The impact of androgen receptor (AR) and histone deacetylase 1 (HDAC1) expression on the prognosis of ductal carcinoma *in situ* (DCIS)

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Research article

Keywords: DCIS, AR (Androgen receptor), HDAC1 (histone deacetylase 1), Prognosis

Posted Date: September 8th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-67580/v1

Abstract

Background Ductal carcinoma *in situ* (DCIS) display favorable outcome but little is known about the factors associated with invasive recurrence. To identify better prognostic biomarkers, we performed gene expression analysis followed by immunohistochemistry (IHC) staining validation.

Methods Differential gene expression (DGE) analysis of 24 pure DCIS patients was performed using a nanostring platform. RNA was extracted from paraffin blocks from age/estrogen receptor matched recurrence-free (n=16) and invasive-recurrence (n=8) cases (disease-free interval >5 years). External validation was done among independent 61 cases, invasive-recurrence (n=16) and recurrence-free (n=45) pure DCIS cases by IHC staining.

Results Eight differentially expressed genes were found statistically significant (log 2-fold change <−1 or >1 and p<0.001). Less than ½ fold expression of *CUL1, AR, RPS27A, CTNNB1, MAP3K1, PRKACA, GNG12, MGMT* genes were observed in REC cases compared to NED cases. Androgen receptor (*AR*) and histone deacetylase 1 (*HDAC1*) were selected for external validation (AR: log 2-fold change −1.35, p<0.001, and HDAC1; log 2-fold change −0.774, p<0.001). AR and HDAC1 protein expression was externally validated by IHC staining of 61 pure DCIS cases (16 invasive-recurrence versus 45 recurrence-free). Absence of AR and high HDAC1 expression was an independent risk factor for invasive recurrence (hazard ratio 5.04, 95% CI: 1.24, 20.4; p=0.023, hazard ratio 3.07, 95% CI: 1.04, 9.04; p=0.042). High nuclear grade (NG 3) was also associated with long term invasive recurrence.

Conclusion Comparative gene expression analysis of pure DCIS revealed 8 genes differentially expressed among recurred cases. Immunohistochemistry validation within an independent cohort suggests that, absence of AR and overexpression of HDAC1 was associated with greater risk of long term invasive recurrence among pure DCIS.

1. Introduction

Ductal carcinoma *in situ* (DCIS) consist upto 20% of newly diagnosed breast cancers owing to widely adopted screening. It is a non-obligate precursor to invasive ductal carcinoma (IDC) exhibiting an excellent prognosis. While surgical resection is the mainstay of therapy, adjuvant radiotherapy (RT) after lumpectomy has long been a controversial issue. Trials showed that the relative reduction in local recurrence rate was 50% in the irradiated group. But, these studies failed to show benefit on distant metastases or overall survival [1]. RT may induce cardiac toxicity, fibrosis of lung and skin changes. It needs daily visits to the radiation center and also costs substantial medical expenses. The risk could outweigh the benefit in patients with a low risk implying the necessity of accurately classify patient groups according to risk of subsequent invasive recurrences. The absolute benefit of chemoprevention is being investigated as well taking into account the side effects of uterine carcinoma and menopausal symptoms. Yet, owing to lack of tool to accurately classify risk and benefit of such treatment, current guideline recommends surgical resection followed by radiation and/or chemoprevention with tamoxifen albeit the indolent clinical course of the disease.

Young age, high histologic grade, large tumor size and absence of hormone receptor are the historical prognostic markers of DCIS [2–4]. Van Nuys prognostic index presents the risk of invasive recurrence into low/intermediate/high risk group by adding scores of patient age, tumor size, margin width and nuclear grade/necrosis [5]. Oncotype DCIS predicts prognosis using gene expression of DCIS tumor samples [6]. DCISionRT is a recently developed radio-genomic tool which predicts the benefit of radiation [7]. However, both requires higher level of evidence and overall feasibility including the cost-effectiveness [8]. For DCIS, it is important to assess the risk of long term recurrence as upto 98% display disease-free within five year after diagnosis. In this study, we aimed

to investigate prognostic biomarkers of DCIS to predicting long term invasive recurrence. We sought to identify markers which could be easily applied in clinic using immunohistochemistry.

2. Materials And Methods

Patient Selection

We performed a retrospective study with mRNA expression profiling and external validation by IHC staining. A discovery cohort was selected for gene expression analysis and a validation cohort was subsequently chosen for IHC staining. Patients histologically diagnosed with pure DCIS without an invasive component after definitive surgery were selected. As to investigate the long term prognosis, all patients underwent surgery before 2008 were analyzed. Patients with clinicopathological data and formalin fixed paraffin embedded available were included.

