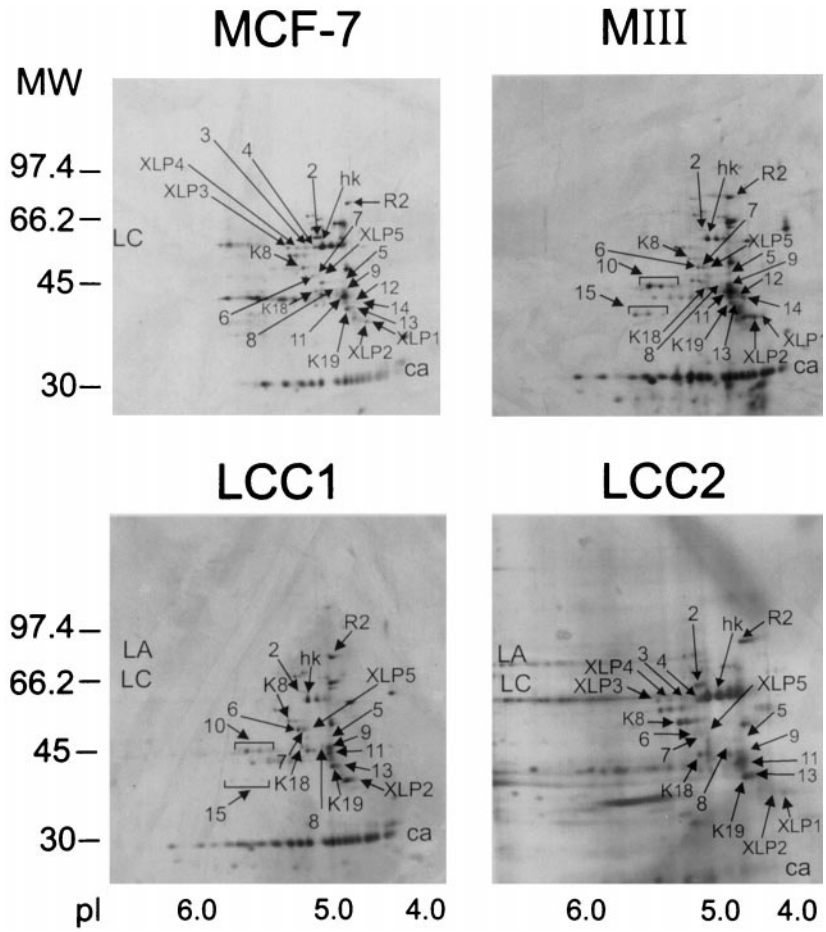

Fig. 2. Proteins cross-linked to DNA by cisplatin *in situ* in MCF-7, MIII, LCC1, and LCC2 breast cancer cell lines. Eighty μg of DNA cross-linked proteins from cells treated with 1 mM cisplatin were electrophoretically resolved on two-dimensional gels. The gels were stained with silver. *ca*, position of the carbamylated forms of carbonic anhydrase. The position of the molecular mass standards (kDa) is shown to the *left* of the two dimensional gel patterns. *LA* and LC, positions of lamins A and C, respectively. *cK8, cK18*, and *cK19*, cytokeratins 8, 18, and 19, respectively. *hK*, position of transcription factor hnRNP K. *R2*, protein displaying similar levels among the four cell lines studied.

patterns but not in the patterns of the other cells. Likewise, XLP2 was abundant in the MIII pattern compared with the other cell lines. The relative levels of XLP5 progressively decreased in the XLP patterns of MIII, LCC1, and LCC2 cells, respectively. In contrast, the levels of XLP3 and XLP4 gradually increased in the MIII, LCC1, and LCC2 XLP patterns, respectively.

