BARX2 Induces Cadherin 6 Expression and Is a Functional Suppressor of Ovarian Cancer Progression

Grant C. Sellari, Li Li, Karen P. Watt, Barry D. Nelkin, Genevieve J. Rabiesz, Euan A. Stronach, Eric P. Miller, David J. Porteous, John F. Smyth, and Hani Gabra

Imperial Cancer Research Fund Medical Oncology Unit and University of Edinburgh Department of Clinical Oncology [G. C. S., L. L., K. P. W., G. J. R., E. A. S., E. P. M., J. F. S., H. G.] and Medical Genetics Section, Department of Medical Sciences [D. J. P.], University of Edinburgh Molecular Medicine Centre, Western General Hospital, Edinburgh EH4 2XU; United Kingdom, and Oncology Centre, Johns Hopkins University School of Medicine, Baltimore, Maryland 21231 [B. D. N.]

Abstract

The human homeobox gene BARX2 is located at 11q24-q25, within a minimal region associated with frequent loss of heterozygosity and adverse survival in epithelial ovarian cancer. BARX2 is a transcription factor that regulates transcription of specific cell adhesion molecules in the mouse. We show that BARX2 and cadherin 6 are expressed in normal human ovarian surface epithelium. BARX2 and cadherin 6 both have significantly lower expression in a clinical sample of endometrioid and clear cell ovarian cancers, as compared with serous or mixed mesodermal tumors. In a series of ovarian cancer cell lines, BARX2 expression showed a significant direct correlation with cadherin 6 expression. In OAW42, an ovarian cancer cell line that does not endogenously express BARX2, in vitro transfection of human BARX2 cDNA induced cadherin 6 expression. Transfection of BARX2 into OAW42 inhibited Matrigel invasion, haptotactic cellular migration to a collagen IV signal, and adhesion to collagen IV-coated plates. Our data demonstrate that BARX2 is expressed in the ovarian surface epithelium and has functional suppressor properties in ovarian cancer cells.

Introduction

EOC is the most common cause of death from gynecological malignancy. EOC arises as a result of multiple genetic lesions accumulated by the ovarian surface epithelium, resulting in either activation of the neoplastic state (oncogenes) or inactivation of genes that maintain the nonneoplastic state (TSGs). Inactivation of TSGs is a frequent and important process in neoplasia, and regions of the genome that contain such genes may be indicated by either cytogenetic or LOH analyses in tumors.

Many regions that contain a high frequency of LOH have been described in EOC, indicating that multiple genes may be inactivated during the initiation or progression of the disease. Frequent LOH of a critical 8.5-Mb interval between D11S934 and D11S1320 has been associated with poor survival (1, 2), suggesting that the putative TSG in this region acts as a suppressor of ovarian cancer progression.

BARX2 is located at 11q24-q25, within this critical LOH region (3, 4). The human gene encodes a 254 amino acid homeodomain transcription factor (4) homologous with the Drosophila Bar class of homeobox-domain-containing proteins. Human BARX2 was identified as a transcription factor that mediates ras/raf dependent transcription of the calcitonin gene via a ras/raf responsive promoter element (4). Clinically, down-regulated expression of the calcitonin gene correlates with poor differentiation of thyroid carcinoma cells and also with adverse patient survival (5). Therefore, loss of BARX2 expression may be involved in tumor progression.

Mouse Barx2 was originally isolated in an assay to identify proteins that bind to the consensus ATTA-containing homeodomain binding site sequence found in the regulatory site of CAM genes (6). It was demonstrated that the L1-170 CAM gene promoter could be activated after cotransfection with Barx2 and that the CCATTAGPyGA motif within the L1 promoter was sufficient for this activation. Deletion of the homeodomain-binding site in Barx2 actually resulted in repression of transcription, suggesting a bifunctional role for Barx2 in the regulation of specific CAMs (7). Binding of Barx2 to the AMP-responsive element binding protein CREB1 and activating transcription factor-1 was also demonstrated, suggesting a possible mechanism through which it mediates transcriptional regulation of specific target genes.

The object of this study was to define whether BARX2 has a role in ovarian cancer progression. We present evidence that BARX2 is expressed in the normal ovarian surface epithelium and that expression of BARX2 is significantly lower in the CC and EM group of ovarian cancers. We show that there is a direct correlation between BARX2 expression and cadherin 6 expression in ovarian cancer cell lines. We also present evidence that BARX2 induces cadherin 6 expression in OAW42 and is a suppressor of tumor progression as defined by functional assays of migration, invasion, and adhesion.