Thirty-six pure DCIS patients were selected for discovery cohort. Twelve patients with invasive recurrence (REC) were chosen. Cases with no recurrence (NED) were selected by 1:2 ratio matching for age and ER. All patients were diagnosed with DCIS and underwent definitive surgery with negative tumor margin after breast-conserving surgery or mastectomy, between 1995 and 2004. Sequencing was successfully performed in 8 REC cases and 16 NED cases. Sixty-one pure DCIS patients were selected as validation cohort for IHC analysis. REC and NED cases were selected as done for discovery cohort. All patients have underwent surgery at Asan Medical Center (AMC) between 1995 and 2008.

RNA Extraction And Quantification

Surgical specimens from the discovery cohort obtained during surgery for routine diagnostic pathologic examinations were used. Areas of tumor component were dissected from formalin-fixed paraffin-embedded (FFPE) tissue. Total RNA was extracted from the sections using MasterPure™ Complete DNA & RNA Purification Kit (Lucigen-Epicentre, Middleton WI, USA), according to the manufacturer's instructions. RNA yield and purity were assessed using a DS 11 Spectrophotometer (Denovix Inc., Wilmington DE, USA). Since nanostring recommends the use of ≈ 300 ng total RNA, 300 ng of total RNA was added to the sample preparation reaction in the available 5 µL volume. An RNA quality check was performed by using Fragment Analyzer (Advanced Analytical Technologies, Ankeny, IA, USA).

mRNA Expression profiling by nanoString nCounter system and data analysis

The digital multiplexed nanoString nCounter human mRNA expression assay (nanoString Technologies, Seattle, WA, USA) was performed with 300 ng total RNA isolated from the tissues. Samples were assessed using the nCounter PanCancer Pathways Panel, which targets 730 genes representing all the major cancer pathways. Hybridizations were carried out by combining 5 µL of each RNA sample with 8 µL of nCounter Reporter probes in hybridization buffer and 2 µL of nCounter Capture probes (for a total reaction volume of 15 µL) overnight at 65℃ for 16–20 h. Excess probes were removed using two-step magnetic bead-based purification on the nCounter Prep Station (nanoString Technologies).

Abundances of specific target molecules were quantified on the nCounter Digital Analyzer by counting the individual fluorescent barcodes and assessing the target molecules. For each assay, a high-density scan encompassing 280 fields of view was performed. Data were collected using the nCounter Digital Analyzer after taking images of the immobilized fluorescent reporters in the sample cartridge with a charged-couple device camera. mRNA data analysis was performed using the nSolver software analysis, freely available from nanoString Technologies. The

mRNA profiling data were normalized using housekeeping genes. R software (R Core Team. (2016). R: A Language and Environment for Statistical Computing. Vienna, Austria. Retrieved from https://www.R-project.org/) was used for the analysis.

Biomarker Determination

Tumor tissues from the validation cohort obtained after surgery for routine diagnostic pathologic examinations were used for IHC studies of the anti-AR (host-Rabbit, clone SP107, 1:100, 200R-16, CELL MARQUE, Roklin, CA, USA). FFPE tissue sections were immunohistochemically stained for anti-AR using a BenchMark XT automatic immunostaining device (Ventana Medical Systems, Tucson, AZ, USA) with OptiView DAB IHC Detection Kit (Ventana Medical Systems), according to the manufacturer's instructions. Four-micrometer-thick sections, obtained with a microtome, were transferred onto silanized charged slides and allowed to dry for 10 min at room temperature, followed by 20 min in an incubator at 65 °C. Sections were obtained by a heat-induced epitope retrieval method using Cell Conditioning 1 buffer for 32 min and incubated for 16 min with antibodies in the autoimmunostainer. Antigen-antibody reactions were visualized using Ventana OptiView DAB IHC Detection Kit (Optiview HQ Linker 8 min, Optiview HRP Multimer 8 min, Optiview H2O2/DAB 8 min, Optiview Copper 4 min). Counterstaining was performed using Ventana Hematoxylin II for 12 min and Ventana Bluing reagent for 4 min. Finally, all slides were removed from the stainer, dehydrated, and coverslipped for microscopic examination.

Biomarker expression was quantified by Allred score. The conventional biomarker ER expression was quantified by Allred score and classified based on the St. Gallen and American Society of Clinical Oncology/College of American Pathologists guidelines (2013). Cases with Allred score 0 was considered AR negative. Allred score > 5 was defined as a HDAC1 high, and score < 6 as HDAC1 low.

Statistical Analyses

Independent Student's t-test and Mann-Whitney test were performed for all continuous variables. Chi-squared test or Fisher's exact test was used to evaluate the relationship between the categorical clinical characteristics and relapse status. Recurrence-free survival was estimated using the Kaplan-Meier method. The prognostic role of risk factors was analyzed using a Cox proportional hazards model. All p values were based on two-sided testing, and values lower than 0.05 were considered statistically significant. Statistical analysis was performed with IBM SPSS Statistics for Windows, version 20 (IBM Corp., Armonk, N.Y., USA).