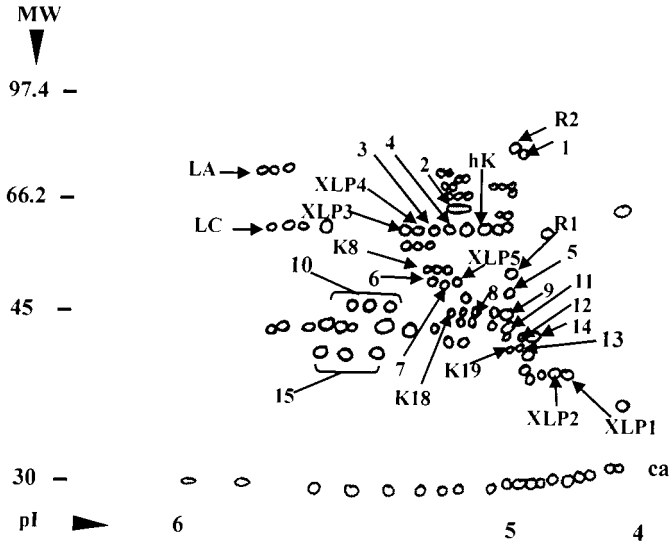

Fig. 3. A schematic representation of the two-dimensional gel pattern data of DNA cross-linked and non-DNA cross-linked proteins from MCF-7, MIII, LCC1, and LCC2 breast cancer cell lines. The position of the molecular mass standards (kDa) is shown to the *left* of the two-dimensional gel patterns. See legends to Figs. 1 and 2 for designation of *ca, LA, LC, hK, cK8, cK18, cK19, R1*, and *R2*.

Cytokeratins 8, 18, and 19 were among the more abundant proteins cross-linked to DNA by cisplatin. Although these proteins were seen in the XLP preparations of the four cell lines, their abundance was consistently lower in the MIII preparations (Fig. 2). The levels of these three intermediate filament proteins progressively increased in the LCC1 and LCC2 XLP preparations.

When comparing the XLP patterns of MIII, LCC1, and LCC2 preparations, a progressive increase in the levels of lamins A and C was also observed (Fig. 2). The levels of lamins A and C were prominent in the XLP patterns of LCC2 cells, whereas lamin C and to a lesser extent lamin A was observed at lower levels in the LCC1 XLP patterns.

DISCUSSION

In the progression of breast cancer, ER-positive breast epithelial cells no longer require estrogens for growth and gain resistance to antiestrogens. The cell line series MIII, LCC1, and LCC2 provide a model system to study breast cancer progression. In comparing the MIII, LCC1, and LCC2 NMPs that were cross-linked to nuclear DNA *in situ*, we observed a selective and progressive change in the interaction of NMPs with nuclear DNA. Among this group of NMPs, a loss in cisplatin cross-linking of the NMPs to nuclear DNA was found. The loss of cisplatin cross-linking may be a consequence of reduced NMP levels, loss of contact with DNA, and/or rearrangement of the NMP relative to DNA such that the NMP is positioned further than 4 Å from nuclear DNA (34).

Table 1 *Relative levels of DNA cross-linked nuclear matrix proteins in two-dimensional patterns of DNA cross-linked protein preparations*

Levels of each protein were assessed based on their relative expression to hnRNP K and R2. ++++, ++, and + designate nuclear matrix proteins present at high, moderate and low levels, respectively. +/− represents proteins present at very low levels in one or more preparations, whereas − designates proteins not detected in all preparations.

			MCF-7	MIII	LCC1	LCC2
Cell line	Mass (kDa)	pI	Estrogen-dependent Estrogen-responsive Anti-estrogen-sensitive	Invasive Metastatic Estrogen-independent Estrogen-responsive Anti-estrogen-sensitive	Invasive Metastatic Estrogen-independent Estrogen-responsive Anti-estrogen-sensitive	Invasive Metastatic Estrogen-independent Estrogen-responsive Tamoxifen-resistant ICI-sensitive
NM proteins						
2	61	5.2–5.3	++++	+	+	+++
3	60	5.35	++	−	+/−	++
4	60	5.25	+++	−	−	+++
5	52	5	+/−	+++	+	+/−
6	50	5.3	++	+++	+++	+
7	50	5.25	++	+++	+++	+
8	46	5.2	+	+	+	+/−
9	45	5	+++	++++	++++	++
10	44	5.45–5.7	+/−	++++	+++	+/−
11	44	5	++++	+++++	++++	++++
12	42.5	4.85	+/−	+++	+/−	+/−
13	42	4.85	+/−	+	++	+++
14	42	4.75–4.8	+/−	++	−	+/−
15	39	5.5–5.75	+/−	+++	+	+/−
NMP 5 levels in NM						
5	52	5	+++++	+++	++	+