Materials and Methods

Expression Analysis

Total RNA Extraction. Total RNAs from human ovarian cancer cell lines and from primary HOSE were extracted using TRI Reagent (Sigma Chemical Co., Poole, United Kingdom) as per the manufacturer’s protocol. Normal primary HOSE samples, as confirmed by a pathologist, were stripped per-operatively from nine independent noncancerous ovaries from patients undergoing total hysterectomy and unilateral salpingo-oophorectomy for uterine leiomyoma. Contaminating genomic DNA was removed by DNase-I digestion. DNase-I-treated total RNA extracted from a histologically reviewed panel of primary human ovarian tumors was provided by Dr. Simon Langdon (Imperial Cancer Research Fund, Edinburgh, United Kingdom).

RT-PCR Analysis. Oligodeoxynucleotide-primed first-strand cDNA was prepared from 1 μg of DNase-I-treated total RNAs using the 1st Strand cDNA Synthesis Kit (Roche Diagnostics, Ltd., Lewes, United Kingdom) as per the manufacturer’s protocol. Control reactions minus reverse transcriptase were also performed. Aliquots (2 μl) of first-strand cDNA, either prepared as described or alternatively commercially available first-strand cDNAs prepared from adult human multiple normal tissue types (BD CLONTECH, Basingstoke, United Kingdom), were used as a template in 25-μl RT-PCR reactions using the following primer sets and amplification conditions, as appropriate.
BARX2 OVEREXPRESSION IN OVARian CANCer CELL LINE OAW42

BARX2 Overexpression. Plasmid DNA from the full-length human BARX2 cDNA (4) cloned into pBABE Hygro (conferring hygromycin resistance) expression vector (pBABE BARX2) was prepared by standard methods (QIagen, Ltd., Crawley, United Kingdom) and linearized by Ncol digestion before transfecion into cell lines. As a control, Ncol-linearized pBABE Hygro parental vector was also transfected into cell lines.

pBABE BARX2-derived expression was specifically detected in OAW42 BARX2 transfectant clones using an RT-PCR assay consisting of a sense primer located in the 3′ untranslated region of BARX2 and an antisense primer designed against sequence from the expression construct that is incorporated in the construct-derived transcript and is not present in the endogenous transcript. The PCR primers used to specifically detect construct-derived BARX2 expression are as follows: Sense: 5′-CGGGCGAGACTTCACCG-3′ (BARX2 AF031924 nuc 1035–1054); antisense: 5′-GTGACGCTATGAAAGCT-3′ (pBABE BARX2 vector-specific sequence), 300-bp cDNA product.

Cell Cycle. Cell lines were maintained in RPMI 1640 or DMEM/heat-inactivated FCS (10%)/l-glutamine (2 mM)/penicillin (100 units/ml)/streptomycin (100 μg/ml) with selective media (hygromycin B and G418 as appropriate) at 37°C in a humidified atmosphere of 5% CO2 in air. Transfected and nontransfected cells were harvested from log phase cultures and prepared as single cell suspensions for use in all functional assays detailed below.

Matrix Invasion Assay. Matrix invasion chambers (Biocoat; Becton Dickinson Labware Europe, Le Pont-De-Clair, France) were prehydrated according to the manufacturer’s recommendations. Cells (50; washed in protease inhibitor stripped 10% FCS DMEM) were dispensed into the top compartment and incubated in a humidified incubator for 48 h. The number of cells on the top and undersurface of the porous membrane was assessed using the MTT assay (8).

Transwell Migration Assay. Cells (5 × 104; 100 μl) prepared in serum-free RPMI were added to 400-μl serum-free RPMI in the top compartment of 8-μm transwell culture inserts (Costar; Western Laboratory Supplies, Aldershot, United Kingdom), the undersides of which were coated with either BSA or collagen IV. The cells were then incubated for 24 h. The number of cells present in the top and bottom compartments was then determined using the MTT assay (8).

Adhesion Assay. Cells (5 × 104) prepared in serum-free RPMI were transferred to uncoated and collagen-coated wells of 24 well trays and incubated for 24 h. The media were then gently removed, and the plates were washed three times in serum-free media. The number of cells on the wells was assessed using the MTT assay (8).

Statistical Analysis for Functional Assays. Comparison of differences between OAW42 and transfected cell lines was performed using the Kruskal-Wallis nonparametric method. Where appropriate, additional statistical analysis using Dunn’s multiple comparison test was performed to determine which cell lines were significantly different.