3. Results

Differentially expressed genes among the discovery cohort

Among the initially selected 36 pure DCIS patients for comparative mRNA expression profiling, sequencing was successfully performed in 8 REC cases and 16 NED cases. Clinicopathological characteristics of the 24 cases (8 REC, 16 NED) are displayed in Table 1. The characteristics of the REC and NED group showed no statistically significant differences. Among the REC patients, 6 cases were local recurrence and 2 were regional recurrence. The median follow-up was 149 months in total: 56 months for the REC group, and 162 months for the NED group. The mRNA expression levels for these two groups were compared. Fold change (FC) was calculated as the ratio of gene expression level REC/NED. Genes with log2_|FC| >1 and p value < 0.001 were selected as differentially expressed genes (DEG). Recurred cases had significantly lower expression of *CUL1, AR, RPS27A, CTNNB1, MAP3K1, PRKACA,*

GNG12, MGMT genes. No gene was found significantly overexpressed among recurred case. List of the differentially expressed genes were described in Table 2.

Table 1
Patient characteristics of discovery cohort

Characteristics	Overall	(n = 24)	NED	(n = 16)	REC	(n = 8)	P-value
Average age(range)	40.3	(25–56)	41.1	(28–56)	38.7	(25–50)	0.667*
≥ 40	12	50.00%	9	56.25%	3	37.50%	
< 40	12	50.00%	7	43.75%	5	62.50%	
	24		16		8		
Nuclear grade							0.333**
1	2	8.33%	2	12.50%	0	0.00%	
2	21	87.50%	14	87.50%	7	87.50%	
3	1	4.17%	0	0.00%	1	12.50%	
	24		16		8		
Tumor size(cm)							0.189
< 2 cm	8	33.33%	7	43.75%	1	12.50%	
≥ 2 cm	16	66.67%	9	56.25%	7	87.50%	
	24		16		8		
ER							0.578
+	20	83.33%	14	87.50%	6	75.00%	
-	4	16.67%	2	12.50%	2	25.00%	
	24		16		8		
PR							1.000
+	19	79.17%	13	81.25%	6	75.00%	
-	5	20.83%	3	18.75%	2	25.00%	
	24		16		8		
HER2							0.137
+	7	29.17%	3	18.75%	4	50.00%	
-	16	66.67%	13	81.25%	3	37.50%	
	23		16		7		
HTx (n = 10)							1.000
+	6	25.00%	5	31.25%	1	12.50%	
-	4	16.67%	3	18.75%	1	12.50%	

*Fisher exact test for ≥ 40 group vs < 40 group. **Fisher exact test for NG 1, 2 group vs 3 group. NED No evidence of disease, REC Recur, ER Estrogen receptor, PR Progesterone receptor, HER2 Human epidermal growth factor receptor 2, HTx Hormone therapy, RTx Radiotherapy, RM Resection margin

Characteristics	Overall	(n = 24)	NED	(n = 16)	REC	(n = 8)	P-value
	10		8		2		
RTx							0.509
+	9	37.50%	6	37.50%	3	37.50%	
-	3	12.50%	3	18.75%	0	0.00%	
	12		9		3		
RM							1.000
+	3	12.50%	2	12.50%	1	12.50%	
-	21	87.50%	14	87.50%	7	87.50%	
	24		16		8		

*Fisher exact test for ≥ 40 group vs < 40 group. **Fisher exact test for NG 1, 2 group vs 3 group. NED No evidence of disease, REC Recur, ER Estrogen receptor, PR Progesterone receptor, HER2 Human epidermal growth factor receptor 2, HTx Hormone therapy, RTx Radiotherapy, RM Resection margin

Table 2
Differentially expressed gene list

Gene	Gene name	Log2 FC	std error	Lower CL	Upper CL	p value
CUL1	Culin1	−−1.13	0.189	−−1.49	−−0.755	0.00000532
AR	Androgen Receptor	−−1.35	0.306	−−1.95	−−0.746	0.000231
RPS27A	Ribosomal Protein S27a	−−1.43	0.33	−−2.07	−−0.78	0.000272
CTNNB1	Catenin Beta 1	−−1.34	0.333	−−1.99	−−0.689	0.00056
MAP3K1	Mitogen-Activated Protein Kinase Kinase Kinase 1	−−1.54	0.383	−−2.3	−−0.792	0.000567
PRKACA	Protein Kinase CAMP-Activated Catalytic Subunit Alpha	−−1.68	0.433	−−2.53	−−0.831	0.000809
GNG12	G Protein Subunit Gamma 12	−−1.27	0.331	−−1.92	−−0.62	0.000902
MGMT	O-6-Methylguanine-DNA Methyltransferase	−−1.04	0.272	−−1.57	−−0.505	0.000938
*FC: fold change, CL: confidence limit						
Cutoff score: Log2-fold change <−1 or > 1 and p < 0.001.						