A parallel change in the abundance of a NMP in the NMP and XLP preparations was noted, but the incidence of this observation was infrequent. For example, NMP5 was less abundant in the LCC1 and LCC2 NMP and XLP preparations compared with the respective MIII preparations. For the remainder of the NMPs exhibiting a change in abundance in the XLP preparations, parallel changes in NMP levels were not found. As examples, NMP10 and NMP15 showed progressive declines in the XLP preparations in the MIII, LCC1 and LCC2 cells, but these changes in NMP10 and NMP15 levels were not observed in the NMP preparations of these cells. The absence of NMP10 and NMP15 in the NMP preparation from MIII cells suggests that although both proteins are associated with DNA, they are either not associated with the nuclear matrix or have a low affinity for the nuclear matrix. In LCC1 cells, NMP10 and NMP15 are associated with the nuclear matrix and DNA. It is conceivable that recruitment by NMP10 and NMP15 of specific DNA sequences to the nuclear matrix of LCC1 cells has functional implications in gene repression or activation (39), which may be involved in progression of human breast cancer.

For five XLPs showing variations in abundance in the cell line preparations, the corresponding protein could not be detected in the nuclear matrix preparation. In the preparation of NMPs, intermediate filament proteins and proteins that are able to assemble into insoluble structures are removed (37). However, this procedure may remove cytoskeletal and perhaps NMPs that are DNA binding proteins. The cytokeratins are examples of DNA attached proteins that are removed from NMP preparations. Thus, the cisplatin cross-linking procedure complements conventional methods to analyze NMPs (40).

Table 2 *Relative levels of differentially abundant DNA cross-linked proteins in two-dimensional patterns of DNA-cross-linked protein preparations*

Levels of DNA-cross-linked proteins were assessed based on their relative expression to hnRNP K and R2. The level of NMP5 within NMP patterns was assessed based on its relative expression to hnRNP K and R1. ++++, ++, and + designate DNA cross-linked proteins present at high, moderate, and low levels, respectively. +/− represents proteins present at very low levels in one or more preparations, whereas − designates proteins not detected in all preparations.

Cell line	Mass (kDa)	pI	MCF-7	MIII	LCC1	LCC2
XLP1	37	4.5	+/−	++	−	++
XLP2	37	4.6	++	+++	+	+
XLP3	60	5.6	++	−	+/−	+
XLP4	60	5.45	++	−	+/−	+
XLP5	50	5.2	+/−	++	+	+/−

In contrast to decreased cisplatin cross-linking of NMPs to DNA in parallel with progression, lamins A and C and cytokeratin cross-linking to nuclear DNA was enhanced. These observations suggest that rearrangements in chromosomal domains have occurred such that contacts between lamins and cytokeratins at the nuclear periphery and chromosomal domains have been augmented. The chromosomal regions in contact with lamins or cytokeratins are not known. Heterochromatin, which is located at the nuclear periphery, would be expected to make contacts with lamins and cytokeratins (41). However, transcriptionally active chromatin regions are sometimes located at or near the nuclear periphery and may be in contact with these proteins (42).

We analyzed previously the proteins cross-linked to DNA *in situ* in T-47D5 and T5-PRF cell lines (34). The T5-PRF is an ER-positive, estrogen-independent cell line that was developed from the T-47D5 parent cell line by chronically depleting the cells of estrogen in long-term culture (43). Thus, similar to the MCF-7, MIII, LCC1, and LCC2 cell line panel, the T-47D5 and T5-PRF cell lines reflect stages of progression in human breast cancer. Compared with the parent cell line, NMP5, NMP6, NMP10, XLP1, and XLP2 increased, whereas XLP3 and XLP4 decreased among the proteins cross-linked to DNA in T5-PRF cells (34). Furthermore, lamin A and cytokeratins 8, 18, and 19 cross-linking to nuclear DNA was enhanced in the T5-PRF cell line (33, 34). Thus, our observed changes in patterns of proteins cross-linked to DNA in the MCF-7, MIII, LCC1, and LCC2 cell line panel are also found in the T-47D5 and T5-PRF cell line panel.

In conclusion, our study provides evidence that breast cancer progression of ER-positive cells is accompanied with a loss of contacts between chromatin and NMPs and an augmentation of protein-nuclear DNA contacts between lamins and intermediate filament proteins at the nuclear periphery. Whether these changes are driven by alterations in NMP and/or chromosomal domain positioning remains to be determined. The function and identity of NMPs exhibiting alterations in cross-linking with nuclear DNA as a function of progression (*e.g.*, NMP5) will be the focus of future studies.

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