Cell Cycle and Annexin-V FACS Analysis. Single cell suspensions for DNA analysis were prepared by standard protocols (9). Nuclei were extracted, fixed, and stained with PI and analyzed using a FacsCeller (Becton Dickinson, High Wycombe, United Kingdom). Relative DNA content and distribution of cells with respect to the cell cycle was analyzed using MODFIT software.

Flow-cytometric detection of phosphatidylserine expression in cell lines was performed according to the manufacturer’s method using the fluorescein-labeled Annexin-V Apoptosis Detection Kit (R & D Systems, Abingdon, United Kingdom; Ref. 10). The measure of early apoptosis derives from the assessment of the proportion of Annexin-V-positive/PI-negative cells.

Results

Expression Analyses

BARX2 and Cadherin 6 Are Expressed in Normal Ovarian Surface Epithelium. We have demonstrated clear expression of BARX2 and cadherin 6 by RT-PCR in nine independent peroperatively harvested primary HOSE specimens (Fig. 1a). RT-PCR analysis of adult human multiple tissue cDNAs (BD CLONTECH) showed clear expression of BARX2 in thymus, prostate, testis, small intestine, colon, pancreas, and kidney (data not shown). Additionally, IHC using a human BARX2 polyclonal antibody (Santa Cruz Biotechnology) demonstrated that the ovarian surface epithelium is the site of normal BARX2 (Fig. 1b) expression in the ovary (Fig. 1b). In keeping with its role as a transcription factor, the immunohistochemical staining for BARX2 in the HOSE was predominantly nuclear.

Transfection of BARX2 Induces Cadherin 6 Expression in OAW42. RT-PCR demonstrated that the ovarian cancer cell line OAW42 was null with respect to BARX2 expression. This cell line provided the ideal background in which to study the transcriptional and functional consequences of BARX2 re-expression. We transfected OAW42 with the expression construct pBABE BARX2, comprising the full-length human BARX2 cDNA (4) cloned into the mammalian expression vector pBABE Hygro or with vector-only controls. The
BARX2 SUPPRESSOR FUNCTION IN OVARIAN CANCER

BARX and Cadherin 6 Expression Are Significantly Correlated in Ovarian Cancer Cell Lines. Quantitative RT-PCR (Light Cycler; Roche) established the relative endogenous levels of BARX2 and cadherin 6 expression in a panel of ovarian cancer cell lines comprising the strong BARX2 expressers PEO1, PEO1 CDDP (an in vitro-derived, platinum-resistant variant of platinum-sensitive PEO1), NIH:OVCAR3, and the weak BARX2 expressers A2780, OAW42, and the OAW42 BARX2 transfectants BX1.2 and BX1.7 (Fig. 2a). Analysis of the ovarian cancer cell lines using Pearson’s linear correlation test shows a highly significant direct relationship between the level of BARX2/Actin expression and the log 10 -transformed cadherin 6/Actin expression (r = 0.9520, r² = 0.9062, P = 0.0034; Fig. 2b). OAW42 does not endogenously express either BARX2 or cadherin 6 and could not be log transformed. It was therefore omitted from the Pearson analysis.

OAW42 parent cell line and the vector-transfected cell line OAW42 H7.5 do not show either endogenous or plasmid-derived BARX2 expression. In contrast, the OAW42 pBABE BARX2 transfectants OAW42 BX1.2 and OAW42 BX1.7 both express BARX2, and this is derived specifically from the transfection construct (Fig. 1c). The 1.5-fold difference in the level of expression between the two transfectants is reflected in the functional analysis (see below).

Because Barx2 is known to regulate the expression of three CAMs during murine development, RT-PCR was performed to evaluate if BARX2 transfection of the OAW42 cell line altered CAM expression. OAW42 does not express either BARX2 or cadherin 6, either by RT-PCR (Fig. 1c) or by Northern blotting (data not shown). In contrast, E-cadherin, N-cadherin, N-CAM, and L1-CAM are expressed in OAW42 (data not shown). RT-PCR analysis of OAW42 and its BARX2-transfected derivatives OAW42 BX1.2 and OAW42 BX1.7 showed that transfection of BARX2 induced expression of cadherin 6 (Fig. 1c). Northern blot analysis of the same cell lines hybridized with probes for BARX2 and cadherin 6, demonstrating that at least three isoforms of cadherin 6 transcript were induced after BARX2 transfection (data not shown).
Endometrioid and CC Ovarian Cancers Have Significantly Lower Levels of BARX2 and Cadherin 6 As Compared with SER and MMT Tumors. BARX2 and cadherin 6 expression in a panel of 22 ovarian cancers was each then examined by gel-based semiquantitative RT-PCR. Low BARX2 expression relative to Actin was significantly associated with EM and CC histology of ovarian cancer as compared with SER and MMT histological types (P = 0.035; Fig. 2c). The median BARX2/Actin ratio was 4.7 for EM/CC tumors compared with 48.4 for SER/MMT tumors. Low cadherin 6 expression, relative to Actin, was also significantly associated with EM and CC histology of ovarian cancer (P = 0.0081; Fig. 2c). The median cadherin 6/Actin ratio was 22.8 for EM/CC cancers and 63.1 for SER/MMT cancers. Together, these studies therefore provide clinical evidence of a significant link between BARX2 and cadherin 6 in these histological subgroups of ovarian cancer.