Among the DEGs, AR, gene coding androgen receptor was chosen as a candidate prognostic biomarker. Androgen receptor (AR) is a hormone receptor that is expressed in over 70% of invasive breast cancers [9]. While it's role during breast cancer carcinogenesis is not clear, AR is suggested to have an inhibitory effect especially in estrogen receptor

(ER)-positive breast cancer. It is an emerging prognostic and predictive marker and a potential therapeutic target for invasive breast cancers [10]. Gene expression data of the discovery cohort presented 1.71 times over expression of *HDAC1 gene*, Histone deacetylase 1 in recurred cases compared to NED group (log2_FC 0.774, $p < 0.001$). HDAC1 is a member of the protein family of HDAC, which is involved in chromatin modification by removing acetyl groups from histones. It is known to contribute to cancer cell development by suppressing the expression of genes such as cell cycle inhibitors, tumor suppressor genes, and apoptosis inducer genes, and by inducing the global loss of acetylation [11]. HDAC1 inhibitors induced cell differentiation and inhibited the growth of breast cancer cells by cell cycle arrest and induction of apoptosis [12]. We evaluated the prognostic impact of both AR and HDAC1 within an independent validation cohort by IHC.

Validation of prognostic biomarker among an independent cases

An independent cohort was selected for validation of AR and HDAC1 as a prognostic biomarker. Samples from 61 pure DCIS surgical specimens were obtained for IHC staining. Among the 61 validation cohort, 16 were REC patients and 45 were NED patients. Clinicopathological characteristics of the validation cohort is described in Table 3. In the REC group, 13 local recurrence cases and 3 regional lymph node recurrence patients were included. The median follow-up time was 110 months in total: 65 months for the REC group, and 114 months for the NED group. Figure 1 shows the IHC staining results for AR. To evaluate factors associated with AR negativity (versus AR positive) and high HDAC1 expression (versus HDAC1 low) the validation cohort was divided according to AR-positive versus AR-negative group, and a high HDAC1 versus low HDAC1 group. As indicated in Table 4, each pair of groups were statistically homogeneous regarding other conventional risk factors.

Table 3
Patient characteristics for the validation cohort

Characteristics	Overall	(n = 61)	NED	(n = 45)	REC	(n = 16)	p-value
Average age, years(range)	43.1	27–65	43.6	27–65	41.9	28–59	0.674
≥ 40	37	60.66%	28	62.22%	9	56.25%	
< 40	24	39.34%	17	37.78%	7	43.75%	
	61		45		16		
Nuclear grade							0.063
1	13	21.31%	9	20.00%	4	25.00%	
2	36	59.02%	30	66.67%	6	37.50%	
3	12	19.67%	6	13.33%	6	37.50%	
	61		45		16		
Tumor size(cm)							0.576
Multiple	7	11.48%	4	8.89%	3	18.75%	
≥ 2	17	27.87%	13	28.89%	4	25.00%	
< 2	37	60.66%	28	62.22%	9	56.25%	
	61		45		16		
ER							1.000
+	46	76.67%	34	75.56%	12	80.00%	
-	14	23.33%	11	24.44%	3	20.00%	
	60		45		15		
PR							1.000
+	45	75.00%	34	75.56%	11	73.33%	
-	15	25.00%	11	24.44%	4	26.67%	
	60		45		15		
HER							0.803
+	27	45.76%	21	46.67%	6	42.86%	
-	32	54.24%	24	53.33%	8	57.14%	

NED No evidence of disease, REC Recur, ER Estrogen receptor, PR Progesterone receptor, HER2 Human epidermal growth factor receptor 2, HTx Hormone therapy, RTx Radiotherapy, RM Resection margin, AR Androgen receptor, HDAC Histone deacetylase

Characteristics	Overall	(n = 61)	NED	(n = 45)	REC	(n = 16)	p-value
	59		45		14		
RTx (n = 57)							0.333
+	50	87.72%	40	90.91%	10	76.92%	
-	7	12.28%	4	9.09%	3	23.08%	
	57		44		13		
HTx (n = 43)							0.295
+	24	55.81%	20	60.61%	4	40.00%	
-	19	44.19%	13	39.39%	6	60.00%	
	43		33		10		
AR							0.108
+	56	91.80%	43	95.56%	13	81.25%	
-	5	8.20%	2	4.44%	3	18.75%	
	61		45		16		
HDAC1							0.164
0–6	47	77.05%	37	82.22%	10	62.50%	
7–8	14	22.95%	8	17.78%	6	37.50%	
	61		45		16		
RM							1.000
+	5	8.20%	4	8.89%	1	6.25%	
-	56	91.80%	41	91.11%	15	93.75%	
	61		45		16		

NED No evidence of disease, REC Recur, ER Estrogen receptor, PR Progesterone receptor, HER2 Human epidermal growth factor receptor 2, HTx Hormone therapy, RTx Radiotherapy, RM Resection margin, AR Androgen receptor, HDAC Histone deacetylase

Table 4
Univariate analysis of clinicopathologic factors

Characteristics	AR+	(n = 56)	AR-*	(n = 5)	p-value	HDAC1 high	(n = 14)	HDAC1 low	(n = 47)	p-value
Average age, years(range)	42	27–65	46	32–55	0.640	39	28–59	44	27–65	0.120
≥ 40	33	58.9%	4	80.0%		6	42.9%	31	66.0%	
< 40	23	41.1%	1	20.0%		8	57.1%	16	34.0%	
	56		5			14		47		
Nuclear grade					0.573					0.124
1	10	17.9%	3	60.0%		3	21.4%	10	21.3%	
2	34	60.7%	2	40.0%		6	42.9%	30	63.8%	
3	12	21.4%	0	0.0%		5	35.7%	7	14.9%	
	56		5			14		47		
Tumor size(cm)					0.634					0.492
Multiple	6	10.7%	1	20.0%		3	21.4%	4	8.5%	
≥ 2	16	28.6%	1	20.0%		3	21.4%	14	29.8%	
< 2	34	60.7%	3	60.0%		8	57.1%	29	61.7%	
	56		5			14		47		
ER					1.000					1.000
+	43	76.8%	3	75.0%		11	78.6%	35	76.1%	
-	13	23.2%	1	25.0%		3	21.4%	11	23.9%	
	56		4			14		46		
PR					0.258					0.734
+	43	76.8%	2	50.0%		10	71.4%	35	76.1%	
-	13	23.2%	2	50.0%		4	28.6%	11	23.9%	
	56		4			14		46		
HER2					0.617					1.000
+	26	47.3%	1	25.0%		6	42.9%	21	46.7%	
-	29	52.7%	3	75.0%		8	57.1%	24	53.3%	
	55		4			14		45		

*Absence of AR positive cells. NED No evidence of disease, REC Recur, ER Estrogen receptor, PR Progesterone receptor, AR androgen receptor, HDAC1 histone deacetylase 1, HER2 Human epidermal growth factor receptor 2, HTx Hormone therapy, RTx Radiotherapy

Characteristics	AR+	(n = 56)	AR-*	(n = 5)	p-value	HDAC1 high	(n = 14)	HDAC1 low	(n = 47)	p-value
RTx (n = 53)					0.07					0.333
+	48	90.6%	2	50.0%		10	76.9%	40	90.9%	
-	5	9.4%	2	50.0%		3	23.1%	4	9.1%	
	53		4			13		44		
HTx (n = 43)					0.079					0.295
+	24	60.0%	0	0.0%		4	40.0%	20	60.6%	
-	16	40.0%	3	100.0%		6	60.0%	13	39.4%	
	40		3			10		33		

*Absence of AR positive cells. NED No evidence of disease, REC Recur, ER Estrogen receptor, PR Progesterone receptor, AR androgen receptor, HDAC1 histone deacetylase 1, HER2 Human epidermal growth factor receptor 2, HTx Hormone therapy, RTx Radiotherapy

Table 5
Survival analysis of independent validation cohort (n = 61)

	Univariate analysis**	Multivariate analysis***		
Variables	p-value	HR	95% CI	p-value
NG	0.006	4.89	1.60–14.9	0.005
1,2 vs 3				
Size	0.617	1.18	0.42–3.37	0.749
Cut off 2 cm				
AR	0.031	5.04	1.24–20.4	0.023
negative*				
HDAC1	0.010	3.07	1.04–9.04	0.042
High				

* Absence of AR positive cell ** Kaplan-Meier test was conducted for univariate analysis. *** Cox regression test was conducted for multivariate analysis

NG nuclear grade, **AR** androgen receptor, **HDAC1** histone deacetylase 1

Survival analysis was done to evaluate AR and HDAC1 protein expression and the risk of invasive recurrence. Figure 2 illustrates Kaplan-Meier survival curve for AR negative versus positive group, and HDAC1 high and low group. 10 year invasive recurrence for AR negative versus positive, and HDAC1 high versus low was 53.3% vs 77.6% and 46.8% vs 83.5%. Factors associated with recurrence by univariate and multivariate Cox regression analysis is described in Table 5. Conventional risk factors eg, age and size, were not significantly associated. Patients with NG

3 had a higher recurrence rate than NG 1or 2 cases indicating high grade DCIS as an independent risk factor (HR 4.89, p = 0.005). Both AR-negativity and high HDAC1 expression were independently associated with invasive recurrence (HR 5.04, p = 0.031, and HR 3.07, p = 0.010, respectively). We then expanded the survival analysis including the IHC result of the discovery cohort. Similarly, both AR negativity and high expression of HDAC1 by IHC was an independent risk factors (p = 0.004, and p = 0.038, data not shown)

4. Discussion

To identify biomarkers for long term invasive recurrence in pure DCIS, we performed differential mRNA expression profiling of cancer panel genes followed by external validation using IHC for selected candidates, *AR* and *HDAC1*. The study was conducted with pure DCIS cases and showed that DCIS cases that progress into invasive carcinoma present different gene expression features compared to DCIS cases that remain in the NED state. Differential gene expression profiling found 8 genes that were found to be less expressed in REC cases ($|FC|>2$, $p < 0.001$). Among them, *AR* and *HDAC1* was selected for external validation by IHC, the most commonly applied methodology in real-world clinic. Survival analysis revealed that the group with the absence of AR cells and group with higher HDAC1 exhibited higher risk of invasive recurrence. These results were statistically significant, suggesting that both *AR* and *HDAC1* may serve as potential prognostic biomarkers for pure DCIS patients.