Functional Analysis of BARX2

In view of the role of Barx2 as a regulator of CAMs, the functional effects of BARX2 were assessed in Matrigel invasion assays, cellular migration assays, and cellular adhesion assays after transfection of BARX2 into the ovarian cancer cell line OAW42 (described above).

BARX2 Suppresses Matrigel Invasion. The OAW42 BARX2-transfected clones OAW42 BX1.2 and OAW42 BX1.7 were directly compared with the parent cell line and vector-only transfected OAW42 BX1.7 for evidence of inhibition of Matrigel invasion (Fig. 3a). OAW42 BX1.2, the higher BARX2 expressing clone, was significantly (P < 0.001) and strongly suppressed for Matrigel invasion compared with controls (Fig. 3a).Activated BARX2 expression was also suppressed for Matrigel invasion but much less than OAW42 BX1.2, and this result was not statistically significant (Fig. 3a). This suggested that the extent of Matrigel invasion was inhibited at the level of BARX2 expression in OAW42.

BARX2 Suppresses Cellular Migration Toward a Collagen IV Signal. The OAW42 BARX2-transfected clones OAW42 BX1.2 and OAW42 BX1.7 were directly compared with the parent cell line and vector-only transfected OAW42 BX1.7 for evidence of inhibition of cellular migration (Fig. 3b). Introduction and expression of BARX2 in OAW42 was significantly (P < 0.001) associated with inhibition of transwell cellular migration to a collagen IV haptotactic signal for both transfecants. Again, the higher expressing BARX2 clone had greater inhibition of cellular migration (Fig. 3b).

BARX2 Suppresses Adhesion to Collagen IV-coated Plates. The OAW42 BARX2-transfected clones OAW42 BX1.2 and OAW42 BX1.7 were directly compared with the parent cell line and vector-only transfected OAW42 BX1.7 for evidence of alteration of cellular attachment to collagen IV-coated plastic (Fig. 3c). For these same transfecants, introduction and expression of BARX2 in OAW42 were significantly associated with inhibition of attachment to collagen IV-coated plastic (Fig. 3c). No such effect was observed on uncoated plastic alone. Additionally, the higher expressing BARX2 clone had greater inhibition of adhesion to collagen IV (40% of control, P < 0.001; Fig. 3c).

BARX2 Causes Accumulation in S Phase of the Cell Cycle. Transfection of BARX2 into OAW42 appeared to cause an accumulation of cells in the S phase of the cell cycle as demonstrated by PI-labeled FACS analysis. This suggested that BARX2 transfection was associated with delay in this part of the cell cycle (Fig. 3d). However, using the annexin V FACS assay, we could show no evidence of either increased cell death or apoptosis after transfection of BARX2 into OAW42 (data not shown).

Discussion

The observation that BARX2 was located in a region of frequent LOH within the 11q24-q25 region associated with adverse survival (1, 2), prompted additional investigation. We identified strong expression of BARX2 in normal ovarian surface epithelium and noted loss of expression in two ovarian cancer cell lines. We also demonstrated significantly lower expression of BARX2 in CC and EM ovarian cancer as compared with other histological subtypes.

Studies of mouse Barx2 have shown it to be a regulator of N-CAM and L1-CAM expression (6, 11). This raised the possibility that CAMs may also be regulatory targets for human BARX2 in ovarian surface epithelium and carcinomas. We analyzed expression of CAMs in OAW42, an
ovarian cancer cell line that does not endogenously express BARX2, and found that cadherin 6, although not expressed in OAW42, was induced in OAW42-derived cell lines transfected with and expressing BARX2.