Unlike ER, the role of AR in the carcinogenesis of breast cancer is not well established. AR is co-expressed with ER in 70–90% of cases and is expressed in 40% of ER-negative cancers. The effect of AR in ER-negative breast cancer is rather mixed, but in ER-positive invasive breast cancers, cross talk between ER signaling and AR signaling do exist. Several studies showed that AR expression is associated with favorable characteristics such as, older age, smaller size, well-differentiated tumors, lower proliferation index, and higher positivity of hormone receptors and as a consequence, better overall survival [13–16]. Results from *in vitro* models support the findings that overexpression of AR in ER-positive cell lines inhibits the proliferative activity of ER [17]. AR appears to have a competitive effect against ER for binding to the estrogen-response element of ER target genes [18].

However the prognostic role of AR in DCIS is more ambiguous. IHC staining of hormone receptors showed that the rate of AR expression was lower in high-grade (HG)-IDC than in HG-DCIS [19]. Other studies showed that AR expression level was higher in DCIS adjacent to IDC compared to pure DCIS [20, 21]. In statistical analyses of the expression of several biomarkers of breast cancer, AR did not show statistical significance as a risk factor for recurrence [22].Recently, based on the concept of competition between AR and ER, Ravaioli *et al.* suggested AR/ER ratio as a prognostic marker for DCIS [23]. AR indeed was associated with a favorable prognosis in this study population which correlates with the previously reported biological effect of AR as an ER signal inhibitor. As the cases were selected according to HR positivity rather than in a consecutive manner, we couldn't directly see the effect of AR confined to HR positive DCIS cases. Also the numbers analyzed were small especially the REC cases.

The HDAC family is an epigenetic regulator of gene expression that plays an important role in both normal cell development and carcinogenesis. In cancer cells, by removing an acetyl group from histones, HDAC causes chromatin condensation resulting in the global gene expression suppression including tumor suppressor genes, cell cycle inhibitors, and differentiation factors or apoptosis inducers. HDAC1 regulates the expression of key proteins involved in the cell cycle, such as p21, p53, and cyclin D1. Also, overexpression of HDAC1 induces hypoxia-inducible factor-1α and vascular endothelial growth factor and increases angiogenesis. Besides specific gene regulation, global loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 are hallmarks of human cancer cells, including breast cancer cells [24]. Moreover, global loss of histone modification was related to poor breast cancer-

specific survival and disease-free survival [25]. Little is known about the correlation between HDAC1 and breast cancer. In an *in vitro* study, overexpression of HDAC1 induced the loss of ER and increased cell proliferation, and the use of an HDAC inhibitor induced ER re-expression [26]. Junko Suzuki reported DCIS showing reduced acetylation compared to normal breast epithelial cells, regardless of HDAC1 expression [27]. Several retrospective studies showed HDAC1 to be associated with a favorable prognosis [28]. A recent meta-analysis argued that HDAC1 overexpression did not correlate with disease-free survival and overall survival in invasive breast cancer [29]. The expression of HDAC1 in DCIS remains controversial.

This study implies the potential role of AR and HDAC1 as prognostic biomarkers for pure DCIS. The strength of this study is that the cohort consist of pure DCIS cases with long term follow up, as most of DCIS patients don't undergo > 5–10 year surveillance. Observation of pure DCIS lesions over a long follow-up period, compared to analyzing synchronous DCIS lesions, enables investigating the natural history more directly. Synchronous DCIS lesions may not be a high-risk DCIS and may possess different biological features. Also, to represent true high risk group, in situ recurrences were excluded and only invasive recurrence were included for REC group. Nonetheless, there are several limitations. The number of cases analyzed in both cohorts were small. The study was not a consecutive case-series analysis. Rather, each of REC and NED cases were selected according to age, hormone receptor status in a 1:2 ratio. But as DCIS is a disease displaying exceptionally favorable outcome, cases with invasive recurrence within certain timeframe is small. The REC and NED cases were selected matched by age, hormone receptor status as to minimize the effect of these known risk factors. The cutoff value of AR and HDAC1 should be furtherly addressed as, currently there is no standard cutoff.

5. Conclusion

In conclusion, we have performed a matched comparative gene expression analysis of pure DCIS cases to identify prognostic indicators of long term invasive recurrence. Our study revealed absence of AR and overexpression of HDAC1 was found to be associated with greater risk of invasive recurrence. Further validation within a larger series are needed.

Declarations

Ethics approval and consent to participate

Clinicopathological information and follow up recurrence data was reviewed within ABLE (Asan Biomedical Research Environment) system which enable anonymous data and sample extraction. The study was approved by the institutional review board (IRB No. 2016-0976) of AMC.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

Funding

This work was supported by the National Research Foundation of Korea(NRF) grant funded by the Korea government(MOE) (No. 2015R1D1A1A01058390).

Authors' contributions

All the authors have made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data. CML participated in the interpretation and analysis of data and drafted the manuscript. YSP and HJL participated in the acquisition and interpretation of pathologic data. GWY, HGJ, and HJP participated in the whole process throughout the research and statistical analysis. SBL, IYC, HJK, BSK, JWL, BHS, and SHA, participated in the interpretation and analysis of the data. As corresponding author, JSK designed and coordinated the research and provided close guidance throughout the process. The authors read and approved the final manuscript.

Acknowledgments

This work was supported by the National Research Foundation of Korea(NRF) grant funded by the Korea government(MOE) (No. 2015R1D1A1A01058390) and by a grant (2018-0783) from the Asan Institute for Life Sciences, Asan Medical Center, Seoul, Korea.

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Figures

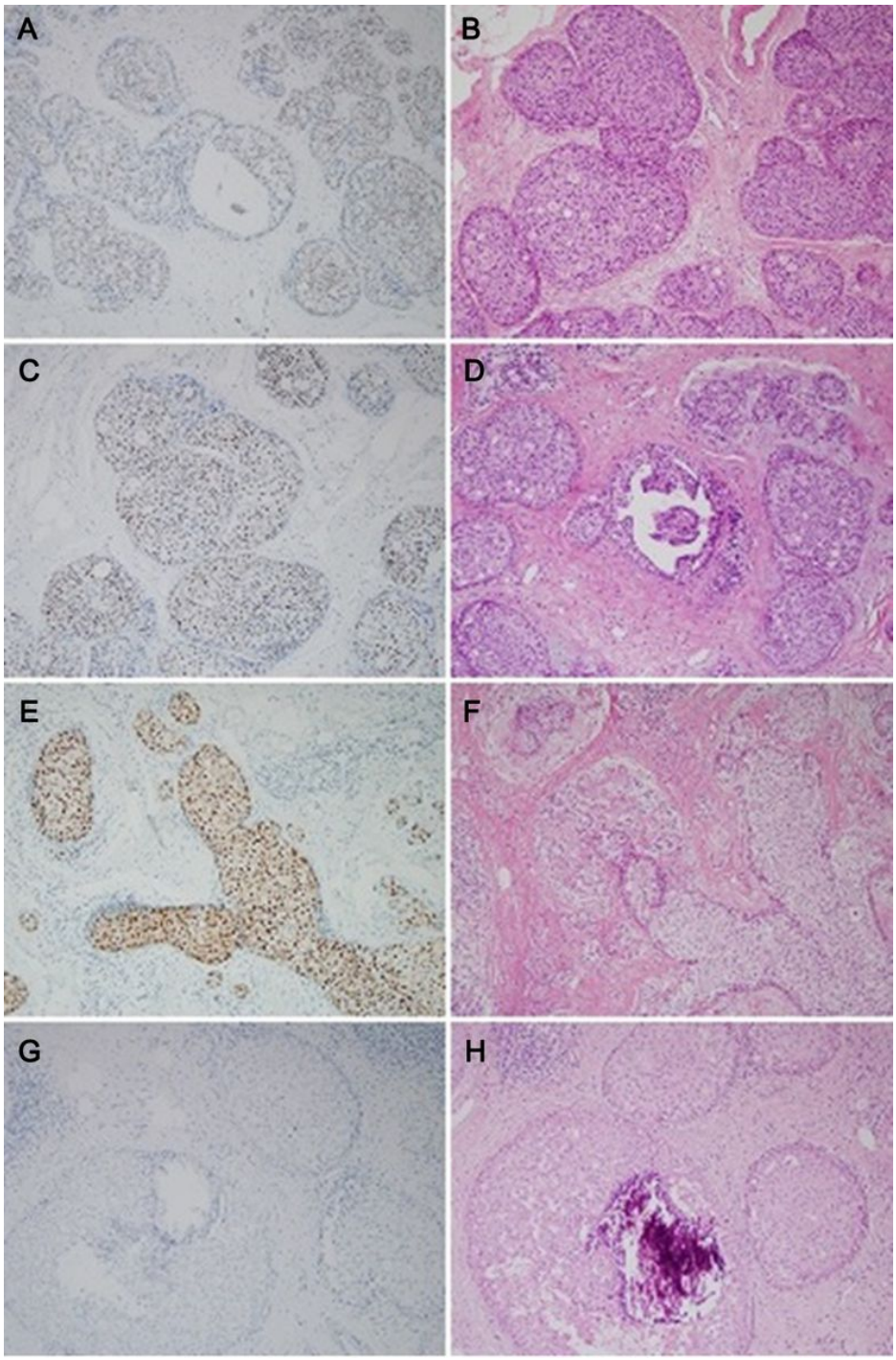
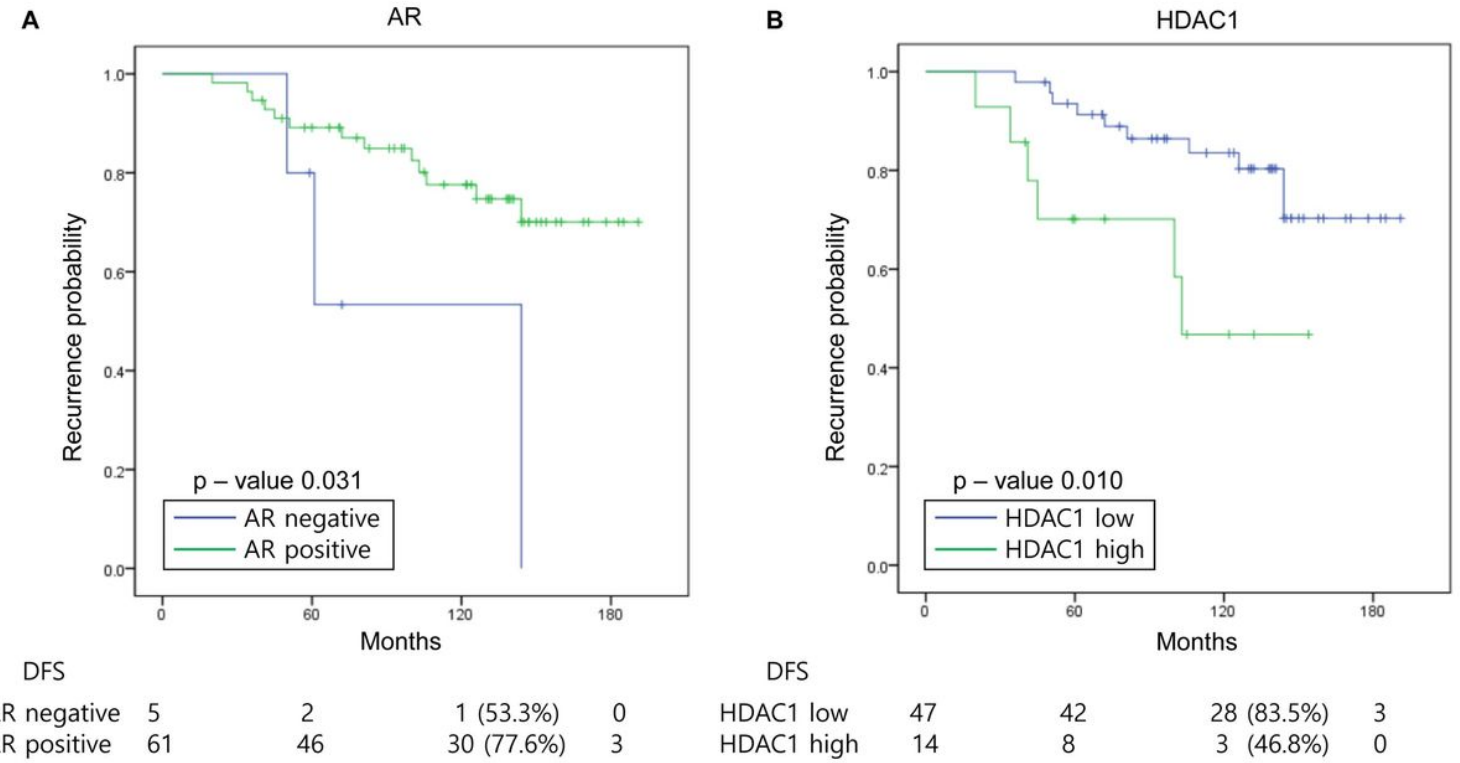


Figure 1

IHC staining of AR and its corresponding H & E staining. The presenting slides are classified by intensity of AR. (g) shows a case with negative AR intensity. (a), (c), (e) show cases with intensity 1, 2, 3, respectively. (b), (d), (f), (h) are the H & E results. IHC Immunohistochemistry AR Androgen receptor



DFS				
AR negative	5	2	1 (53.3%)	0
AR positive	61	46	30 (77.6%)	3

DFS				
HDAC1 low	47	42	28 (83.5%)	3
HDAC1 high	14	8	3 (46.8%)	0

Figure 2

Kaplan-Meier survival analysis. Recurrence-free survival curve. (a) Group with AR negative showed poor prognosis (p-value 0.031, 10yr DFS 53.3%, 77.6% for AR negative and positive group respectively).(b) Group with high HDAC1 showed higher recurrence rate (p - value 0.010, 10yr DFS 46.8%, 83.5% for HDAC1 high and low group respectively). DFS Disease free survival, AR androgen receptor, HDAC1 histone deacetylase 1