The transcriptional regulation of cadherin 6 by a homeobox transcription factor has been documented recently. Cadherin 6 was identified as one of a number of genes differentially expressed in response to overexpression of Hoxa1 (12). In addition, studies of Hoxa-1 knockout mice indicate that Hoxa-1 may be required for early cadherin 6 expression in the posterior hindbrain of the developing mouse (13). On the basis of this documented relationship and of the known functions of Barx2, the induction of cadherin 6 may well be attributable to direct transcriptional activation, although this remains to be proven.

We have shown that cadherin 6 is also specifically expressed in the normal ovarian surface epithelium. Cadherin 6 expression was significantly lower in CC and EM ovarian cancer mirroring the pattern of BARX2. Quantitative RT-PCR in a series of ovarian cancer cell lines showed a highly significant Pearson linear correlation between BARX2 and log-transformed cadherin 6 expression, providing additional evidence of a relationship between these molecules. The involvement of cadherin 6 in the development or progression of ovarian cancer has not been documented previously. Previous studies have reported that aberrant cadherin 6 expression detected immunohistochemically correlates with adverse patient survival in renal cell carcinoma (14, 15).

Cell biological assays for identifying interactions between the cell and the extracellular matrix showed that BARX2-transfected OAW42 cells were significantly inhibited as compared with controls in their ability to invade Matrigel, to migrate to a collagen IV haptotactic signal, and to adhere to collagen IV-coated plates. The magnitude of the phenotypes observed after BARX2 transfection of OAW42 was more marked with higher BARX2 levels.

We have therefore observed clear suppressor functions for BARX2 that are compatible with a role in ovarian cancer progression. Studies conducted previously in developmental biology may shed light on possible mechanisms to explain these observations. During murine development, Barx2 is coexpressed with Barx1, particularly in the central and peripheral nervous system and in craniofacial structures (6). Expression of Barx2 was limited to ectodermal borders adjacent to regions of mesenchymal Barx1 expression, where tissues were undergoing remodeling. Additionally, focal Barx2 expression was observed at sites undergoing epithelial-mesenchymal transition, such as developing lung buds. Barx2, in common with other Bar class genes, is expressed during the development of anterior embryonic structures (6). Other homebox proteins which share homology with the BARX2 homeodomain, such as Xvent-1 in Xenopus (16), are also regulators of mesodermal cell fate. The mesodermal origin of the ovarian surface epithelium suggests that these molecules may have a regulatory role in its development.

The role of CAMs in epithelial-mesenchymal transition is well documented in cancer progression, e.g., it has been proposed that epithelial tumor cells lose cell-cell and cell-matrix contact in the later stages of tumorigenesis, in association with down-regulation of CAMs, such as E-cadherin (17). Inactivation of E-cadherin has been demonstrated to be of causal importance in the adenoma-to-carcinoma transition in murine transgenic models (18) and is inactivated by mutation in human familial gastric cancer (19). The loss of epithelial and acquisition of mesenchymal characteristics in late tumorigenesis has been compared with epithelial-mesenchymal transitions occurring during development.

In the Madin-Darby canine kidney system, cadherin 6-down-regulated cells were found to be flatter, formed less compacted colonies, and were more migratory (20), and this latter migratory phenotype is consistent with the phenotype observed in this study. However, the down-regulation of cadherin 6 did not affect the initiation of cell-cell contact or the formation of epithelium (20).

Taken together, these observations suggest that BARX2 has a role in the maintenance of the normal HOSE, and its disrupted expression may have a role in the progression of EOC.

In conclusion, we have shown that BARX2, a positional candidate suppressor gene identified through LOH at 11q24-25 and the association of LOH with adverse survival in this region, is expressed in the normal ovarian surface epithelium. BARX2 expression is lost in two ovarian cancer cell lines, induces cadherin 6 expression, is associated with particular histological subgroups of ovarian cancer, and is a functional suppressor of ovarian cancer cell invasion, migration, and adhesion.

Acknowledgments

We thank Prof. Nick Hastie, director of the Medical Research Council Human Genetics Unit, Edinburgh, United Kingdom, and Prof. Ian Hart, the Richard Dimbleby Imperial Cancer Research Fund Department of Cancer Research, UMDN, London, United Kingdom, for their constructively critical review of this work.

References


6981
BARX2 Induces Cadherin 6 Expression and Is a Functional Suppressor of Ovarian Cancer Progression

Grant C. Sellar, Li Li, Karen P. Watt, et al.

Cancer Res 2001;61:6977-6981.